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This supplementary material has been provided by the authors to give readers additional information about their work.
eMethods. Study design

Study design.

Monocentric study conducted in the department of Dermatology and Neurology in the University hospital of Nice, France, between March 2008 and August 2012. An observational follow-up of the cohort of all patients with MS treated with natalizumab was performed. Institutional review board waived. Oral informed consent was obtained for each patient.

Patients.

All the patients with the intention of being treated with natalizumab for MS were included. The decision to treat with natalizumab was based on the French guidelines for all the patients (at least 1 relapse during a well 1 year-conducted interferon-β (IFNβ) therapy plus 9 T2 lesions and/or gadolinium enhanced lesion(s) on brain MRI or 2 relapses plus T2 lesion load increasing and/or gadolinium enhanced lesion(s)). Natalizumab was infused intravenously once every 4 weeks in the MS unit. All patients were followed up clinically (complete clinical and neurological evaluation for relapses; disability scored on the Expanded Disability Status Scale [EDSS] every 6 months). For each patient, age, sex, date of the onset of treatment, history of other previous immunosuppressive treatments, JC virus serology, history of cancers and risk factors of melanoma (frequent sunburns, especially in childhood; familial and personal history of melanoma; dysplastic nevi; and having >50 nevi) were collected.

Procedures.

French health authorities recommend a dermatological examination before the first infusion of natalizumab. In our prospective follow-up, all patients had a complete clinical and dermoscopic skin examination every 6 months. If natalizumab had to be stopped for any reason, a last visit was mandatory for a final assessment of their melanocytic lesions. At each
visit, a total body cutaneous examination was performed, and melanocytic nevi were compared side by side with baseline photographs. If the patients had only a few nevi, all the nevi were followed. If the nevi were numerous, only the most atypical were chosen. We used the 2-step method of digital follow-up for each patient. The first step was a clinical and digital total body mapping. The second step was a digital computerized dermoscopy (Fotofinder Systems, Bad Birnbach, Germany). Three trained experienced investigators (M.P, E.C. and P.B.) reviewed all the monitoring melanocytic skin lesions. Only patients that have at least 2 follow-up visits were kept for the analysis.

Criteria Definitions.

We used the same criteria for changes as defined by Kittler et al 1. Substantial and nonsubstantial modifications were differentiated. Substantial criteria were enlargement, changes in shape, regression, color changes (appearance of new colors), and structural dermoscopic changes (appearance of dermoscopic structures known to be associated with melanoma; eg, appearance of irregularly distributed black dots in the periphery, radial streaming, whitish veil, grayish-blue area, pseudo pods, and appearance of a prominent and irregular pigment network). Nonsubstantial criteria were darker or lighter overall pigmentation of a lesion without other modifications, increase or decrease in the number of brown globules, inflammation, disappearance or decrease in the number of initially present black dots, and replacement of parts of the pigment network by a diffuse light brown pigmentation. Excision of the lesions was proposed on clinical (ABCDE rules) and dermoscopic criteria, such as asymmetric enlargement, focal changes in pigmentation and structure, regression features, or change in color (appearance of a new color) 1,2.

Immunofluorescence.
Tissue sections were obtained from formalin-fixed and paraffin-embedded nevi collected from patients with MS or primary cutaneous melanoma. Informed consent was sought before patient materials were obtained. Formalin-fixed tissue sections were deparaffinized with xylene and EtOH-dehydrated followed by microwave treatment. The sections were treated with 1% BSA for 30 min at 37°C to block nonspecific binding sites. Overnight incubation at 4°C with the primary antibodies (anti-S100 1:100, Abcam; anti-SPARC 1:50, R&D Systems; anti-integrin β3 1:10, Abcam) was followed by incubation with with Alexa Fluor-conjugated secondary antibodies (1:400; Molecular Probes) for 1 hour at room temperature. Images were captured on a Zeiss LSM 510 META laser scanning confocal microscope, with sequential fluorophore excitation. Negative controls (no primary antibody added) showed low background staining.

Cell culture.

Human melanoma cell lines were obtained from the ATCC and cultured in Dulbecco’s modified Eagle Medium (DMEM) supplemented with 7% fetal bovine serum (FBS) as previously described. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza and grown according to manufacturer's recommendations.

Cell proliferation assay.

Cell proliferation was determined by a colorimetric assay that is based on the cleavage of yellow tetrazolium salt (XTT) to form an orange formazan dye by mitochondrial dehydrogenases (Cell Proliferation Kit II, Roche Diagnostics). The absorbance of the formazan dye was measured at 490nm. All experimental values were determined from quadruplicate wells.
Migration assay.

Chemotactic migration assays were performed using Boyden chambers with filter inserts (8.0 µm pore size, Transwell, Corning) coated with 10 µg/ml fibronectin (BD Biosciences) in 24-well dishes as previously described 3. Before performing the migration assay, cells were pretreated for 90 min with blocking α4 integrin antibody natalizumab or isotype control antibody (IgG).

Three-dimensional (3D) melanoma tumor spheroid invasion.

Spheroids were generated using the liquid overlay technique and implanted into a gel of collagen I (0.9 mg/mL) as previously described 4. The suspension was poured into 6-well plates pre-coated with 1.5% agar (5-10 spheroids per well) and growth medium was overlaid on top of solidified collagen lattices. Spheroids were incubated 3 days and tumor cell outgrowth was visualized by phase contrast microscopy.

Transendothelial cell migration assay:

1×10^5 HUVECs were plated in the upper chamber of a gelatin-coated Transwell insert (8.0 µm pore size, Corning) and grown in complete endothelial medium to confluence. The endothelial monolayer was then left untreated or treated with human TNF-α (10 ng/ml) overnight. Melanoma cells labeled with 5 µM CellTracker Green CMFDA dye (Molecular Probes) were nonenzymatically detached, treated for 90 min with blocking α4 integrin antibody natalizumab or isotype control antibody in the endothelial medium and plated on top of the endothelial monolayer at 1×10^5 cells/insert. Melanoma cells were allowed to migrate for 7 hours at 37°C in 5% CO2. After gently scraping the upper side of the inserts with cotton swabs, cells on the underside of the filter were fixed in 3% paraformaldehyde, permeabilized with 0.5% Triton X-100 and the filter was mounted onto glass slides for microscopic analysis.
Transmigrated cells were visualized on a Zeiss immunofluorescent microscope. At least 10 random fields per samples were scored.

Statistical analysis.
Experiments shown were representative of at least 3 independent experiments. All data were presented as means ± standard deviation (SD). Statistical analysis was performed using the Student $t$ test with $P<.05$ deemed as statistically significant.
eReferences.


eFigure 1. Dermoscopy findings in a representative nevus


B, Dermoscopy of the same nevus in June 2012, showing features of regression with whitish area and telangiectasias. The lesion was removed and the histopathological analysis showed a benign nevus with central irritating phenomena.
eFigure 2. Effect of natalizumab on proliferation of melanoma cells

Human A375 melanoma cells were treated with the indicated concentrations of isotype-matched control IgG or natalizumab (NTZ). Cell proliferation was measured by XTT assay at different times after treatment. Columns, mean of four independent determinations; error bars, SD. Similar results were obtained in another melanoma cell, WM793 (not shown).
eFigure 3. Natalizumab blocks melanoma cell migration across tumor necrosis factor (TNF)–activated endothelial cells

Primary human endothelial cells were seeded into gelatin-coated Transwell inserts, allowed to growth to confluence and activated or not with TNFα (10 ng/mL) overnight. A375 cells labeled with the fluorescent CellTracker dye CMFDA were incubated with isotype-matched IgG (75 µg/ml) or the indicated concentrations of Natalizumab for 90 minutes, seeded in the upper compartment of the Transwell and left to migrate across the endothelial monolayer for 7 hours. Transmigrated cells were visualized under a fluorescent microscope and counted in at least 10 independent fields per well (three wells par condition). Columns, mean of 3 independent determinations; error bars, SD. *, P< .01 (Student’s test).