A Key Role for Matrix Metalloproteinases and Neutral Sphingomyelinase-2 in Transplant Vasculopathy Triggered by Anti-HLA Antibody

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Background—Outcomes for organ transplantation are constantly improving because of advances in organ preservation, surgical techniques, immune clinical monitoring, and immunosuppressive treatment preventing acute transplant rejection. However, chronic rejection including transplant vasculopathy still limits long-term patient survival. Transplant vasculopathy is characterized by progressive neointimal hyperplasia leading to arterial stenosis and ischemic failure of the allograft. This work sought to decipher the manner in which the humoral immune response, mimicked by W6/32 anti-HLA antibody, contributes to transplant vasculopathy.

Methods and Results—Studies were performed in vitro on cultured human smooth muscle cells, ex vivo on human arterial segments, and in vivo in a model consisting of human arterial segments grafted into severe combined immunodeficiency/beige mice injected weekly with anti-HLA antibodies. We report that anti-HLA antibodies are mitogenic for smooth muscle cells through a signaling mechanism implicating matrix metalloproteinases (MMPs) (membrane type 1 MMP and MMP2) and neutral sphingomyelinase-2. This mitogenic signaling and subsequent DNA synthesis are blocked in smooth muscle cells silenced for MMP2 or for neutral sphingomyelinase-2 by small interfering RNAs, in smooth muscle cells transfected with a vector coding for a dominant-negative form of membrane type 1 MMP, and after treatment by pharmacological inhibitors of MMPs (Ro28-2653) or neutral sphingomyelinase-2 (GW4869). In vivo, Ro28-2653 and GW4869 reduced the intimal thickening induced by anti-HLA antibodies in human mesenteric arteries grafted into severe combined immunodeficiency/beige mice.

Conclusions—These data highlight a crucial role for MMP2 and neutral sphingomyelinase-2 in vasculopathy triggered by a humoral immune response and open new perspectives for preventing transplant vasculopathy with the use of MMP and neutral sphingomyelinase inhibitors, in addition to conventional immunosuppression. (Circulation. 2011;124:2725-2734.)

Key Words: anti HLA class I mAb ■ metalloproteinases ■ proliferation ■ smooth muscle cells ■ transplant vasculopathy

Progress in surgical techniques, organ preservation, and immunosuppressive drugs has improved the outcome of organ allografts. However, the late outcome of organ transplants is impaired by transplant vasculopathy (also known as transplant arteriosclerosis or chronic vascular rejection), which occurs in up to 50% of heart transplant recipients within 5 years after transplantation. Transplant vasculopathy is mediated by immune and nonimmune mechanisms (such as ischemia/reperfusion, inflammation, viral infection, and hypertension) that trigger diffuse and concentric intimal hyperplasia and lead finally to arterial stenosis, chronic ischemia, and functional loss of the grafted organ.

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Both cell-mediated and humoral immunologic mechanisms are thought to participate in the pathogenesis of transplant vasculopathy. Experimental and clinical studies suggest that B cells and donor-specific alloantibodies play a role in
long-term allograft rejection and induce intimal proliferation of smooth muscle cells (SMCs), a key event in the genesis of transplant vasculopathy. Anti-HLA antibodies trigger the production of growth factors and inflammatory cytokines (fibroblast growth factor, interferon, tumor necrosis factor-α [TNF-α]), the expression of antiapoptotic members of the Bcl-2 family, and activation of signaling pathways involved in the migration and proliferation of vascular cells. Moreover, the proinflammatory environment elicited by immune system activation is able to dedifferentiate SMCs that acquire a synthetic phenotype and produce cytokines and extracellular matrix. On the basis of the response of vascular cells to anti-HLA monoclonal antibody (mAb) W6/32 reported by Reed et al, we recently developed an original model of human mesenteric arteries grafted into severe combined immunodeficiency (SCID)/beige mice, in which passive transfer of W6/32 mAb toward HLA class I antireactive antibodies in human arteries grafted into SCID/beige mice.

The sphingomyelin/ceramide signaling pathway is involved in various stress-induced responses and plays a role in the mitogenic response of vascular SMCs to TNF-α, oxidized low-density lipoprotein, and H₂O₂. In this pathway, the activation of neutral sphingomyelinase (nSMase2), which converts sphingomyelin into ceramide, requires the activation of the matrix metalloproteinases (MMPs) membrane type 1 MMP (MT1-MMP) and MMP2. Moreover, MMPs are involved in extracellular matrix degradation, cell migration, and proliferation and could be implicated in cardiac transplant vasculopathy and chronic allograft nephropathy. Because the precise mechanism of action of MMPs remains elusive, we hypothesized that MMPs and the sphingomyelin/ceramide pathways are activated by alloantibodies and thus contribute to the mitogenic signaling leading to intimal hyperplasia and vascular wall remodeling of human arteries grafted in SCID/beige mice. This led us to investigate whether MMP and nSMase2 inhibitors may prevent alloantibody-induced SMC proliferation and transplant vasculopathy. This study was conducted in vitro in human cultured SMCs, ex vivo on human arterial segments, and in vivo on human arteries grafted into SCID/beige mice.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Cell Culture and Ex Vivo Experiments**

Human mesenteric SMC primocultures were isolated from mesenteric arteries and characterized as described in the online-only Data Supplement. Human aortic SMCs (haSMC, CRL-1999 cell line) and murine aortic SMC (MOVAS, CRL-2797) were from ATCC (Manassas, VA). Cells were stimulated by W6/32 mAb, irrelevant mAb (IgG2a, same isotype as W6/32), or anti-mouse major histocompatibility complex mAb (28-14-8, recognizing H-2Db), as reported in the online-only Data Supplement. Human aortic SMCs (haSMC, CRL-1999 cell line) and murine aortic SMC (MOVAS, CRL-2797) were from ATCC (Manassas, VA). Cells were stimulated by W6/32 mAb, irrelevant mAb (IgG2a, same isotype as W6/32), or anti-mouse major histocompatibility complex mAb (28-14-8, recognizing H-2Db), as reported in the online-only Data Supplement.

Ex vivo experiments were performed on human artery rings. After their recovery from mesenteric artery, the rings were immediately incubated in serum-free Dulbecco’s modified Eagle’s medium and stimulated by W6/32 mAb (1 μg/mL) for 24 hours, in the presence of absence of pharmacological agents.

**DNA Synthesis, Small Interfering RNA Targeting, and Cell Transfection**

DNA synthesis was evaluated by [³H]thyidine incorporation, as described. Cell counting was performed with the use of a Z1 Beckman cell counter, as described in the online-only Data Supplement. SMC transfections by small interfering RNA (siRNA) targeting human nSMase2, MMP2, scrambled siRNA, or MT-E240A mutant vector coding for the catalytically inactive MT1-MMP (a generous gift from Dr Alex Strongin, La Jolla, CA) were done as reported.

**Determination of MT1-MMP, MMP2, and nSMase2 Activities**

MT1-MMP activity was determined in SMC extracts with the use of the MT1-MMP fluorogenic substrate dansyl-Pro-Leu-Ala-Arg-NH₂ and MMP2 activity was determined in SMC supernatant and in the plasma from transplanted mice with the use of the MMP2 fluorogenic substrate MCA-Pro-Leu-Ala-Dpa-Ala-Arg-NH₂. The activity of nSMase2 was measured in SMC extracts with the use of radiolabeled sphingomyelin, as reported.

**Human Mesenteric Arterial Segments Grafted Into SCID/Beige Mice**

This experimental animal system has been characterized extensively and its use with anti-HLA mAbs has been reported in a recent methodological article and is briefly described in the online-only Data Supplement. The use of human mesentery from cadaveric organ donors was approved by the French Agence de Biomédecine and the Ethics Committee of the University Hospital of Toulouse. Experimental protocols on animals were conducted in accordance with the French legislation and the local ethical committee for animal experiments.

**Histological Analysis and Histomorphometric Reconstitution of Arteries**

Six weeks after transplantation, the mice were euthanized, and the grafted human arterial segments were removed, fixed, and embedded in paraffin for histological analysis or in Tissue-Tek, and they were snap-frozen in liquid nitrogen for morphometric analysis.

**Statistical Analyses**

Data are presented as mean±SD. Estimates of statistical significance were performed by t test or ANOVA followed by a multiple comparison procedure (with the use of SigmaStat 3.5, Systat software). In populations normally distributed and with equal variances, differences between mean values were evaluated by unpaired t test (2 groups) or by 1-way ANOVA (≥2 groups). When a difference in the groups was detected by 1-way ANOVA, multiple comparisons (generally versus a control group) were performed by the Holm-Sidak test. Values of P<0.05 were considered significant.

**Results**

Anti-HLA mAbs Trigger the Activation of MT1-MMP, MMP2, and nSMase2-Mediated Mitogenic Signaling in Human Cultured SMCs: Inhibition by MMP and nSMase2 Inhibitors

SMC primocultures prepared from human mesenteric arteries as well as the human aortic SMC cell line CRL-1999 (haSMC CRL-1999) express smooth muscle α-actin (Figure I in the online-only Data Supplement). HLA class I antigen recognized by W6/32 mAb was also expressed at the surface of both SMC primocultures prepared from human mesenteric arteries and haSMC CRL-1999 (Figure II in the online-only Data Supplement), as well as in mesenteric arterial segments
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used for grafts in mice (Figure III in the online-only Data Supplement), as reported.33

Dose-response experiments (mitogenic effect of increasing W6/32 concentration) showed that 1 μg/mL W6/32 mAb was optimal to trigger [3H]thymidine incorporation in cultured SMCs (Figure IVA in the online-only Data Supplement) and cell proliferation (increased cell number), as assessed by cell counting (Figure IVB in the online-only Data Supplement). This W6/32 concentration (1 μg/mL) exhibited no cytotoxic effect (Figure IVC in the online-only Data Supplement) and was therefore utilized throughout the experiments (unless otherwise indicated). In contrast, the same concentration (1 μg/mL) of an irrelevant mAb of the same isotype class as W6/32 (IgG2a) induced no mitogenic response (Figure IVA).

Interestingly, the anti-mouse major histocompatibility complex 28-14-8 mAb triggered DNA synthesis in murine aortic SMCs (MOVAS, CRL-2797) but not in human SMCs, and, conversely, W6/32 mAb elicited no [3H]thymidine incorporation (Figure 1A). Interestingly, the anti-mouse major histocompatibility complex 28-14-8 mAb triggered DNA synthesis in murine aortic SMCs (MOVAS, CRL-2797) but not in human SMCs, and, conversely, W6/32 mAb elicited no [3H]thymidine incorporation (Figure 1A).

To exclude any activation resulting from Fc receptors or from the Fc portion of the anti-HLA mAb, we investigated the mitogenic effect of F(ab)2 fragments from the W6/32 mAb (kit Pierce F(ab)2, Thermo Scientific). As shown in Figure 1B, both F(ab)2 fragments and intact mAb exhibited a similar mitogenic effect, indicating that [3H]thymidine incorporation induced by W6/32 mAb is dependent on its anti-HLA specificity and independent of the Fc portion or of Fc receptors. These data are consistent with the lack of effect of irrelevant mAb.

Because several stress-inducing agents (eg, TNF-α, oxidized low-density lipoprotein) trigger SMC proliferation via a signaling mechanism implicating the metalloproteinases MT1-MMP and MMP2 and the sphingolipid pathway,19,20 we investigated whether this signaling is involved in the W6/32 anti-HLA mAb-induced mitogenic effect. As shown in Figure 1C through 1H, mitogenic concentrations of W6/32 mAb activated MT1-MMP, MMP2, and nSMase in haSMCs (under the conditions used, no activation of acidic SMase was observed; data not shown). Interestingly, the activation of MMP2 and [3H]thymidine incorporation by W6/32 mAb was inhibited by MMP2-specific siRNA and in haSMCs expressing dn-MT1, a dominant-negative form of MT1-MMP20 (Figure 1E and 1H). Moreover, nSMase2-specific siRNA abrogated the activation of nSMase2 and [3H]thymidine incorporation by W6/32 mAb (Figure 1G and 1H).

Altogether, these data suggest that W6/32 anti-HLA mAb is mitogenic for human vascular SMCs via the activation of the MMP/nSMase2 signaling pathway. This led us to anticipate that pharmacological inhibitors of MMPs and nSMase2 should inhibit the mitogenic effect of W6/32 mAb.

We first investigated the relative efficacy of 2 MMP inhibitors: batimastat, a broad-spectrum MMP inhibitor, and Ro28-2653, which is more specific for MMP2 and MT1-MMP.19 Dose-response experiments allowed selection of the nontoxic concentration effective to inhibit the mitogenic effect of W6/32 mAb (10 nmol/L for Ro28-2653 and 100 nmol/L for batimastat) (Figures V and VI in the online-only Data Supplement). The 2 MMP inhibitors efficiently inhibited the activation of MT1-MMP and MMP2 (Figure 2A and 2B), thereby confirming the results observed in MMP2-silenced haSMCs (Figure 1). MMP inhibition by batimastat or by Ro28-2653 also blocked the activation of nSMase2 (Figure 2C) and [3H]thymidine incorporation elicited by W6/32 anti-HLA mAb (Figure 2D).

We then tested the effect of the SMar inhibitor GW4869,34 which efficiently blocked the activation of nSMase2 and [3H]thymidine incorporation elicited by W6/32 mAb (Figure 2C and 2D) (as observed in SMCs silenced for nSMase2 by siRNA). Note that GW4869 had no inhibitory effect on MT1-MMP and MMP2 activation by W6/32 mAb (data not shown). Likewise, batimastat, Ro28-2653, and GW4869 inhibited [3H]thymidine incorporation elicited by W6/32 mAb in SMC primocultures prepared from human mesenteric arteries (Figure 2D).

Altogether, these data suggest that MT1-MMP and MMP2 are required for the activation of nSMase2 by the anti-HLA W6/32 mAb and its subsequent mitogenic signaling. This indicates that W6/32 anti-HLA mAbs behave like stress-inducing agents and activate the stress-induced mitogenic response mediated by the MMP/sphingolipid pathway in SMCs, as shown previously with stress-inducing agents.19–21 Interestingly, pharmacological inhibitors of MMPs and nSMase2 effectively abrogated DNA synthesis mediated by W6/32 mAb.

Histological Study of Human Mesenteric Arterial Segments Grafted Into SCID/Beige Mice

As indicated above, HLA class I antigens recognized by W6/32 mAb were expressed in human mesenteric arteries utilized for xenotransplantation (Figure III in the online-only Data Supplement). As expected, the endothelial cell lining was intensely stained by W6/32, whereas the media was moderately labeled by W6/32 mAb. After subtraction of the yellow autofluorescence of the arterial wall, the green fluorescence of the media strongly suggests that SMCs of the media express HLA class I antigens (Figure III in the online-only Data Supplement).

Immunodeficient SCID/beige mice, grafted with human mesenteric artery segments, were injected weekly with W6/32 anti-HLA mAb (1 μg mAb per 10 g body wt for 5 weeks), irrelevant mAb (same protocol as W6/32), or vehicle (phosphate-buffered saline) only.17 Histological studies were performed on the grafted human mesenteric artery segments recovered at the end of the treatment. In mice injected with phosphate-buffered saline vehicle ("untreated") or irrelevant mAb, we observed neither major histological alteration nor thrombosis (Figure 3A). Human mesenteric arteries grafted into mice treated by W6/32 mAb for 5 weeks developed lesions of transplant vasculopathy, characterized by a dramatic intimal hyperplasia (Figure 3A), as reported previously.17 Mouse aortic segments taken near the graft did not exhibit any histological alteration, in particular no intimal thickening (Figure 3B). This is consistent with the specificity of the W6/32 mAb for human HLA antigens but not for murine major histocompatibility complex antigens35,36 and is in agreement with the lack of effect of W6/32 anti-HLA mAb on mouse SMCs (Figure 1A).
Figure 1. Mitogenic effect of W6/32 anti-HLA monoclonal antibody (mAb) to cultured smooth muscle cell (SMC) and inhibition by small interfering RNA (siRNA) specific for matrix metalloproteinase (MMP)2. A, [3H]Thymidine incorporation induced by anti-HLA mAb W6/32 (HLA), anti-mouse major histocompatibility complex mAb 28-14-8 (MHC), and irrelevant antibody IgG2a (Irr) was evaluated by [3H]thymidine incorporation (see Methods) in human mesenteric SMC primoculture (phmSMC), in human aortic SMC (haSMC, CRL-1999 cell line), and in murine aortic SMCs (MOVAS, CRL-2797). Cells were starved in serum-free Dulbecco’s modified Eagle’s medium for 24 hours, then stimulated by the different antibodies (1 μg/mL) for 48 hours. In each experiment, [3H]thymidine incorporation was normalized to the negative control (ie, expressed as percentage of the untreated control). [3H]Thymidine incorporation ranged between 5460 and 8220 dpm per well in untreated phmSMC, 9120 and 15 060 dpm per well in untreated haSMC CRL-1999, and 37 150 and 48 210 dpm per well in untreated MOVAS CRL-2797 (in the set of experiments reported here). B, [3H]Thymidine incorporation induced by intact W6/32 mAb (Ig) and F(ab’2) purified fragments (1 μg/mL) in human haSMC CRL-1999 cell line. C through E, MT1-MMP and MMP2 in haSMC CRL-1999. In C and D, time course of activity of membrane type 1 (MT1)-MMP and MMP2 in haSMC CRL-1999. In C and D, time course of activity of membrane type 1 (MT1)-MMP and MMP2 in haSMC CRL-1999 treated by W6/32 mAb or irrelevant mAb (1 μg/mL) is shown. Cells were incubated without or with W6/32 mAb (1 μg/mL) at the indicated time and then were harvested, and cell extracts were used for enzymatic assays. In E, untransfected haSMCs (dotted bar) or haSMCs expressing a mutant dominant-negative MT1-MMP (dn-MT1) or transfected by MMP2 siRNA (MMP2-si), neutral sphingomyelinase-2 (nSMase2-) siRNA (nSM2-si), or scrambled siRNA (scr) were incubated without or with W6/32 mAb (1 μg/mL) for 30 minutes. Then cells were washed and harvested, and cell extracts were used for Western blotting or enzymatic determination. In insert, Western blots show the lack of MMP2 expression in MMP2-silenced SMC. Enzyme activities were determined with the use of fluorogenic substrates, as described in Methods. F through H, Activity of nSMase in haSMCs treated under the same conditions as in C and D. F, Time course of nSMase activity on treatment with W6/32 or irrelevant mAb. G, Inhibitory effect of dn-MT1 and siRNAs on nSMase activation by W6/32. H, Effect of dn-MT1 and siRNAs on [3H]thymidine incorporation by W6/32 mAb in haSMCs. In C through H, enzyme activities are expressed as percentage of basal activity (unstimulated control). Data are mean±SD of 3 to 5 separate experiments. *P<0.05. Statistical analyses are as follows: In A, each cell type (each panel) was analyzed separately by 1-way ANOVA and pairwise multiple comparison by Holm-Sidak method; B, t test analysis; C, D, F, t test analysis to compare the effect of irrelevant vs anti-HLA mAb at each time point (each point was evaluated on separate culture dishes); E, G, H, 1-way ANOVA and comparison with the positive control (cells treated with anti-HLA mAb [black bar]) by the Holm-Sidak method.
Neointimal thickening of human arterial segments grafted in SCID/beige mice treated with W6/32 anti-HLA mAb was apparently not altered (Figure 3), thereby confirming that these antibodies are not cytotoxic and that intimal thickening may occur independently of media alteration. No inflammatory infiltration by lymphocytes and macrophages was detected in human mesenteric arteries before and after the vessels were grafted into SCID/beige mice (data not shown).

**W6/32 Anti-HLA mAbs Induce MMP2 Expression, Mitogenic Signaling, and Intimal Thickening in Human Arterial Segments Transplanted Into SCID/Beige Mice: Prevention by Ro28-2653 and GW4869**

Increased MMP2 expression was observed in the neointima of grafted human arterial segments (Figure 4A and 4B), in agreement with in vitro and ex vivo data and with a previous observation by Tsukio et al25 in a model of cardiac transplantation. Altogether, these data suggest that W6/32 anti-HLA mAbs enhance MMP2 expression in grafted arterial segments. Ro28-2653 prevented the rise in MMP2 expression in grafted human mesenteric segments (Figure 4).

The experimental animal model (SCID/beige mice grafted with human mesenteric arteries and treated with W6/32)17 was used to evaluate the in vivo efficacy of MMP and nSMase inhibitors in preventing intimal hyperplasia.

The mitogenic marker proliferating cell nuclear antigen (PCNA) was significantly increased in the intima and in the media of human arterial segments grafted in mice treated with anti-HLA mAb (Figure 5A and 5B), consistent with cell proliferation leading to neointimal thickening and indicating that some SMCs are activated in the tunica media. The MMP inhibitor Ro28-2653 and nSMase2 inhibitor GW4869 prevented, in part, the increased PCNA expression induced by W6/32 anti-HLA mAb (Figure 5A and 5B).

Morphometric analyses of serial histological sections allowed us to reconstruct the 3-dimensional details of the arterial wall (Figure 6A). This clearly shows the dramatic neointimal thickening of the human artery grafted in mice treated with W6/32 anti-HLA mAb. Interestingly, the MMP inhibitor Ro28-2653 and nSMase2 inhibitor GW4869 blocked the W6/32-induced neointimal thickening of the grafted arterial segments (Figure 6A and 6B).

In summary, our data show that, in this experimental model system, both MMP and nSMase2 inhibitors prevent the neointimal thickening occurring in vivo in grafted human arterial segments treated with anti-HLA mAb. The data support a role for MMP2 and nSMase2 in the pathogenesis of transplant vasculopathy and demonstrate the efficacy of these pharmacological inhibitors for preventing this severe complication.

**Discussion**

The innate and adaptive immune systems are thought to play a role in transplant vasculopathy, but the pathophysiological mechanism of arterial wall alteration remains unclear, and conventional immunosuppression is poorly effective in pre-
venting transplant vasculopathy.9–11 HLA alloantibodies have been implicated in chronic allograft rejection and vascular cell proliferation,13–15,17 but thus far their mechanism of action is only partly understood.13–15 The rationale of this study came from our previous work on the stress-induced sphingomyelin/ceramide pathway, which indicated a role for MMPs (namely, MT1-MMP and MMP2) and nSMase2 in stress-induced SMC proliferation.19,20 We hypothesized that this signaling may also be triggered by anti-HLA mAbs, which behave like stress-inducing agents. Our results show that W6/32 anti-HLA mAb activates the MMP/nSMase2-dependent pathway that is involved in DNA synthesis and in intimal hyperplasia. Moreover, the hypothesis is also supported by the protective effect of MMP and nSMase2 inhibitors Ro28-2653 and GW4869, which prevent the neointimal thickening of human arterial segments induced by W6/32 mAb.

The first striking result is that HLA humoral immunity (mimicked by W6/32 anti-HLA mAb stimulation) leads to SMC proliferation in vitro and is sufficient for inducing neointimal hyperplasia through the activation of MMP2 and nSMase2. The implication of MMPs in transplant vasculopathy development is often correlated with vascular remodeling occurring during neointimal proliferation.26–28,37 Moreover, the concomitant inhibition of anti-HLA mAb-induced MMP2 activation, mitogenic signaling, SMC proliferation, and neointimal hyperplasia by the MMP inhibitor Ro28-2653 is consistent with recent observational studies from Lutz et al.,24 Tsukiioka et al.,25 and Hariya et al.,26 suggesting a strong association between MMP2 and transplant vasculopathy. These data and reports from Reed et al11–14,35 indicate that multiple signaling pathways act together to trigger the mitogenic effect mediated by W6/32 mAbs. Note that the anti-HLA mAb concentration used in our experiments for triggering SMC proliferation was lower than that used by Reed,35 but the dose-response curve showed that the W6/32 mAb concentration used here induces a proliferation in SMCs similar to that obtained with higher dose (Figure IV in the online-only Data Supplement). In addition, differences in the cell type (SMCs versus endothelial cells) may also play a role because, in our experience, the stress-induced mitogenic response is higher in SMCs than in endothelial cells.

A major outcome is the efficacy of MMP inhibitors to reduce the neointimal thickening induced by anti-HLA mAb. This suggests that, in addition to other previously identified mitogenic pathways, MMP2 (and other MMPs targeted by Ro28-2653) plays a crucial role not only in cultured SMCs stimulated by various stress agents19,20 but also in vivo in the progression of transplant vasculopathy. MMP inhibitors tested in clinical trials for vascular diseases and cancer therapy exhibit nonnegligible toxicity, possibly because of their broad spectrum of activity.38,39 However, the MMP inhibitor Ro28-2653 is more specific and acts mainly on

Figure 3. Histology of human mesenteric arteries grafted into severe combined immunodeficiency/beige mice treated with W6/32 anti-HLA monoclonal antibody (mAb). A, Hematoxylin-eosin (HE) staining of histological sections from human mesenteric arteries grafted into severe combined immunodeficiency/beige mice either untreated (phosphate-buffered saline vehicle only) or treated with irrelevant mAb or with W6/32 anti-HLA mAb (1 μg/10 g body wt) injected intravenously weekly for 5 weeks. B, HE staining of sections of the abdominal aorta of mice (just above the grafted human artery), which were untreated (vehicle only) or treated with anti-HLA mAb, as in A. C, Smooth muscle α-actin staining of grafted human arteries in mice treated with anti-HLA mAb or control, as in A. D, Masson trichrome staining of grafted human mesenteric arterial sections from mice that were either untreated or treated with anti-HLA mAb, as in A. The intimal hyperplasia staining of grafted human mesenteric arterial sections from mice that were either untreated or treated with anti-HLA mAb.
MMP2, MMP9, and MT1-MMP. This agent fits well with the specificity required for inhibiting the MMP/sphingolipid pathway and could be less toxic because of its relatively narrow specificity. Moreover, it is of interest to inhibit both MMP2 and MMP9, which share various biological properties and could play a role in transplant vasculopathy. Finally, this compound was easily administered to mice and was nontoxic over the treatment period.

Another important result is the demonstration that nSMase2 is implicated in SMC proliferation elicited by anti-HLA mAb. In agreement with our previous reports, nSMase2 activation requires MMP2 and MT1-MMP, as assessed by the inhibitory effect of MMP inhibitors, siRNA directed to MMP2, or overexpression of an inactive form of MT1-MMP (which acts in our system as a dominant-negative form). The precise molecular mechanisms by which MT1-MMP and MMP2 mediate the activation of nSMase2 on stimulation by anti-HLA mAb and subsequently the mitogenic signaling involved in transplant hyperplasia are not known. One hypothesis is that MMP2 and MT1-MMP form a complex at the plasma membrane, as suggested by Strongin, which in turn may activate nSMase2 and sphingosine kinase-1, which finally generates sphingosine-1 phosphate (S1P).

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Interestingly, GW4869 significantly reduced the progression of transplant vasculopathy induced by W6/32 in SCID/beige mice grafted with human mesenteric arteries, confirming the role of nSMase2 and of the sphingolipid pathway in the pathophysiology of transplant vasculopathy. Our data demonstrate that the sphingolipid pathway is involved in the vascular response triggered by W6/32 HLA mAb in human arterial segments grafted in immunodeficient SCID/beige mice. Although there are thus far no known links directly connecting nSMase2 and sphingosine kinase-1, it is likely that S1P is involved in W6/32-induced intimal hyperplasia because S1P is able to mediate SMC migration and proliferation. Moreover, the role of S1P in transplant vasculopathy has been reported on the basis of the inhibitory effect on transplant rejection of FTY720, a structural analog of S1P that targets the S1P receptor S1P1 and acts as an immunosuppressor. The role of this pathway in our model is currently under investigation.

A number of articles have highlighted the role of inflammatory cytokines such as TNF-α and interferon-γ in transplant vasculopathy. This has been investigated by Tel- lides and in SCID/beige mice grafted with human artery segments and “humanized” by injection of peripheral blood mononuclear cells allogeneic to the artery donor. Because interferon-γ is mainly produced by CD4 positive T-helper lymphocytes and a role of interferon-γ is a priori unlikely in our model, which only explores the humoral response. However, anti-HLA mAb may induce the expression and secretion of various inflammatory factors and cytokines including TNF-α, which activates nSMase2 and formation of sphingolipid mediators. Interestingly, TNF-α induces SMC proliferation dependent on the MMP/nSMase2 signaling, which is inhibited by MMP inhibitors. In the local inflammatory context of transplant vasculopathy, inflammatory cytokines might contribute to the mitogenic signaling mediated by anti-HLA mAb via the MMP/nSMase2 pathway, and their mitogenic effect could be inhibited by MMP and/or nSMase2 inhibitors.

In conclusion, we show that W6/32 anti-HLA mAb triggers SMC proliferation and intimal hyperplasia, independently of any cellular immune response. This is in agreement with clinical observations suggesting that B cells and donor-specific antibodies can induce intimal hyperplasia and transplant vasculopathy in animal models and compromise long-term allograft survival. Our data emphasize the critical role of the stress-induced MMP/sphingolipid pathway in the mitogenic signaling and the efficacy of MMP and nSMase2 inhibitors (Ro28-2653 and GW4869). In addition to classic immunosuppression, these agents could constitute a supplementary treatment for preventing transplant vasculopathy by reducing the response of vascular cells to alloantibody-induced stress.

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Disclosures

Dr Krell was an employee of Roche Diagnostics GmbH, Penzberg, Germany. The other authors report no conflicts.

References

CLINICAL PERSPECTIVE

Despite major advances in clinical organ transplantation, the late outcome of transplanted patients is impaired by chronic rejection, which mainly affects the blood vessels of the transplanted organ, so-called transplant vasculopathy. This pathology is characterized by a progressive neointimal hyperplasia with proliferating smooth muscle cells and secretion of extracellular matrix, leading to arterial stenosis and ischemic failure of the allograft. The mechanism of transplant vasculopathy is only partly understood and involves cellular and humoral immune responses that target the transplanted organ. We wanted to elucidate the manner in which the humoral immune response contributes to transplant vasculopathy. In vitro studies indicated that anti-HLA class I antibodies are mitogenic for human smooth muscle cells through a mechanism that involves matrix metalloproteinases and neutral sphingomyelinase-2. Pharmacological inhibitors of metalloproteinases (batimastat and Ro28-2653) and of neutral sphingomyelinase-2 (GW4869) blocked signaling through these pathways. To explore these findings in vivo, we developed a model for human transplant vasculopathy, consisting of human mesenteric arterial segments grafted into severe combined immunodeficiency/beige mice. Mice injected weekly with monoclonal antibody toward HLA class I developed neointimal thickening of the grafted human arterial segment, whereas mice injected with an irrelevant antibody accepted the graft permanently. Treatment of the animals with Ro28-2653 and GW4869 led to a net reduction of the intimal hyperplasia induced by monoclonal antibody in the human mesenteric artery grafts. These data highlight a crucial role for metalloproteinases and neutral sphingomyelinase-2 in vasculopathy generated by a humoral immune response, opening new therapeutic perspectives for preventing this pathology, in addition to treatment with conventional immunosuppression.
A Key Role for Matrix Metalloproteinases and Neutral Sphingomyelinase-2 in Transplant Vasculopathy Triggered by Anti-HLA Antibody

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SUPPLEMENTAL MATERIAL

Expanded Methods

Reagents

$[^{3}H]$Thymidine (5 Ci/mmol) and [choline-methyl-$^{14}$C]sphingomyelin were from Perkin Elmer (Wellesley, MA). Anti-HLA (clone W6/32), and irrelevant mAb (clone B/22, IgG2a isotype) were from Diaclone (Besançon, France), anti-H2-D$^{b}$ mAb (clone 28-14-8) was from Fitzgerald (Interchim France), PCNA antibody from Dako (Trappes, France). Goat anti-MMP-2 was from Santa Cruz Biotechnologies (Santa Cruz, CA), anti-phospho and anti-total ERK1/2 antibodies were from Cell Signaling (Ozyme, St Quentin en Yvelines), mouse anti-smooth muscle $\alpha$-actin antibody (Sigma, France), the EnVision kit for immunofluorescence studies was from Dako (Trappes, France) and AlexaFluor®-488 (green) and -546 (red)-conjugated antibodies were from Invitrogen (Cergy Pontoise, France). Fluorogenic substrates for MT1-MMP (DANSYL-Pro-Leu-Ala-Cys($p$-OMeBz)-Trp-Ala-Arg-NH$_2$) and MMP-2 (MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$) were from Calbiochem (Darmstadt, Germany). SYTO-13 and propidium iodide (PI) were from Molecular Probes/Invitrogen (Cergy Pontoise, France). Sphingomyelin and GW4869 were from Sigma, Batimastat and Ro 28-2653 were from Roche Diagnostics (Dr Krell, Penzberg, Germany).

Cell Culture.

Human aortic SMCs (CRL-1999, haSMC) and murine aortic SMC (CRL-2797) were obtained from ATCC (Manassas, USA). Under standard conditions, cells were seeded at 30,000 cells/well in 12 multiwell plates and grown in DMEM containing glutamax® and supplemented with 10% fetal calf serum (FCS-Invitrogen, France), penicillin (100 units/ml), and streptomycin (100 μg/ml), at 37°C in a humidified 5% CO2 atmosphere, for 24 h. Cells were synchronized by 24 h starvation in serum-free DMEM, before stimulation by anti-HLA antibody (W6/32 mAb) (1 µg/ml), anti-mouse MHC antibody (monoclonal anti-H2-Db) (1 μg/ml) or irrelevant antibody (monoclonal IgG2a, same isotype as W6/32) (1 μg/ml).

Human mesenteric SMC primocultures (phmSMC) were prepared from mesenteric artery using the following explant technique. Human mesenteric arteries were recovered from the organ donor, dissected and kept in preservation solution (Celsior solution) under the previously described conditions 1,2. Arteries were cut into 1 mm$^2$ squares, placed luminal surface down in 60 mm culture dishes containing 1 ml DMEM + 10% FCS culture medium, and incubated to yield SMC culture, according to 3. Confluent phmSMC monolayers were harvested from explants by 0.1% trypsin/EDTA treatment, and immediately used for experiments up to 5-6 passages, to preserve their phenotype. The SMC phenotype was characterized in CRL-1999 and in phmSMC primocultures, using a monoclonal mouse antibody against human smooth muscle $\alpha$-actin.
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Evaluation of live and dead cells.
Cells (200/well) were counted after double staining by two fluorescent DNA intercalating agents SYTO-13/PI (0.6 µmol/l SYTO-13, permeant green-colored intercalating probe, and 15 µmol/l propidium iodide, PI, a non permeant red intercalating probe), using an inverted fluorescence microscope (Fluovert FU, Leitz). Living, apoptotic and necrotic cells were characterized on the basis of their morphological features of the nucleus. Normal living cells exhibit green loose chromatin. Apoptotic nuclei display green/yellow condensed (pyknotic) chromatin and/or fragmented nuclei (the green color indicates that the plasma membrane or the membrane of apoptotic bodies is not permeabilized), while post-apoptotic necrotic cells exhibited the same morphological features (condensed fragmented nuclei), but were red-colored due to the permeabilization of plasma membrane surrounding apoptotic bodies and PI penetration into the cell, indicating a necrotic process). Cells undergoing primary necrosis exhibit red/orange nuclei with loose chromatin.

DNA synthesis and cell counting
DNA synthesis was evaluated by[^3]H]thymidine incorporation, under previously described conditions, modified as follows: After 48h culture, cells were labeled for 4 h with[^3]H]thymidine (0.5 μCi/ml[^3]H]thymidine and 10 µmol/l of unlabeled thymidine). Then the cells were harvested and were washed three times with PBS. After addition of 5 % trichloroacetic acid and sodium pyrophosphate (10 mmol/l), the acid-precipitable material was dissolved for 2 h in 0.25 N NaOH and the acid-precipitable radioactivity was evaluated by liquid scintillation counting.

Cell counting was done after 48h incubation with the indicated concentration of W6/32 mAb. The culture medium was discarded, SMCs were washed twice with PBS and harvested in 0.5 ml trypsin 0.1%. The cell suspension was immediately diluted in 10 ml Isoton and counted using a Z1 Beckman Cell counter, as reported.

siRNA targeting and cell transfection
MMP2 (SMARTPool MMP2, cat number L-005959), and scrambled siRNA (PRKAA2) were purchased from Dharmacon (Lafayette, CO), siRNAs targeting human nSMase-2 (sequence AAt-gctactggctggtggacc) were from Eurogentec (Belgium). SMC were transfected with 20 nmol/l siRNAs in DMEM mixed with 2,5 mol/l CaCl2. After transfection, cells were incubated for 48h in FCS-containing DMEM, and in serum-free DMEM for 12 h, before experiments. Mutant catalytically inactive MT1-MMP SMC (iMT1/SMC) were obtained by transfection with a pcDNA3.1 vector containing a resistance gene to zeocin and the cDNA of MT1-MMP with substitution E240A in the active site (the mutant vector MT-E240A was generously given by Pr. A. Strongin, La Jolla, US).

Determination of MMP and nSMase-2 activities.
MMP activity was determined in the media of cultured SMC, of human arterial segments and in the plasma from transplanted mice using the fluorogenic substrate for MMP-2 (MCA-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2). After 2 hours of incubation (37°C), the fluorescence was read (excitation and emission wavelengths 325 nm and 393 nm, respectively). MT1-MMP activity was determined on cultured SMC using the fluorogenic substrate for MT1-MMP (dansyl-Pro-Leu-Ala-Cys(p-OMeBz)-Trp-Ala-Arg-NH2) (excitation and emission wavelengths, 280 nm and 340 nm, respectively). The activity of nSMase-2 was determined in SMC extracts. The assay mixture contained [choline-methyl-14C]sphingomyelin (120 000 dpm/assay) and unlabelled sphingomyelin 20
mmol/l, in 20 mM HEPES buffer pH 7.4, containing 0.1% Triton X-100, 1 mM MgCl\(_2\) and 50 µg protein from cell homogenate in a final volume of 100 µL. After 2 h incubation at 37°C, the liberated [methyl-\(^{14}\)C] choline was quantified by liquid scintillation counting \(^5\).

**Western blot experiments**

Western blots were performed under the previously used conditions \(^1,4\). Briefly, human artery rings were homogenized using a Precellys® system (Bertin Technology, MI) in solubilizing buffer (50 mmol/l Tris pH 7.4, 250 mmol/l NaCl, 5 mmol/l EDTA, 1 mmol/l sodium vanadate, 10 mmol/l sodium pyrophosphate, 160 mmol/l sodium fluoride, 2.5 mmol/l phenylmethylsulfonyl fluoride, 10 µmol/l leupeptin, 2 µmol/l pepstatin A, 10 mg/l aprotinin, 1% triton X-100) at 4°C. After centrifugation (15,000g, 10 min), supernatants were used for western-blot experiments \(^1,4\). Protein concentration was determined using the Bradford reagent (Biorad).

**Immunofluorescence**

Cells grown on glass cover slides, were washed with PBS and fixed in cold methanol for 10 min, then incubated with the respective primary and AlexaFluor®-conjugated secondary antibodies, and visualized using a widefield epifluorescence Diaplan Leitz, or a Zeiss LSM 510 fluorescence confocal microscope.

**Animals**

C.B-17 SCID/beige mice were from the Bomholtgaard Breeding and Research center (Ry, Denmark). Twelve week old males were used for the transplant experiments, and were kept in micro-isolator cages with sterilized water and mouse chow *ad libitum*. Plasmatic immunoglobulin level was measured by ELISA as reported \(^1,2\). Mice with a residual immunoglobulin level lower than 0.5 µg/ml were used for the experiments. All experimental protocols were approved by the INSERM Institutional Committee for animal experiments.

**Mesenteric artery harvesting.**

All procedures concerning the use of human tissues were approved by the French “Agence de Biomédecine” and the Ethics Committee of the University Hospital of Toulouse (France). According to the French laws on Bioethics, the registry for refusal of organ donation was consulted and informed consent was obtained from relatives for the use of human tissue for medical research. After organ removal, a piece of mesentery was recovered from the donor, under the previously described conditions \(^1,2\). One cm-long segments of terminal collaterals of the superior mesenteric artery of a suitable diameter (0.8-1 mm) were dissected and kept in preservation solution (Celsior solution) at 4°C until use (up to 24h).

**Ex vivo experiments on human mesenteric arterial rings**

For *ex vivo* experiments, human mesenteric arterial rings were prepared from dissected mesenteric arteries and immediately incubated in serum-free DMEM. The rings were stimulated for 24 h with W6/32 or irrelevant mAbs (1 µg/ml), with or without Ro28-2653, as indicated.

**Transplantation operative methods.**

Mesenteric artery transplantation was performed as previously reported \(^1,2\). Briefly, after intraperitoneal injection of Ketamin/Xylazin/Atropin solution, completed with gaseous anesthesia (Isofluran) a mid-line laparotomy approach was used. The abdominal aorta was clamped between, proximally, the renal arteries, and distally, the iliac bifurcation. A human mesenteric arterial segment (between 0.8-1 cm-long and similar in diameter to the mouse aorta, i.e. approximately 0.8 mm of internal diameter) was then inserted. After restoring
vascular blood flow, hemostasis was verified and the peritoneal cavity flooded with warm, sterile solution. After wound closure, animals were kept under a heating lamp until complete recovery. Back leg activity and mobility were a reliable indicator for early graft success. In cases of paralysis, the mice were excluded from the protocol.

**Cell stimulation by W6/32 and irrelevant mAb.**

Cells were stimulated with 1 µg/ml of W6/32 anti-HLA mAb. The same concentration was used for *ex vivo* stimulation of mesenteric artery sections. For *in vivo* stimulation, the mice received weekly intravenous injection of anti-HLA mAb (1 µg/10 g body weight), or irrelevant mAb of same isotype (same mAb concentration) for 5 weeks (beginning one week after transplantation).

**Immunohistochemistry and histomorphometric studies.**

Histological analysis was performed on human arterial rings used for *ex vivo* experiments and on human arterial segments grafted into SCID/beige mice and recovered at the end of the experiments (6 weeks after transplantation). After fixation in formaldehyde (4% in PBS), arterial segments were paraffin embedded for histological analysis. Sections were stained by hematoxylin/eosin or labeled with the indicated primary antibodies and revealed by a peroxidase-conjugated secondary antibody (immunoperoxidase kit, StrepABComplex/HRP Duet, Dako). Controls were done by omitting the primary antibody to determine nonspecific binding. Nuclei were counterstained with hematoxylin.

Morphometric analysis of human arterial segments was performed on sequential sections (40 sections, at around 200 µm intervals). Arterial sections were stained as indicated and analyzed morphometrically. Intima hyperplasia of the grafted arterial segment was quantified by the neointimal index, calculated according to the method of Armstrong et al., that indicates the degree of stenosis in a blood vessel due to intimal thickening. The area of intima (between endothelial layer and internal elastic lamina) and lumen were circumscribed manually and evaluated using the CyberView v3 program (Cervus International, France), as previously used. The average neointimal index of the grafted arterial segment was presented as a reconstructed virtual longitudinal section using a previously described home-made program.

**Statistical analyses.**

Data are presented as mean ± SD. Estimates of statistical significance were performed by *t*-test or analysis of variance followed by multiple comparison procedure (using the SigmaStat 3.5, Systat software). Tests for normality and equal variance were performed by the Kolmogorov-Smirnov test and by the Levene median test, respectively. When the populations were normally distributed and exhibited equal variances, differences between means values were evaluated by unpaired *t* test, to compare two groups, or by one-way ANOVA (experiments were designed to study the effect of one factor) to compare several experimental groups with different treatments. When a difference in the groups was detected by one-way ANOVA, multiple comparisons (generally versus a control group) were performed by the Holm-Sidak test, as indicated in the legend to the Figures. Values of *p* < 0.05 were considered significant.
Supplemental Results

**Human smooth muscle cells**

Human mesenteric SMC (phmSMC) primocultures and human aortic SMC CRL-1999 cell line (haSMC) express smooth muscle α-actin (supplemental Fig.I) and HLA class I antigens recognized by W6/32 mAb (supplemental Fig.II).

**HLA class I antigens recognized by W6/32 mAb are expressed in mesenteric artery.**

HLA class I antigens recognized by W6/32 mAb are expressed in human mesenteric arteries used for transplantation in SCID/beige mice (supplemental Fig.III). Both the endothelial cell lining and the media were labeled by W6/32 (revealed by the secondary AlexaFluor®-488 green antibody) (supplemental Fig.IIIA). It may be noted that, as expected, endothelial cells were more brightly stained than the media. The auto-fluorescence (independent of W6/32 staining) of internal elastic lamina and other structures appeared yellow-colored in wide field fluorescence microscopy (supplemental Fig.III).

**Dose-response experiments of W6/32 mAb in human SMC**

The optimal mitogenic concentration of W6/32 mAb on phmSMC (primocultures) and haSMC (CRL-1999), was determined by dose-response experiments. As shown on supplemental Fig.IV-A,B, W6/32 mAb was mitogenic at 0.1 µg/ml and induced a near maximal DNA synthesis and cell proliferation at 1 and 10 µg/ml on both cell types. The standard concentration of W6/32 mAb (1 µg/ml) was used throughout the manuscript. At this concentration (1 µg/ml), W6/32 mAb induced neither apoptosis nor necrosis, as shown by double fluorescent labeling by SYTO13/PI intercalating probes (supplemental Fig.IV-C).

**Dose-response experiments and toxicity of pharmacological inhibitors**

To evaluate the efficiency of pharmacological inhibitors of MMPs (Batimastat and Ro28-2653) and of nSMase-2 (GW4869) in blocking DNA synthesis elicited by W6/32 (1 µg/ml), we performed dose-response experiments (supplemental Fig.V). This permitted to select the effective concentration of MMP inhibitors utilized under standard conditions, i.e. 10 nmol/l for Ro28-2653, 100 nmol/l for Batimastat, and 5 µmol/l for GW4869 (red symbols in supplemental Fig.V and VIA). It is to note that Batimastat and Ro28-2653 are not toxic up to 1 µmol/l, and GW4869 up to 20 µmol/l (supplemental Fig.VIA) and that the standard concentration of the inhibitors (red symbols) efficient to inhibit DNA synthesis elicited by W6/32 mAb were not toxic (nor apoptotic) for haSMC, as evidenced by MTT (data not shown) and SYTO13/PI assays (supplemental Fig.VIB,C).

**W6/32 anti-HLA mAb triggers MMP2 expression and induces a MMP-mediated PCNA expression in human arterial rings ex vivo. Inhibition by Ro28-2653.**

In human mesenteric arterial rings incubated for 24 h ex vivo in the presence of W6/32 anti-HLA mAb (1 µg/ml), we observed an increased expression of MMP2 (supplemental Fig.VII) associated with an increased PCNA expression (supplemental Fig.VIII). In contrast, the irrelevant mAb had no effect (data not shown). PCNA expression was blocked by the MMP inhibitor Ro28-2653 (supplemental Fig.VIII). This suggests that W6/32 anti-HLA mAb triggers a MMP2-mediated mitogenic signaling of SMC of human arterial rings, like in cell culture, and that this effect is inhibited by the MMP inhibitor Ro28-2653.
Supplemental Figure I. Expression of α-actin in human SMCs.
The expression of α-actin in human SMC primoculture from mesenteric arteries (phmSMC), and in CRL-1999 (haSMC) was detected by immunofluorescence using a primary mouse antibody towards human smooth muscle α-actin (1/100) and a goat anti-mouse IgG AlexaFluor®-546-conjugated secondary antibody (Sigma, France) (1/1000), revealed by wide-field and confocal fluorescence microscopy, in upper and lower panels, respectively.
Supplemental Figure II. HLA-class I antigens expression on cultured human SMC.
Immunofluorescence detection of HLA class I antigen reacting with the W6/32 mAb on phmSMC primoculture (left panels) and haSMC CRL-1999 (right panels). SMC were fixed and incubated with either the W6/32 mAb or with an irrelevant (clone B/Z2, IgG2a isotype) mAb (1/50, 60 minutes) and with a goat anti-mouse IgG AlexaFluor®-488 conjugated secondary antibody (1/500) and imaged by wide-field epifluorescence and confocal fluorescence microscopy.
Supplemental Figure III

Expression of HLA-class I antigens in human mesenteric artery sections.

Immunofluorescence of HLA class I antigen reacting with the W6/32 mAb in human mesenteric artery sections. Arterial rings were fixed, embedded in Tissue-Tek, snap-frozen in liquid N2, and incubated with either W6/32 mAb or irrelevant mAb (1/50 60 min) and AlexaFluor-488 conjugated secondary antibody (1/500). The expression of the HLA-class I antigens on endothelial cell lining is indicated by the white arrowheads, and on cells of the media by arrows. These pictures are representative of 4 separate experiments.
Supplemental Figure IV

A, B - The dose-response of W6/32 mAb on DNA synthesis was evaluated in phmSMC primocultures and in haSMC CRL-1999 by determining the [3H]thymidine incorporation (acid-precipitable) and by cell counting, as described in “Expanded Methods” section. [3H]thymidine incorporation ranged between 5890 and 7860 dpm/well in control untreated phmSMC, and between 11470 and 16 450 dpm/well in untreated haSMC CRL-1999 (in the set of experiments reported here). In A and B, the data are mean ± SD of 3 to 5 separate experiments. Each assay with inhibitor was compared to the negative (untreated) control by the t-test. * p < 0.05.

C - Viability of CRL-1999 haSMC grown under standard culture conditions in the presence or absence (none) of W6/32 mAb (1 µg/ml). Fluorescence microscopy of cells treated under standard conditions and stained with SYTO13/PI under conditions of the Live/Dead assay. W6/32 mAb induced no toxic effect under the used standard conditions (1 µg/ml).
Supplemental Figure V. Inhibitory effect of MMP and nSMase inhibitors on SMC proliferation induced by W6/32 mAb.

Dose-effect of Batimastat (squares), Ro28-2653 (triangles) and GW4869 (circles) on the mitogenic response induced by W6/32 mAb (1 µg/ml) in CRL-1999 (haSMC). DNA synthesis was evaluated by [3H]thymidine incorporation, as described. The standard concentration of MMPs inhibitors (100 nmol/l for Batimastat and 10 nmol/L for Ro28-2653) and nSMase inhibitor (5 µmol/l for GW4869) selected for the experiments is indicated by red symbols. The data are mean ± SD of 3 to 5 separate experiments. Each concentration was compared to the untreated (negative) control (i.e. 100 %) by one-way ANOVA and multiple comparison vs a control group (Holm-Sidak method). * p < 0.05.
Supplemental Figure VI

A - Toxic effect (colored symbols; right scale) of increasing concentrations of MMP inhibitors (Batimastat, green squares, and Ro28-2653, blue triangles) and nSMase inhibitor GW4869 (purple circles) on haSMC CRL-1999 grown under mitogenic conditions (i.e. in the presence of 1 µg/ml of W6/32 mAb). In order to compare the two effects of inhibitors, i.e. the inhibition of DNA biosynthesis (suppl. Fig.V - empty symbols, left scale) and the toxic effect (colored symbols, right scale), the data were reported on the same Figure. The concentrations of inhibitors used in the experiments (empty red symbols) were selected relatively far from the toxic concentration.

B, C - Lack of toxicity of inhibitors used under standard experimental conditions in the presence or absence of W6/32 anti-HLA mAb. The toxicity was evaluated by the Live/Dead SYTO13/PI assay. In A, B, the data are expressed as mean ± SD of 3 to 5 separate experiments. The toxic effect of each concentration (colored symbols) was compared to the untreated (negative) control by one-way ANOVA and multiple comparison vs a control group (Holm-Sidak method). * p < 0.05.
Supplemental Figure VII. Increased MMP2 expression elicited by W6/32 in human mesenteric arterial rings ex vivo.

A - MMP2 expression was analyzed in arterial sections incubated ex vivo for 24 h, under the conditions described in the Methods section, without antibody (PBS vehicle only) or with W6/32 mAb (1 µg/ml) and labeled with a goat anti-MMP2 primary antibody and with a anti-goat IgG secondary antibody conjugated to streptavidin-HRP. Counterstaining by hematoxylin. Arrows show some MMP2-positive cells.

B - Count of MMP2-positive cells (percentage of MMP2-positive cells/total cells), expressed as mean ± SD of 3 separate experiments. Comparison by t test. * p < 0.05.
Supplemental Figure VIII. PCNA expression (mitogenic signaling) induced by W6/32 mAb in human mesenteric arterial rings ex vivo. Inhibition by Ro28-2653.

A, B - PCNA expression in arterial rings treated ex vivo for 24h with the W6/32 mAb (1 µg/ml) and with or without Ro28-2653 (10 nmol/l), under the ex vivo conditions described in the Methods section. In A, after 24h treatment, arterial rings were fixed and sections were labeled with a mouse anti-PCNA primary antibody and with a goat anti-mouse IgG secondary antibody conjugated to streptavidin-HRP. Counterstaining by hematoxylin. Arrows show some PCNA-positive nuclei. In B, count of PCNA-positive cells (percentage of PCNA-positive nuclei/total cell nuclei), expressed as mean ± SD of 3 separate experiments. One-way ANOVA, and comparison to the positive (HLA-treated) control by Holm-Sidak method. * p < 0.05
Supplemental References


