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Platelets Play an Important Role in TNF-Induced Microvascular Endothelial Cell Pathology

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Tumor necrosis factor-α (TNF) is known to be an important mediator in the pathogenesis of several inflammatory diseases. Vascular endothelial cells represent a major target of TNF effects. Platelet sequestration has been found in brain microvessels during experimental cerebral malaria and lung in experimental pulmonary fibrosis, implying that it may participate in TNF-dependent microvascular pathology. In this study, we investigated the mechanisms of platelet-endothelial interaction, using co-cultures between platelets and TNF-activated mouse brain microvascular endothelial cells (MVECs). Adhesion and fusion of platelets to MVECs was evidenced by electron microscopy, dye transfer, and flow cytometry. It was induced by TNF and interferon-γ and depended on LFA-1 expressed on the platelet surface and ICAM-1 expressed on MVECs. The adhesion and fusion also led to the transfer of platelet markers on the MVEC surface, rendering these more adherent for leukocytes, and to an enhanced MVEC sensitivity to TNF-induced injury. These results suggest that platelets can participate in TNF-induced microvascular pathology. ***(Am J Pathol 1997, 151:1397–1405)***

Tumor necrosis factor-α (TNF), an important inflammation mediator, has been involved in the pathogenesis of endotoxic shock,[1] cerebral malaria (CM),[2] graft-*versus*-host disease,[3] pulmonary fibrosis (PF),[4] disseminated intravascular coagulation,[5] and acute respiratory distress syndrome.[6–8] In these diseases, vascular endothelial cell activation is a common pathological feature. TNF is known to induce profound alterations of vascular endothelial cells, including morphological changes, induction of major histocompatibility complex class I antigens, production of some cytokines, expression of adhesion molecules, and modulation of coagulant and fibrinolytic activity.[9,10]

Endothelial cell damage and functional changes often induce interactions with platelets. It has been well recognized that platelets adhere to impaired endothelium and subendothelial tissue,[11–13] but the role of platelets in the formation of microvascular lesions has not been given enough attention. Recent studies indicated that platelets may play an important role in experimental CM[14] and experimental PF.[15] In these diseases, leukocyte sequestration and microvascular necrosis hemorrhages in relevant organs are important pathological features, and platelet sequestration was found in the microvessels of diseased organs. Electron microscopic examination clearly indicated that platelets adhere and fuse to microvascular endothelial cells. Depletion of platelets by *in vivo* injection of anti-platelet monoclonal antibody (MAb) or treatment with an anti-mouse lymphocyte function antigen 1 (LFA-1) MAb not only suppressed the deposition of platelets in these organ microvessels but also prevented the development of those complications and their associated mortality or morbidity.[14,16] These findings led us to investigate and evaluate the role of platelets in the formation of these microvascular lesions, with particular attention to the role of TNF.

Microvascular endothelial cells (MVECs) are known to differ from macrovascular endothelial cells in morphology and functions.[17] Moreover, MVECs also exhibit some organ-specific characteristics.[18,19] To understand the mechanisms of microvascular pathology of experimental CM, we isolated and cultured brain MVECs from CM-susceptible mice (CBA/J) to further analyze the interactions between platelets and TNF-activated endothelial cells *in vitro*.

Materials and Methods

Isolation and Culture of Brain MVECs

Brain MVECs were isolated from 10 4-week-old female CBA/J mice (originally obtained from IFFA CREDO, Les Oncins, France, and bred in our animal facilities) using a modification of the previously described method.[20] Briefly, the cerebral cortex was dissected and minced

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into approximately 2- to 3-mm^3 pieces. The tissue was digested in 0.1% trypsin (Sigma Chemical Co., St. Louis, MO)/0.1% EDTA solution for 30 minutes at 37°C and then further digested in 0.2% collagenase (Sigma, type II) for 15 minutes. The tissue was homogenized and then passed through a 200-μm nylon filter. The homogenate was mixed with 5 ml of fetal calf serum (FCS; Flow Laboratories, McLean, VA) and centrifuged at 500 × *g* for 5 minutes at room temperature. The pellet was resuspended in 5 ml of Dulbecco's modified Eagle's minimal essential medium (DMEM) and added onto 20 ml of 20% Percoll (Pharmacia, Uppsala, Sweden) in DMEM. The density centrifugation was carried out at 1500 × *g* for 15 minutes, and MVECs and microvessels were collected in the pellet. The cells and microvessels were washed two times in DMEM by centrifugation and resuspended in complete culture medium containing 10% inactivated human AB serum, 10% FCS, 100 μg/ml streptomycin/penicillin, 2 mmol/L L-glutamine (Gibco BRL, Gaithersburg, MD), 100 μg/ml endothelial cell growth supplement (Sigma), and 40 U/ml heparin (Liquemine, Hoffman-La Roche, Basel, Switzerland). The cells were cultured in gelatin-precoated 25-cm^2 T25 plastic tissue culture flasks (Falcon) in a 37°C, 5% CO_2 incubator. The cells were cultured for 24 hours and washed with DMEM to discard nonadherent cells, and the medium was changed every 2 days.

The isolated brain MVECs were cultured for approximately 1 week, and the colonies with cobblestone morphology were selected and transferred to a new flask with a glass micropipette after local trypsin/EDTA digestion and weeding of nonendothelial cells around MVEC colonies by manual manipulation under an inverted light microscope. The cells were cultured to confluence and then passaged by trypsin/EDTA (3:1 ratio). The purified brain MVECs were identified by immunocytochemistry for von Willebrand factor (vWF) and platelet endothelial cell adhesion molecule (PECAM, CD31). In addition, cellular surface microvilli and tight junctions, which are important markers of brain MVECs,[21] were demonstrated by transmission electron microscopy. The brain MVECs used in these experiments were at passages 2 to 5.

Platelet Adhesion Assay

Mouse platelets were isolated from EDTA anti-coagulated blood by centrifugation at 150 × *g* for 10 minutes. The platelet-rich plasma was collected and washed three times in adenosine/citrate dextrose (ACD) solution. Platelets were labeled with ^{111}Indium oxine (Amersham, Little Chalfont, UK) for 60 minutes at 37°C, washed three times, and resuspended in 1 ml of ACD solution (10^{10} platelets/ml) for adhesion assay. Mouse MVECs were seeded in a 96-well plate, grown to subconfluence, and stimulated with different doses of recombinant murine TNF (specific activity, 10^8 U/mg protein, a kind gift of Dr. Bernard Allet, Glaxo IMB), in the presence or absence of murine interferon (IFN)-γ (Holland Biotechnology) for 20 hours. MVECs were then washed twice with DMEM, and ^{111}Indium-labeled platelets were added (platelet/MVEC ratio was 1000:1). The cells were co-incubated for 60 minutes at 37°C, and then the plate was washed three times with DMEM on a Vari-Shaker (Dynatech, Oslo, Norway) to remove unbound platelets. The cells in each well were removed with trypsin/EDTA (200 μl/well), and adherent platelets were quantitated in a gamma counter (Packard Instruments, Zürich, Switzerland).

To evaluate inhibitors of platelet adherence, MVECs were prepared and activated in a 96-well plate by 20 hours of incubation in the presence of TNF (500 U/ml) and IFN-γ (100 U/ml) as positive controls. Rat MAbs to murine LFA-1, H35.89.9 (to epitope D) or H155-78 (to epitope E, both from Dr. M. Pierres, Marseille, France) or to murine ICAM-1 (YN 1/1, from Dr. F. Takei, Vancouver, Canada), at 100 μg/ml (final concentrations), were added to TNF- plus IFN-γ-activated MVECs, simultaneously with ^{111}Indium-labeled platelets. After 1 hour of co-incubation, the plate was washed and the radioactivity in each well was measured as described.

Transmission Electron Microscopy

Mouse brain MVECs were plated in cell-culture inserts (Falcon 3095) precoated with 2% gelatin (10^4/well in 1 ml of complete DMEM) and cultured to subconfluence. The cells were stimulated by TNF (500 U/ml) and IFN-γ (100 U/ml) for 20 hours, washed, and then co-incubated with platelets, at a platelet/MVEC ratio of 1000:1, for 4 hours. The cells were washed with PBS, pH 7.2, fixed with 2.5% glutaraldehyde for 30 minutes at room temperature, and post-fixed with 1% osmium tetroxide for 20 minutes. The cells were then dehydrated with different ethanol concentrations and embedded in Epon 812. Selected areas of monolayer cell cultures were stained with uranyl acetate and lead citrate and examined in a Philips EM400 electron microscope.

Dye Transfer

Mouse platelets were labeled with calcein, a fluorescent dye (Molecular Probes, Eugene, OR), at 37°C for 60 minutes, washed twice with ACD solution, and incubated for another 30 minutes at 37°C to discard non-de-esterified dye. The labeled platelets were washed twice again and co-cultured with thrombin (3 U/ml for 30 minutes) or TNF (1000 U/ml for 20 hours) pretreated brain MVECs in a 24-well plate for 4 hours. These MVECs had been washed three times with DMEM before the contact with platelets. The plate was washed three times to remove platelets in suspension, and then the dye transfer was examined under a Zeiss Axiophot fluorescence microscope. For quantitation, these MVECs were detached by trypsin/EDTA after co-incubation with calcein-labeled platelets and then washed twice with PBS containing 0.5% bovine serum albumin (BSA) by centrifugation at 450 × *g* for 5 minutes. Cells were resuspended in 200 μl of PBS/BSA, and endothelial fluorescence intensity was determined by flow cytometric analysis on a Becton Dickinson FACScan (Mountain View, CA).

Flow Cytometric Analysis

After co-incubation of platelets and TNF-pretreated MVECs as described above, MVECs were detached with trypsin/EDTA, washed, and stained with anti-mouse LFA-1 (epitope D) or anti-mouse platelet MAb CV5-H7,[22] at 10 μg/ml, for 30 minutes on ice. Cells were washed two times and incubated with fluorescein-isothiocyanate-conjugated goat anti-rat or goat anti-mouse IgG antibody for another 30 minutes. Cells were washed three times and resuspended in 200 μl of PBS/BSA, and the fluorescence intensity was analyzed by flow cytometry on a FACScan.

Leukocyte Adhesion Assay

Mouse leukocytes were isolated by Ficoll-Paque (Pharmacia) density centrifugation. The isolated peripheral blood mononuclear cells were washed twice in DMEM and then labeled with ^{51}Cr (Amersham) in DMEM containing 10% FCS for 60 minutes at 37°C. Cells were washed three times and resuspended in DMEM containing 10% FCS (5×10^6 cells/ml) for adherence assay. MVECs were seeded in a 2% gelatin-coated 96-well plate (10^4 cells/well) and cultured to subconfluence. The cells were stimulated with TNF (1000 U/ml) for 20 hours, washed, and then co-cultured with platelets for 4 hours. The plate was washed to discard unbound platelets and then incubated with ^{51}Cr-labeled murine peripheral blood leukocytes for 60 minutes. The plate was washed three times on a Vari-Shaker, and the bound leukocytes in each well were detached by trypsin/EDTA. The radioactivity of adhesive leukocytes in each well was measured in a gamma counter, and the results are expressed as cpm/well.

Cytotoxicity Assay

MVECs were plated in an eight-well chamber slide (Nunc 4808) and grown to subconfluence. Cells were stimulated with TNF (1000 U/ml) for 20 hours, washed twice with DMEM, and then co-cultured with platelets at 1000 platelets/MVEC ratios for 20 hours. The resting MVECs as well as MVECs stimulated with TNF alone were used as controls. After co-culture, the endothelial monolayer was examined on an inverted phase-contrast microscope. In addition, the effect of platelet adhesion and fusion on MVEC injury was further investigated by a ^{51}Cr release experiment. First, we investigated TNF-induced MVEC injury in the presence or absence of platelets. MVECs were cultured in a 96-well plate and labeled with ^{51}Cr (Amersham; 1 μCi/well) for 60 minutes at 37°C. After washing three times, the labeled MVECs were stimulated with different doses of TNF in the presence or absence of platelets (1000 platelets/EC). After a 20-hour incubation, the plate was centrifuged at $500 \times g$ for 10 minutes. The supernatant was collected and the radioactivity was measured in a gamma counter. Second, we investigated the effect of platelet numbers on resting or TNF-activated MVEC injury. MVECs were pretreated with TNF (1000 U/ml) for 20 hours in a 96-well plate and labeled with ^{51}Cr for 60 minutes at 37°C. After three washes with DMEM, ^{51}Cr-labeled MVECs were co-incubated with different numbers of platelets for 20 hours at 37°C. The plate was centrifuged at $500 \times g$ for 10 minutes. The supernatant in each well was collected and radioactivity was quantitated in a gamma counter (Packard). The results are expressed as cpm/well.

Statistics

All results are shown as the mean ± SD. The nonparametric Mann-Whitney U test was used to determine the significance of difference between groups.

Results

Identification of Brain MVECs

The purified brain MVECs presented characteristics of monolayer growth and contact inhibition. Cells expressed endothelial markers vWF (more than 95% of positive cells by flow cytometry) and CD31 as well as ELAM-1 inducibility in response to TNF. Transmission electron microscopy demonstrated abundant microvilli on the cytoplasmic membrane and tight junctions between cells (Lou J, Gasche Y, Zheng L, Critico B, Monso-Hinard C, Juillard P, Buurman WA, Grau GE, submitted).

Induction and Modulation of Platelet Adherence to Brain MVECs

Using the co-culture of ^{111}Indium-labeled platelets and brain MVECs, an increased adherence of platelets was found in TNF-pretreated brain MVECs. This increased platelet adherence was dose dependent and amplified by IFN-γ, as shown in Figure 1A. The increased platelet adherence on TNF-activated brain MVECs was blocked by anti-ICAM-1 or LFA-1 (D epitope) MAb. In contrast, anti-LFA-1 (E epitope) MAb, which is not significantly expressed on mouse platelets, or irrelevant antibodies had no significant inhibitory effect, as shown in Figure 1B.

TNF-Induced Platelet Adhesion and Fusion to MVECs

The platelet fusion on TNF-stimulated MVECs was evidenced by three different methods. First, transmission electron microscopy showed different stages of fused platelets in TNF alone or TNF- plus IFN-γ-stimulated brain MVECs (Figure 2, A–D). Initially, platelets adhered to the MVEC surface and formed dense areas (Figure 2A), and then portions of platelet and endothelial membranes fused and dense granules disappeared (Figure 2, B and C), and eventually platelets completely vanished, with parts of the limiting membrane of the platelet constituting a portion of the luminal endothelial membrane (Figure 2D). In contrast, in resting brain MVECs, a few adherent platelets were found, but no fusion was detectable (data not shown).

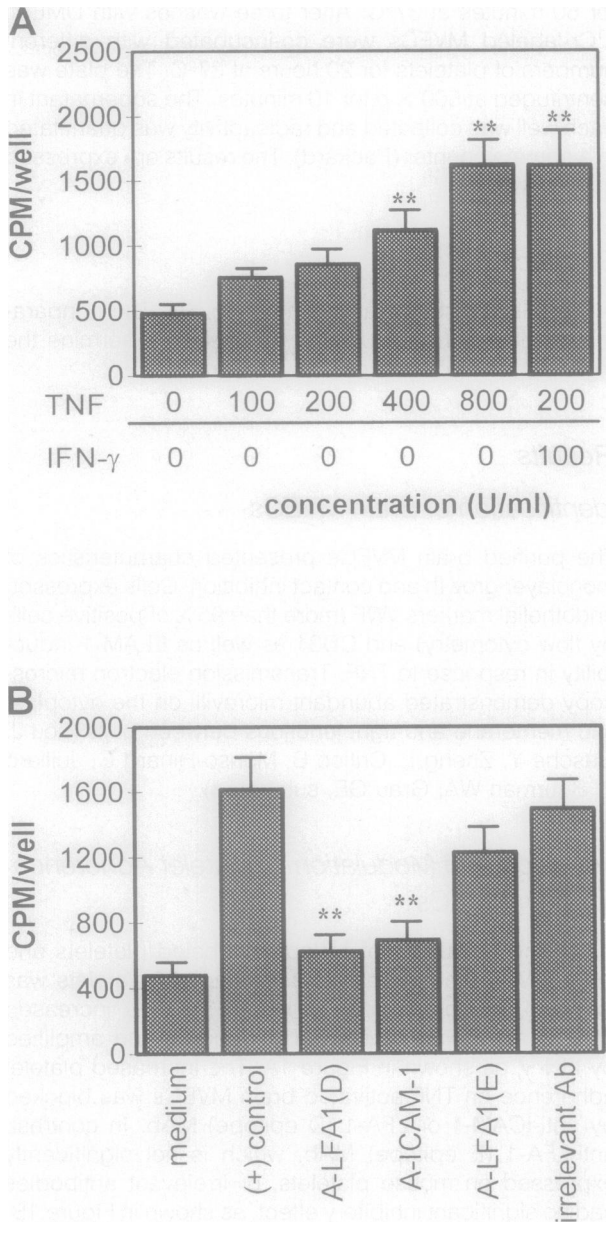

Figure 1. A: Induction of platelet adherence to brain MVECs by TNF. Bars represent SD of four experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with resting conditions. **B:** Modulation of platelet adherence to brain MVECs: importance of the LFA-1/ICAM-1 interaction. Bars represent SD of four experiments. $^{**}P < 0.01$ compared with the positive control.

Second, a dye transfer experiment was performed. As platelet fusion must be accompanied by the transfer of cytoplasm, we investigated whether calcein, a fluorescent dye, can be transferred from labeled platelets into endothelial cells during this process. After 4 hours of co-incubation, only a few calcein-labeled platelets bound to resting brain MVECs (Figure 3A) whereas increased platelet numbers adhered to thrombin-stimulated brain MVECs (Figure 3B). No dye transfer was found in either of these two conditions. In contrast, in TNF-activated brain MVECs, the addition of calcein-labeled platelets resulted in a cytoplasmic pattern of fluorescence in endothelial cells, with cell nuclei remaining negative, indicating that fluorescent dye was transferred from platelets into MVEC cytoplasm (Figure 3C). This dye transfer between calcein-labeled platelets and TNF-activated brain MVECs was quantitated by flow cytometric analysis and found to be proportional to the number of platelets added (Figure 3D). As control, there was no dye transfer when the supernatant of thrombin-activated calcein-labeled platelets was added to TNF-activated MVECs, ruling out a possible re-uptake of fluorescent dye by MVECs (data not shown). Third, flow cytometry was used to detect platelet-specific markers on the MVEC surface after adhesion and fusion. On co-incubation of platelets and TNF-pretreated MVECs, these latter cells were stained for platelet surface molecules such as LFA-1 (D epitope) or the molecule recognized by CV5H7 anti-platelet MAb.[22] Resting or TNF-stimulated brain MVECs did not express these platelet surface molecules. In contrast, both LFA-1 and the antigen recognized by CV5H7 MAb were found on TNF-stimulated brain MVECs after co-culture with platelets, suggesting that these molecules were transferred from platelets (Figure 4, A and B).

Functional Consequences of Platelet Adhesion and Fusion to Endothelial Cells

We then investigated whether the transfer of platelet material to brain MVECs can affect these cells. First, by co-culture with ^{51}Cr-labeled peripheral blood mononuclear cells, we found that adherence of leukocytes was significantly higher on platelet-bound or -fused brain MVECs, compared with normal or TNF-pretreated brain MVECs in the absence of platelets. The increase of leukocyte adherence depended on the number of platelets involved in the adhesion and fusion with MVECs (Figure 5). Second, the effect of platelets on MVEC viability was investigated. On a 20-hour incubation, TNF induced obvious morphological changes but no significant cytotoxicity to MVECs as compared with resting conditions (Figure 6, B *versus* A). However, when platelets were added to TNF-pretreated brain MVECs, their monolayer loosened and some cells detached (12% of trypan-blue-positive cells; data not shown), indicating that cytotoxicity was induced in these brain MVECs. This platelet-related endothelial injury was also quantitated by ^{51}Cr release. As shown in Figure 6, D and E, TNF or platelets alone weakly induced ^{51}Cr release from brain MVECs. In contrast, the ^{51}Cr release was significantly increased when TNF-pretreated brain MVECs were co-cultured with platelets. This increase was both TNF and platelet number dependent.

Discussion

In this study, we investigated the interaction between platelets and TNF-stimulated brain MVECs and showed that this interaction leads to endothelial phenotypic and functional changes. Adherence of ^{111}Indium-labeled platelets to brain MVECs was induced by TNF in a dose-dependent manner and amplified by IFN-γ. As LFA-1 is

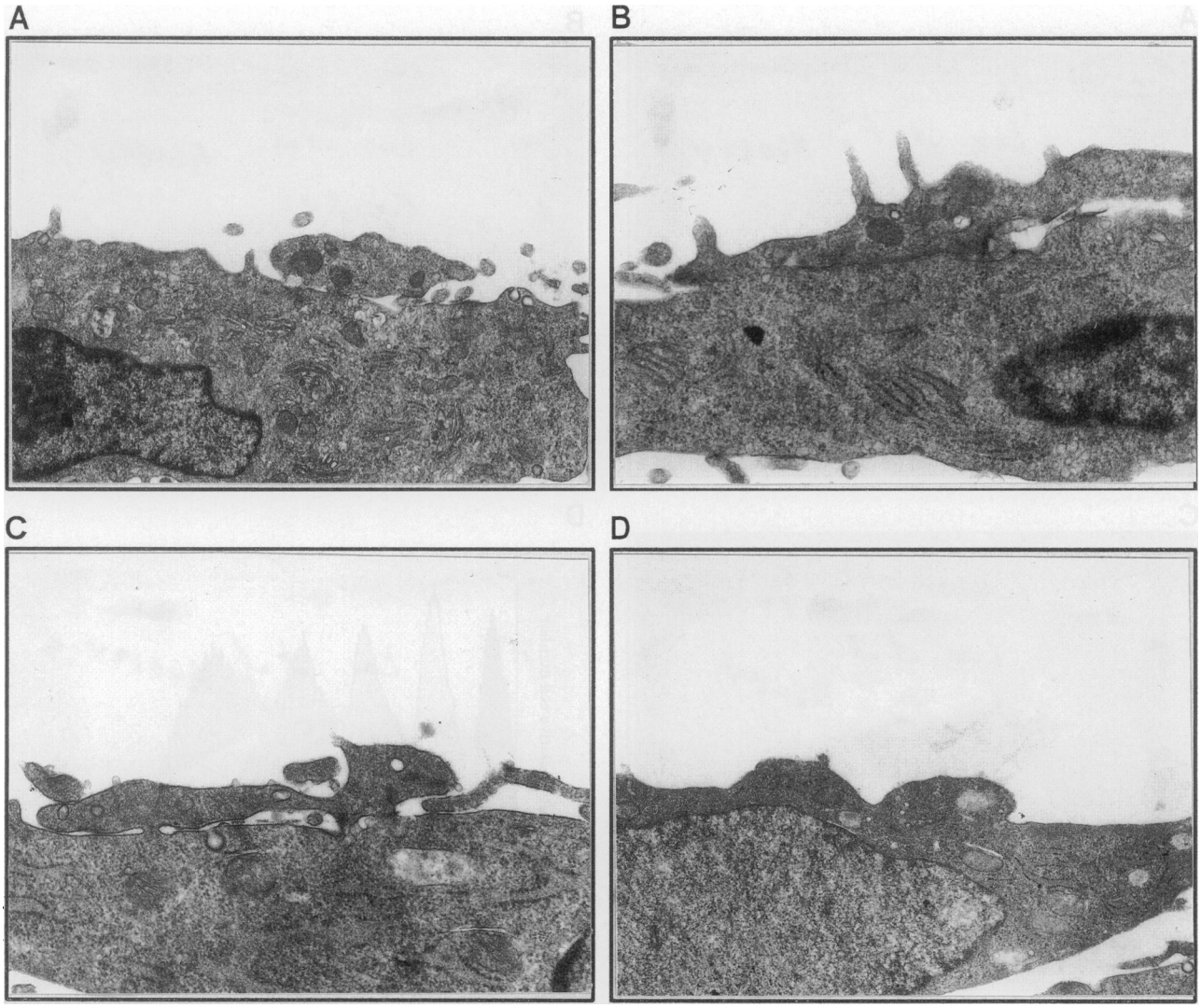

Figure 2. Evidence for platelet fusion to TNF-activated brain MVECs by transmission electron microscopy examination. **A:** Platelet adhered to MVEC and formed dense areas. **B:** A part of platelet and endothelial membrane disappeared and some platelet granules were lost. **C:** Platelet started to fuse into MVEC cytoplasm, and the dense granules were lost completely. **D:** Platelet completely fused to MVEC, and the membrane of platelet has combined with the membrane of endothelial cell.

present on the mouse platelet surface,[14,23] we further investigated the role of this integrin, and the one of its ligand ICAM-1, in the interactions between platelets and endothelial cells. TNF-induced platelet adhesion on MVECs was suppressed by MAbs to either LFA-1 D epitope or ICAM-1. In contrast, MAb to LFA-1 E epitope, which is not expressed on mouse platelets,[14] had no effect. This result indicated that TNF-induced adherence of platelet on brain MVECs is LFA-1/ICAM-1 dependent.

Various hypotheses have been proposed to explain the mechanisms of platelet adherence to endothelial cells.[24–27] In our experiments, the importance of the interaction between ICAM-1 on MVECs, and its ligand LFA-1 present on platelets, is consistent with the prevention of platelet sequestration obtained *in vivo* with anti-LFA-1 MAb.[14,16,28] Although TNF-induced adhesion of platelets was blocked by anti-ICAM-1 MAb, the role of other CAMs potentially up-regulated on the surface of MVECs by various stimuli, such as ELAM-1, VCAM-1, or P-selectin, cannot be ruled out. In our experiment, as brain MVECs were stimulated for 20 hours, ICAM-1 expression had reached its maximum. At other stimulation times, other adhesion molecules might be prominent. It has been reported that TNF can induce adherence of megakaryocytes to endothelial cells, and this adherence was partially blocked by antibodies to LFA-1, VCAM-1, or VLA-4, indicating that several adhesion molecules may be involved in this process.[29] These possibilities are under current investigation.

When analyzed by transmission electron microscopy, platelets showed adhesion and some fusion to TNF-activated MVECs, similar to those originally described.[30] Platelets were always found to be degranulated on contact with activated MVECs, suggesting that their activation did not occur before their adhesion. Interestingly, we did not find any platelet fusion when MVECs were activated by thrombin (data not shown), indicating that fusion does not necessarily follow platelet adhesion. To further

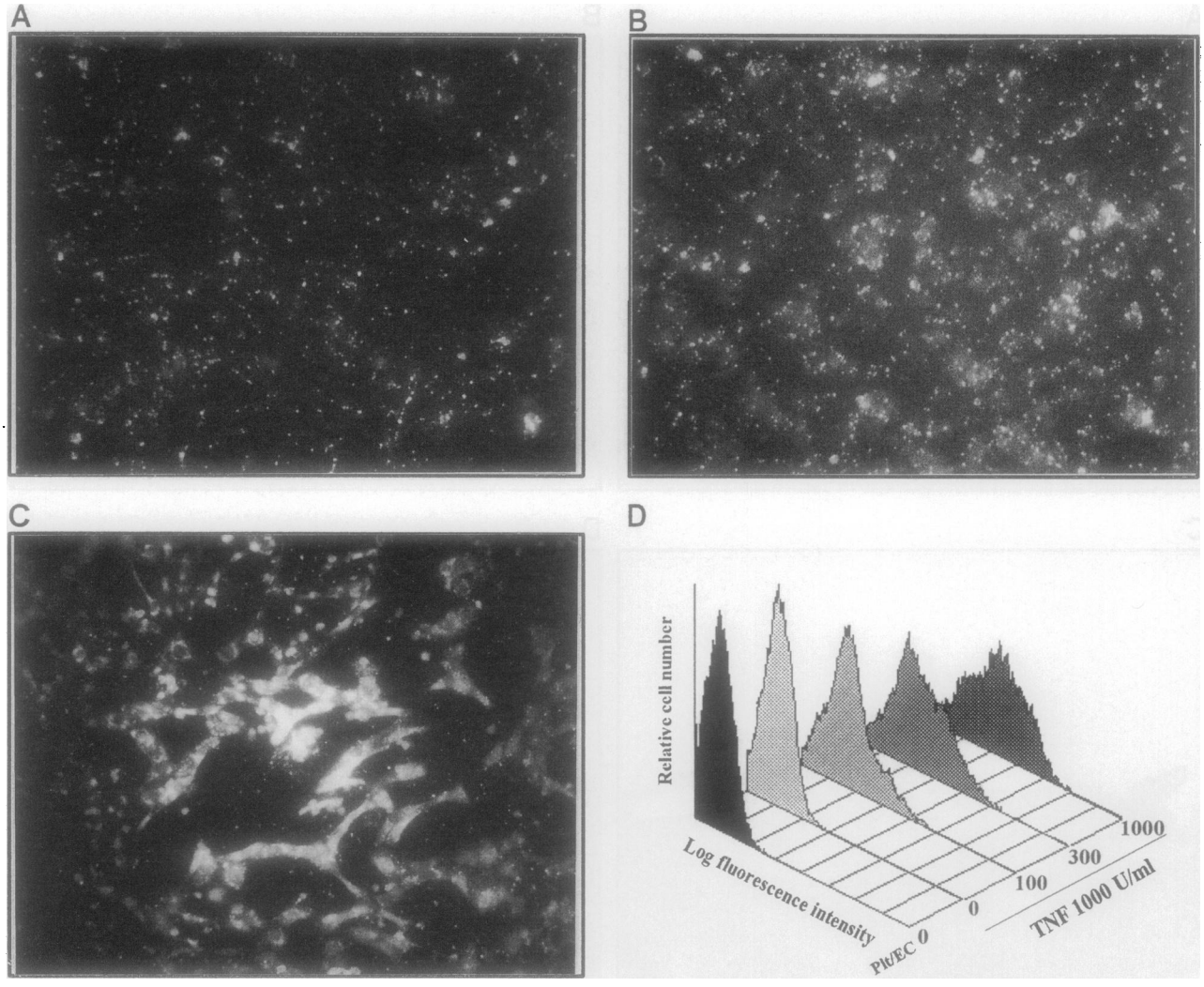

Figure 3. Evidence for platelet fusion to TNF-activated brain MVECs by dye transfer from platelets into brain MVEC. **A:** Co-culture of calcein-labeled platelets with resting brain MVECs. **B:** Co-culture of calcein-labeled platelets with thrombin-stimulated brain MVECs. **C:** Co-culture of calcein-labeled platelets with TNF pretreated brain MVECs. **D:** Quantitation of dye transfer by flow cytometric analysis.

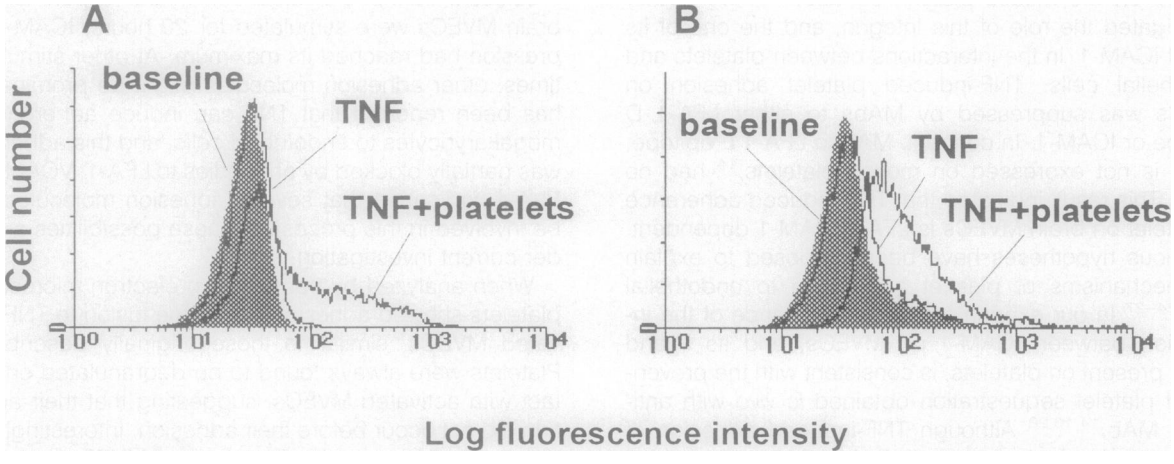

Figure 4. Evidence for platelet fusion to TNF-activated brain MVECs by identification of platelet surface markers on brain MVECs by flow cytometric analysis. The platelet surface molecules did not express on either resting (shaded area) or TNF-stimulated brain MVECs, but these molecules were expressed on platelet-adhered or fused-brain MVECs. **A:** Control and anti-LFA1 MAb. **B:** Control and anti-platelet CV5H7 MAb.

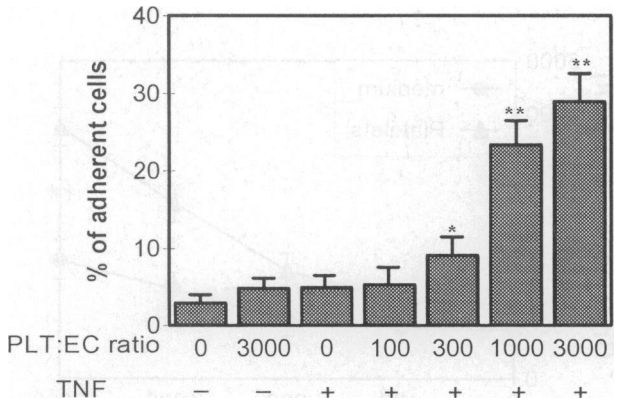

Figure 5. Increased leukocyte adherence to brain MVECs promoted by fused platelets. ^{51}Cr-labeled leukocytes were co-cultured with brain MVECs that had been pretreated with TNF and platelets as described in Materials and Methods. The adherent leukocytes were quantitated by measurement of radioactivity, and results are expressed as cpm/well. Bars represent SD of four experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

assess the process of fusion between platelets and MVECs, we investigated whether fluorescent dye may be transferred from labeled platelets into MVEC cytoplasm. When calcein-labeled platelets were co-cultured with TNF-activated MVECs, a distinct dye transfer was detectable in the latter cells. These MVECs presented a cytoplasm fluorescence pattern, with the nuclei remaining negative. The difference of fluorescence pattern with that of thrombin-stimulated MVECs confirms that fusion occurs only in particular conditions. Flow cytometric analysis showed that TNF-induced dye transfer was directly related to platelet numbers in the co-culture and revealed that platelet adhesion and fusion led to the transfer of platelet markers, such as LFA-1 or the antigen recognized by the CV5H7 MAb,[22] onto MVEC membrane. These results indicated that platelet adhesion or fusion indeed induced endothelial surface phenotypic changes.

Although platelet-endothelial fusion has been reported to support the integrity of endothelium in physiological conditions,[24] little is known about its significance in pathology. As platelet fusion is accompanied by the transfer of cytoplasm and surface molecules from platelet to endothelial cells, we therefore investigated whether this transfer may induce some endothelial functional changes. Leukocyte adhesion assay indicated that adhesion and fusion of platelets into MVECs resulted in increased adhesiveness for leukocytes. The mechanism of increased leukocyte adherence to MVECs on adhesion and fusion is not known, but the adherence of platelets to leukocytes has been reported,[31–33] indicating the presence of ligands of leukocyte adhesion molecules on platelets. It is likely that these platelet molecules are transferred to MVEC surfaces on adhesion and fusion, which may then bind additional relevant CAMs expressed on leukocytes. LFA-1 and P-selectin represent two of these molecules, as their ligand is expressed on leukocytes.

Another consequence of platelet-MVEC adhesion and fusion was the enhancement of TNF-activated endothelial cell injury. Although TNF caused morphological changes in MVECs, their monolayer was not impaired, as compared with resting cells. In contrast, on a 20-hour coincubation of TNF-stimulated MVECs with platelets, MVEC monolayer was damaged and some cells were detached. This enhancement of MVEC injury was quantitated using a ^{51}Cr release assay. Platelets alone were able to induce endothelial injury, as previously described,[34] but MVEC injury was significantly increased by TNF pretreatment. In addition, this effect was platelet number dependent, as increasing platelet/MVEC ratios led to increased ^{51}Cr release from MVECs. We propose that these MVEC alterations, increased adhesiveness and injury, may be important in the development of microvascular hemorrhages and leukocyte sequestration *in vivo*, which are major pathological features in TNF-mediated diseases such as CM and endotoxic shock, where the pathogenic role of platelets has been demonstrated.[14,16]

The original observation of platelet-endothelial fusion was made in the context of an endothelium-supporting role of platelets. This platelet fusion occurs at a low rate and is difficult to find in physiological conditions. In the case of thrombocytopenia, this fusion was found in the whole vascular system and did not exhibit any organ or tissue affinity after labeled platelet transfusion.[30] In contrast, platelet fusion in pathological conditions exhibited some obvious different features. First, this adhesion and fusion was found in diseased organs or tissues but not in others. Second, the fused platelet number was much higher and easy to detect by electron microscopy. Third, the platelet fusion led to endothelial functional changes and was accompanied by leukocyte sequestration or microvessel necrosis and hemorrhage.[14,15]

In vitro, an active role for platelets in endothelial damage has also been proposed, but in these cases, the microvascular injury was induced when platelets were activated.[13,34,35] In the process of the fusion described here, endothelial cell changes are primary and the addition of normal platelets is sufficient to induce endothelial changes. We propose that the TNF-induced ICAM-1 upregulation on endothelial membranes is responsible for an exaggeration of the fusion phenomenon. In man, cerebral malaria has been associated with accumulation of platelets in brain microvessels, although the nature of the platelet-endothelial interactions in this setting has not been investigated in detail.[36] Also, platelet sequestration in lesions of brain vasculitis in patients with systemic lupus erythematosus and in reperfusion injury have been reported.[37,38] Interestingly, these syndromes are consistently associated with leukocyte sequestration.

Taken together, the data presented here suggest that, first, platelet adhesion and fusion to endothelial cells can be induced by TNF and amplified by IFN-γ; second, TNF-induced platelet adhesion and fusion depend on the LFA-1/ICAM-1 interaction; and third, the consequences of platelet fusion into endothelial cells are enhancement of cell injury and of adhesiveness for leukocytes. Therefore, platelets display an important role in TNF-induced microvascular pathology.

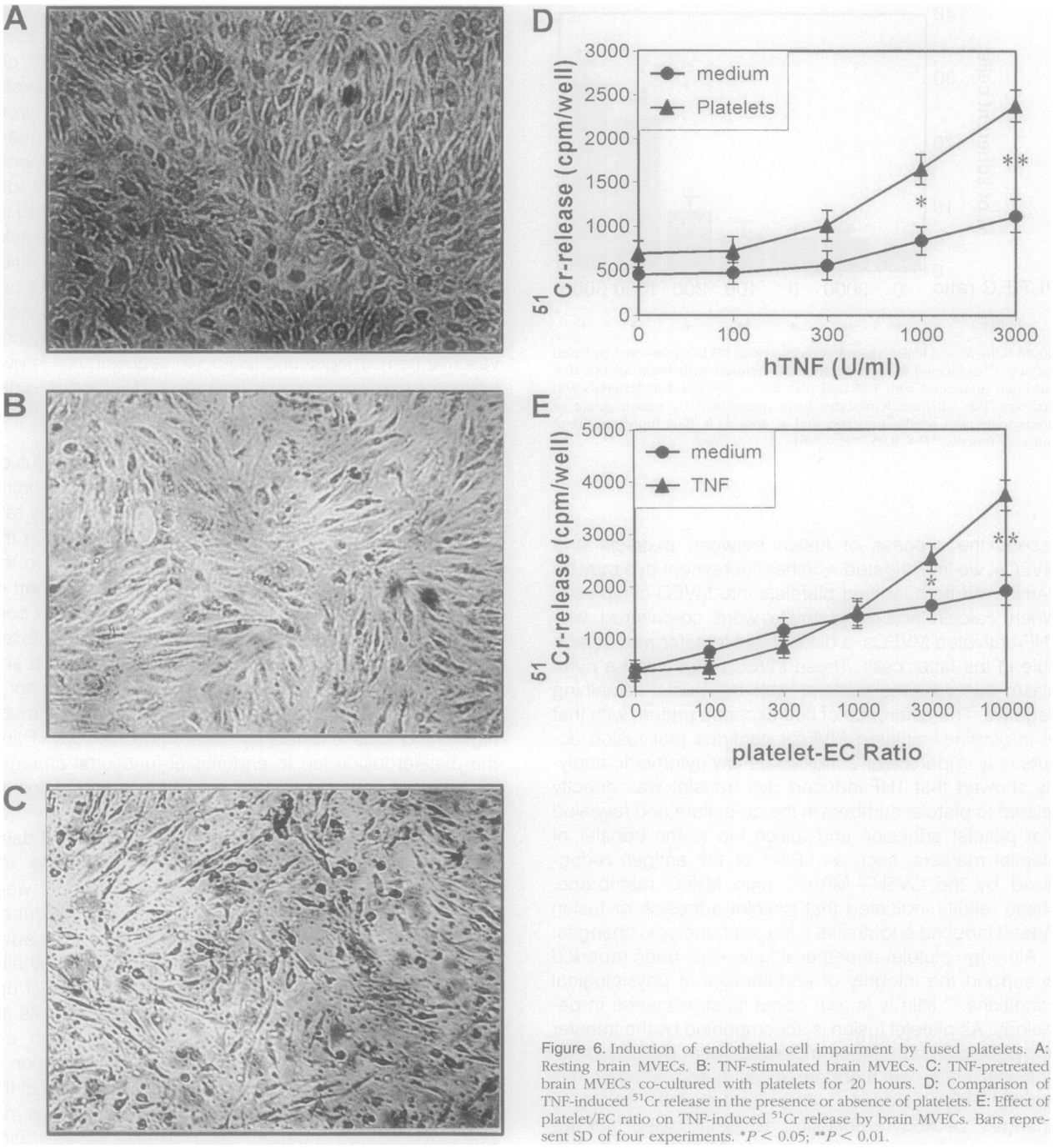

Figure 6. Induction of endothelial cell impairment by fused platelets. A: Resting brain MVECs. B: TNF-stimulated brain MVECs. C: TNF-pretreated brain MVECs co-cultured with platelets for 20 hours. D: Comparison of TNF-induced ^{51}Cr release in the presence or absence of platelets. E: Effect of platelet/EC ratio on TNF-induced ^{51}Cr release by brain MVECs. Bars represent SD of four experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

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