

Title page

Mechanisms of human smooth muscle cell proliferation and transplant vasculopathy induced by HLA class I antibodies: *in vitro* and *in vivo* studies

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Short title:

Antibody-induced transplant vasculopathy

Abstract

Vascular smooth muscle cells (SMC) play an important role in the pathophysiology of transplant vasculopathy (TV), a major cause of late death in patients receiving an organ transplant. In this review we describe the proliferative effect *in vitro* and *in vivo* of HLA class I antibodies on human SMC. We have developed an experimental model using segments of human mesenteric arteries transplanted in the position of the infrarenal aorta in immunodeficient mice (SCID/beige). Weekly injections of transplanted mice with a monoclonal antibody towards HLA class I provoked typical lesions of TV after 6 weeks in the human graft while transplanted mice receiving an irrelevant antibody did not develop any significant lesion. *In vitro*, the anti-HLA antibodies were mitogenic to SMC and we showed that they activate a stress-induced signaling pathway implicating matrix metalloproteinases (MMP) and neutral sphingomyelinase 2 (nSMase-2). The proliferative effect of anti-HLA antibodies could be blocked by pharmacological inhibitors or by siRNA. Administration of pharmacological inhibitors diminished the development of TV in grafted mice injected with anti-HLA antibodies demonstrating an important role of the MMP/nSMase-2 pathway in antibody-induced TV. This observation opens new perspectives for the management of TV in clinical settings.

Keywords

Antibodies
Vasculopathy
Signaling
Smooth muscle cells
Animal models

Abbreviations

TV: Transplant Vasculopathy
HLA: Human Leucocyte Antigen
SMC: Smooth Muscle Cells
EC: Endothelial Cells
MMP: Matrix MetalloProteinases
MMP-2: Matrix MetalloProteinase 2
FAK: Focal Adhesion Kinase
mTOR: mammalian Target Of Rapamycin
PI3K: Phosphatidyl Inositol 3 Kinase
ERK: Extracellular signal-Regulated protein Kinase
MHC: Major Histocompatibility Complex
MICA: Major histocompatibility complex class I-related Chain A
TNF- α : Tumor Necrosis Factor α
S1P: Sphingosine-1-Phosphate
nSMase-2: type 2 neutral SphingoMyelinase
MT1-MMP: Membrane Type 1 Matrix MetalloProteinase
MAPK: Mitogen-Activated Protein Kinase
oxLDL: oxidized Low-Density Lipoprotein
siRNA: small interfering RiboNucleic Acid
DNA: DesoxyriboNucleic Acid
SCID: Severe Combined ImmunoDeficiency
PCNA: Proliferating Cell Nuclear Antigen
 α -SMA: α Smooth Muscle Actin
SL: SphingoLipid

1. Introduction

Advances in immunosuppressive treatment, surgical techniques and patient care have greatly improved the early survival of patients receiving an organ allograft. In spite of this progress, too many patients die after some years, mainly due to chronic rejection. The rejection of organ allografts is mediated by many different components of the adaptive and innate immune system. Acute rejection in patients that are not sensitized towards donor alloantigens typically occurs 1-2 weeks after organ transplantation. It is characterized by graft cell necrosis and/or graft vessel thrombosis caused by the immune system of the host. Rejection episodes may be prevented or reversed by treatment with immunosuppressive agents [1]. Chronic rejection is a more insidious process that develops months to years after the graft in spite of immunosuppressive treatment and it is more likely to occur if the patient has suffered from acute rejection episodes. One of the principal elements of chronic rejection is transplant vasculopathy (TV)[1]. Experimental models in rats have been developed more than 20 years ago in order to describe the sequential events of TV, mainly using an aorta allograft interposition model [2, 3]. Later on, progress in microsurgery has allowed the carrying out of aortic allografts in mice [4, 5]. In these rodent models, chronic lesions develop 1-2 months after transplantation that are very similar to TV observed in humans. The use of mutant mice strains with immunologic defects has shown the importance of CD4+ helper cells, humoral antibodies and macrophages for the development of TV [6]. In immunocompetent animal models of allografts, an infiltration of T cells and monocytes in grafted arteries is accompanied by the appearance of lymphoid follicles with B cells in the adventitia, suggesting a local production of alloantibodies [7]. Proliferation of smooth muscle cells (SMC) and modulation of extracellular matrix in the neointima are important events in the development of vasculopathy, because the lumen of the arteries of the graft narrows, the blood flow slows down and the organ becomes increasingly ischemic. Clinical studies underline a strong correlation between the presence of antibodies towards HLA class I and the development of chronic rejection [8]. Signaling induced by anti-HLA antibodies on EC has been studied by many groups, and the secretion of cytokines *in vivo* has probably a strong impact on the SMC in the grafted vessel. However, the direct action of antibodies on SMC is much less well known. We hypothesize that anti-HLA antibodies act as stress agents on SMC, inducing activation and proliferation of these cells. We focus on a signaling pathway involving matrix metalloproteinases (MMP) and neutral sphingomyelinase 2 (nSMase-2), already described by our team to be activated by other stress-inducing agents such as oxLDL and TNF- α , leading to SMC proliferation [9, 10].

In this review, the role of antibodies towards HLA class I in the development of TV will be discussed. In the following sections we give details on our experimental models *in vitro* and *in vivo*, the mechanisms of action of anti-HLA antibodies and the possible clinical implications of our work.

2. Alloantigens on vascular cells

Patients receiving an organ allograft are confronted with incompatible alloantigens on cells and tissues of the donor. The most immunogenic antigens are those of the major histocompatibility complex (MHC), called HLA in humans. While endothelial cells express HLA class II antigens constitutively [11], SMC only express HLA class II in certain pathological states [12]. HLA class I antigens may be divided into classical antigens (A, B and C) which are expressed by most nucleated cells in the body, and non-classical antigens [13]. The W6/32 monoclonal antibody recognizes a monomorphic epitope shared among all

native HLA class I molecules [14, 15]. The epitope probably includes residue 121 in the α 2 domain and residue 3 on beta-2 microglobulin as shown in Fig 1 [16]. The antibody binds to a discontinuous epitope that is situated under the antigen presenting groove. Both endothelial cells and SMC express classical HLA class I antigens and are thus potential targets of the W6/32 monoclonal antibody. The mechanism of action of anti-HLA class I antibodies has often been viewed as strictly complement-dependent. However, we and others have shown that these antibodies have direct effects on vascular cells *in vitro* [17-19] and *in vivo* [18, 20] in the absence of complement. This may be explained by their ability to crosslink HLA molecules at the surface of cells thereby recruiting other proteins as for instance integrin β 4 to transduce signals into the cells [21]. Fig. 2 shows the labeling of HLA class I molecules on human SMC *in vitro* and on sections of human arteries using the W6/32 antibody.

It has been reported that the presence of antibodies towards MICA (Major histocompatibility complex class I-related Chain A) antigens in patients could be correlated with graft rejection [22]. However, in contrast to the effect of anti-HLA class I antibodies, we found that anti-MICA antibodies neither provoked development of TV in our animal model, nor induced SMC proliferation *in vitro* [18]. Antibodies towards HLA class II antigens certainly also play a role in chronic rejection [8], but in this review we will only address the mechanisms implicating HLA class I antibodies.

3. Mechanisms implicated in migration and proliferation of vascular cells induced by anti-HLA antibodies *in vitro*

It is well documented *in vitro* that antibodies toward HLA class I are able to provoke proliferation in human endothelial cells (EC) [19]. They induce phosphorylation of protein kinases such as c-Src and Fyn of the Src family and of proteins associated with the cytoskeleton such as FAK and paxillin. In addition, it was shown that the PI3K/Akt pathway was activated by Src and FAK, leading to increased expression of pro-survival proteins Bcl-2 and Bcl-xL [23] and of antioxidant defense such as HO-1 [24] in EC stimulated by anti HLA antibodies. Furthermore, they induce ERK phosphorylation by an mTOR-dependent mechanism [25, 26]. mTOR also appears to be involved in the phosphorylation of Akt and in the proliferative effect of anti-HLA antibodies on EC, but its activation is dependent on Src and FAK [25, 26]. Upstream of this signaling, the interaction between HLA class I and integrin β 4 is essential for the phosphorylation of these proteins and the proliferation of EC in response to anti-HLA antibodies [21]. Recently, Reed's team has shown that anti-HLA antibodies induce stress fiber formation in EC [27]. Besides, anti-HLA antibodies induce increased expression of VEGF and endothelial permeability [28]. EC stimulated by anti-HLA antibodies secrete the proinflammatory cytokines IL-1 β , IL-6, IL-8 and TNF α [29].

In vivo, anti-HLA antibodies might indirectly trigger SMC proliferation via the production of growth factors and inflammatory cytokines by EC [23, 29]. *In vitro* studies show that anti-HLA antibodies directly activate signaling pathways involved in the migration and proliferation of SMC [17, 18, 30, 31]. HLA class I antibodies increase the expression of the fibroblast growth factor receptor of SMC and a subsequent tyrosine phosphorylation of proteins involved in SMC proliferation [32]. These events are potentiated by a pre-treatment of cells with TNF- α and interferon γ [17]. As reported in EC the signaling cascade implicating FAK, Akt and ERK is involved in the proliferative and migratory properties of anti-HLA antibodies in SMC [30]. Results from our team indicate that the matrix metalloproteinases/type 2 nSMase (MMP/nSMase-2) signaling pathway plays an important role in SMC proliferation elicited by pro-atherogenic agents and we will therefore shortly describe some basic elements of the biomolecules involved in this pathway.

3.1.Sphingolipids

Sphingolipids (SL) are characterized by the presence of a long-chain sphingoid backbone. Most of them are located in membrane bilayers, especially in the plasma membrane. They are present in microdomains involved in signal transduction such as rafts or caveolae [33]. Ceramide can be formed through hydrolysis of sphingomyelin by sphingomyelinases [34](see Fig.2). This molecule can be degraded into sphingosine by ceramidases, while sphingosine phosphorylation by sphingosine kinases generates sphingosine 1-phosphate (S1P), another important bioactive SL. S1P can be exported to the extracellular compartment where it may bind members of the S1P receptor family which are G protein-coupled receptors located at the plasma membrane [35]. Bioactive SL such as ceramide and S1P play a key role in physiological and pathological cellular responses. Sphingolipid mediators are often generated in response to stress-inducing agents and are implicated in various cellular responses that may lead to cell death or survival, differentiation, proliferation, inflammation, autophagy or angiogenesis. Ceramide and S1P exhibit sometimes opposite effects: for instance, ceramide is anti-proliferative and proapoptotic [36], while S1P promotes cell proliferation and counteracts apoptotic stimuli. S1P is also involved in cell migration and angiogenesis [37, 38]. The balance between ceramide and S1P levels (the so-called ceramide/S1P rheostat) conditions the cell's fate towards apoptosis or survival and proliferation [39]. This rheostat constitutes a potential therapeutic target in diseases implicating sphingolipid mediators (e.g. metabolic diseases, cancer) [40, 41].

3.2. Enzymes involved in the MMP/nSMase-2 pathway

Several sphingomyelinases have been cloned and are characterized by their optimum pH, biochemical properties, and subcellular localization [42]. Neutral sphingomyelinases (nSMase) hydrolyze sphingomyelin at neutral pH (7.4) into ceramide. Type 2 nSMase (nSMase-2) is ubiquitously expressed, with a high expression in the brain. It is coded by the *smpd3* gene and its enzyme activity depends on the presence of Mg^{2+} [43]. nSMase-2 plays a key role in sphingomyelin hydrolysis for cell stress-induced ceramide generation, cell signaling and cell cycle regulation [44]. nSMase-2 is present in sphingomyelin-rich rafts and in caveolae, where local ceramide generation modulates the biophysical properties of the membrane and contributes to cell signaling [45-47]. nSMase-2 is normally present in the Golgi apparatus from where it translocates to the plasma membrane upon stimulation by stress-inducing agents [48, 49]. The translocation mechanism involves various signaling pathways, among them p38-MAPK [48] and protein kinase C δ [50]. However, the upstream mechanisms leading to nSMase-2 activation remain unclear so far. Reports from our group indicate that the activation of nSMase-2 by stress-inducing agents (oxLDL, TNF- α , oxidative stress) depends on the action of MT1-MMP and MMP-2 [9, 10, 51]. nSMase-2 is involved in SMC proliferation, but not in apoptosis elicited by stress-inducing agents [52].

3.3.Matrix metalloproteinases

Matrix metalloproteinases (MMP) are a family of Zn-dependent proteinases, which contribute to the renewal of the extracellular matrix [53]. MMP activity is regulated by cleavage of the pro-enzyme by serine-proteases or membrane-associated MMP such as MT1-MMP. MMP are inhibited by the tissue inhibitors of metalloproteinases, which are glycoproteins ubiquitously present in tissues [54]. MMP such as MMP-2 are involved in extracellular matrix degradation [53], but also in stress-induced proliferation [9, 10]. Inhibition of MT1-MMP and MMP-2 by pharmacological drugs (Batimastat, Ro28-2653), specific siRNA or by mutation, abrogates activation of nSMase-2 and subsequent cell proliferation in response to stress-inducing

agents. This suggests that these MMP are required for triggering nSMase-2 activation, ceramide generation and subsequent mitogenic cell signaling [9, 10].

3.4. The MMP/nSMase-2 pathway and anti-HLA antibodies *in vitro*

Our data suggest that anti-HLA antibodies behave as stress-inducing agents on SMC, activating the MMP/nSMase-2 pathway [31]. As reported for other stress-inducing agents (TNF- α , oxLDL, H₂O₂), anti-HLA antibodies (1 μ g/mL) rapidly activate nSMase-2 *via* an upstream activation of MT1-MMP and MMP-2. This pathway is mitogenic, as assessed by the inhibition of DNA synthesis and SMC proliferation by pharmacological inhibitors of MMP (Ro28-2653 at 10 nM) or nSMase (GW4869 at 5 μ M). Moreover, the mitogenic signaling induced by antibodies was inhibited in human SMC silenced for MMP-2 or for nSMase-2 by specific siRNA, and in SMC transfected with a vector coding for a dominant-negative form of MT1-MMP. It is important to notice that these effects of anti-HLA antibodies are independent of complement action (inactivation by heating). As described below, the role *in vivo* of the MMP/nSMase pathway for the development of TV was supported by the use of MMP or nSMase inhibitors that inhibited intimal hyperplasia in our experimental model.

4. Animal model to study TV induced by anti-HLA antibodies

We have developed an experimental animal model that allows elucidating the role of various components of the immune system implicated in TV of human arteries. Several immunodeficient mice strains exist that accept transplantation of normal or tumor tissue to the animal from other species [55]. We have chosen the SCID/beige mice that lack functional T and B cells due to mutation in a gene involved in recombination of T and B cell receptors [56], associated with a defect of the innate immunity resulting from the beige mutation [57].

4.1. Establishment of the transplantation model

Transplantation of human arteries into SCID/beige mice was first reported in 1999 by Lorber et al [58] who showed that a human artery grafted into the place of the infrarenal aorta was accepted permanently. Injection of peripheral blood lymphocytes from blood donors provoked typical lesions of TV in the arteries. The arteries were recovered from surgical waste, typically after coronary by-pass operations. However, the arteries were of variable quality and only a few suitable arteries could be obtained from each patient. We have improved the model by using human mesenteric arteries from deceased organ donors. These arteries are of excellent quality and of homogeneous size, and a great number of small arteries of a diameter compatible with the murine aorta can be dissected out (see Fig.4). We applied a rapid sleeve technique for the insertion of each artery in the place of the infrarenal aorta in SCID/beige mice [59] and up to 8 mice have been grafted with arteries from the same donor. In a first series of experiments, the mice were injected with human spleen cells from deceased organ donors, and we have shown that immunological reconstitution provokes a typical TV after 6 weeks when the cell donor is different from the arterial donor (allogeneic combination) and little or no TV if cells and artery come from the same donor. The immunological reconstitution concerned both the cellular and humoral response, but it was not possible in this model to determine which component was the most important for the development of lesions. In addition, there was a problem of xenoreactivity of the human lymphoid cells against the mouse tissue and graft versus host reactions were seen in liver, spleen and other organs [60].

4.2. Development of the model for studying humoral alloreactivity

After these preliminary experiments we then turned to studies of the role of humoral alloreactivity in transplant rejection. A minor problem was the presence of low levels of endogenous immunoglobulin in a number of mice, the so-called leaky phenotype. We systematically selected mice for experiments and for breeding that had a level of endogenous immunoglobulin below 0.5 µg/mL (corresponding to about 1/1000 of normal mouse serum levels). In this way, potential interference of mouse immunoglobulin with injected antibodies or transplanted tissue was minimized. As commented upon in the following section, weekly intravenous injections of the monoclonal anti-HLA antibody (W6/32) were able to provoke TV in the grafted artery after 6 weeks while no changes were noted in the mouse aorta adjacent to the transplanted segment [18]. Irrelevant antibody had no effect. It could be shown that the reaction took place without the action of complement *in vivo* by the treatment of the mice with cobra venom factor. The lesions were quantified on transversal sections made with 200 µm intervals and a virtual longitudinal section was constructed on the basis of the measures using a homemade developed software [59].

4.3. Implication *in vivo* of the MMP/nSMase pathway

We have shown that repeated intravenous injections of anti-HLA antibodies (1 µg/10 g body weight) in SCID/beige mice grafted with a human mesenteric artery segment induced the development of intimal hyperplasia specifically at the human graft (see Fig. 5). The vascular lesions were concentric, diffuse, and cellular with positive staining for α -smooth muscle actin (α -SMA), suggesting a role for SMC in the development of TV in this model. In addition, some SMC present in the lesions expressed PCNA, a nuclear marker of proliferation, indicating the proliferative effect of anti-HLA antibodies on human SMC *in vivo* in this model. To assess whether the MMP/nSMase pathway is involved in TV, we used pharmacological inhibitors of MMP and nSMase which were injected in the grafted mice all along with the treatment with anti-HLA antibodies. We used the MMP inhibitor Ro28-2653 (oral gavage twice a week at 1 µg/10g body weight) and the nSMase inhibitor GW4869 (intraperitoneal injection twice a week at 1 µg/1g body weight). Both inhibitors efficiently reduced the development of TV induced by anti-HLA antibodies after 6 weeks of treatment [31]. GW4869 protected the human graft from intimal hyperplasia at about 50 % and Ro28-2653 induced a decrease of more than 60 % of the vascular lesions induced by anti-HLA antibodies. These results suggest a major involvement of the MMP/nSMase pathway in the development of TV. As no apparent toxic side effects were noted in the mice, the results open new prospects in therapeutic strategies that might be used to limit antibody-mediated allograft rejection.

5. Discussion

In the preceding sections we have described the role of anti-HLA antibodies in SMC proliferation *in vitro* and *in vivo*, and the implication of the MMP/nSMase-2 signaling pathway (see Fig. 6). To our knowledge this is the first report showing a role for this pathway in TV. Reports from Reed's group indicate a role for FAK (and Akt and ERK)[30](see Fig.6). It is likely that links exist between FAK activation and the MMP/nSMase-2 pathway. The signaling response induced by anti-HLA antibodies is initiated very quickly, within minutes after the addition of antibodies in the culture medium of SMC. Data in the literature show that proliferative and migratory effects of oxLDL are accompanied by the activation of MMP-2 and the phosphorylation of FAK [61]. Integrin α_5 inhibition decreases the phosphorylation of FAK and the secretion of MMP-2[62]. MT1-MMP activation induces the cleavage of FAK and the activation of MMP-2 in human SMC [63]. Moreover, it has been shown in EC that

S1P can induce the phosphorylation of FAK [64]. Thus, FAK may play a role at several steps in the MMP/nSMase-2 pathway leading to the proliferation of SMC.

It might be argued that stimulation of human SMC by a mouse monoclonal antibody does not reflect the situation in patients where the alloantibodies are polyclonal and directed towards specific HLA epitopes. However, it has been shown that monoclonal antibodies of murine or human origin directed towards specific HLA class I antigens stimulated human SMC *in vitro* in the same way as the W6/32 antibody, and the stimulation was dose dependent [30]. In a model of aortic allografts into immunodeficient rats, the injection of rat alloantisera provoked typical TV lesions in the graft [65]. It is therefore likely that polyclonal antibodies towards HLA class I would behave similarly, *in vitro* and *in vivo*. Our model may thus be very relevant for testing of new therapeutic principles in the treatment of TV.

Like Reed's group, we have found that anti-HLA antibodies exert a migratory effect on human SMC that is decreased by the inhibitors of the MMP/nSMase-2 pathway. However, we do not know the precise mechanisms by which the pathway is activated by anti-HLA antibodies, or how MMP activate sphingolipid metabolism or even how the SL transduce the mitogenic signals of anti-HLA antibodies in human SMC. Our preliminary results suggest an important role of S1P in the proliferation and migration of SMCs in response to anti-HLA antibodies (Trayssac et al., in preparation). Inhibitors targeting the fixation of S1P on its receptors are now considered as a new class of immunosuppressive agents [66]. The preventive effect of FTY-720 [67] and KRP-203 [68] against TV and chronic rejection could thus be related to a direct effect on SMC in the graft in addition to their effect on cells of the immune system [69].

To which extent the endothelial cells and SMC in vascular allografts are replaced by cells of recipient origin remains a still open question [70]. We assume that in our xenograft model the proliferating cells are of human origin although it cannot be excluded that a part of them is of murine origin. To further explore the question about the origin of the vascular cells in an allograft model and the mechanisms of lesion development, we are currently developing an allograft model of TV, by grafting an aortic segment from C57BL/6 mice (H-2^b) into SCID/beige mice (H-2^d) followed by injections of antibodies towards MHC class I (H-2K^bD^b). This model should allow characterizing the involvement of MMP/nSMase-2 pathway in the development of TV induced by anti-MHC antibodies through the use of mice mutated for this pathway (deletion of genes coding for MMP-2 or nSMase-2).

Many classical immunosuppressive agents, such as cyclosporine or mycophenolic acid, exhibit anti-proliferative effects on vascular SMC that could be responsible for a part of their efficacy in delaying the progression of TV [71]. On the basis of these observations it could be proposed that new inhibitors of SMC proliferation (MMP and nSMase inhibitors) might be used in combination with conventional immunosuppressive drugs in order to limit the development of TV.

6. Conclusion

We and others have shown that vascular SMC in organ allografts are direct targets for HLA class I antibodies. The antibodies act as stress-inducing agents on SMC by activating a signaling pathway involving MMP and nSMase leading to SMC proliferation *in vitro* and *in vivo*. TV develops in human arteries grafted into immunodeficient SCID/beige mice that are injected with HLA class I antibodies. Pharmacological inhibitors of MMP and nSMase prevent SMC proliferation and TV induced by HLA class I antibodies. The pharmacological inhibitors did not seem to have side effects on the animals in doses that significantly reduced

TV. This opens up for new therapeutic strategies to improve long-term survival of solid allografts.

Acknowledgements

The authors thank Prof. Robert Salvayre for critical reading of the manuscript. We also thank Denis Calise for photographs of surgical procedures.

M. Trayssac was funded by a Ph.D. fellowship from Ministère de l'Enseignement Supérieur et de la Recherche. This work was also supported by INSERM (Institut National de la Santé Et de la Recherche Médicale), Agence de la Biomédecine and University Paul Sabatier Toulouse III.

References

- [1] Libby P, Pober JS. Chronic rejection. *Immunity*. 2001;14:387-97.
- [2] Hayry P, Mennander A, Tiisala S, Halttunen J, Yilmaz S, Paavonen T. Rat aortic allografts: an experimental model for chronic transplant arteriosclerosis. *Transplant Proc*. 1991;23:611-2.
- [3] Plissonnier D, Levy BI, Salzmann JL, Nochy D, Watelet J, Michel JB. Allograft-induced arterial wall injury and response in normotensive and spontaneously hypertensive rats. *Arteriosclerosis and thrombosis : a journal of vascular biology / American Heart Association*. 1991;11:1690-9.
- [4] Koulack J, McAlister VC, Giacomantonio CA, Bitter-Suermann H, MacDonald AS, Lee TD. Development of a mouse aortic transplant model of chronic rejection. *Microsurgery*. 1995;16:110-3.
- [5] Dambrin C, Calise D, Pieraggi MT, Thiers JC, Thomsen M. Orthotopic aortic transplantation in mice: a new model of allograft arteriosclerosis. *J Heart Lung Transplant*. 1999;18:946-51.
- [6] Shi C, Lee WS, He Q, Zhang D, Fletcher DL, Jr., Newell JB, et al. Immunologic basis of transplant-associated arteriosclerosis. *Proc Natl Acad Sci U S A*. 1996;93:4051-6.
- [7] Thauinat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF, et al. Lymphoid neogenesis in chronic rejection: evidence for a local humoral alloimmune response. *Proc Natl Acad Sci U S A*. 2005;102:14723-8.
- [8] Terasaki PI, Cai J. Human leukocyte antigen antibodies and chronic rejection: from association to causation. *Transplantation*. 2008;86:377-83.
- [9] Auge N, Maupas-Schwalm F, Elbaz M, Thiers JC, Waysbort A, Itohara S, et al. Role for matrix metalloproteinase-2 in oxidized low-density lipoprotein-induced activation of the sphingomyelin/ceramide pathway and smooth muscle cell proliferation. *Circulation*. 2004;110:571-8.
- [10] Tellier E, Negre-Salvayre A, Bocquet B, Itohara S, Hannun YA, Salvayre R, et al. Role for furin in tumor necrosis factor alpha-induced activation of the matrix metalloproteinase/sphingolipid mitogenic pathway. *Mol Cell Biol*. 2007;27:2997-3007.
- [11] Hirschberg H, Bergh OJ, Thorsby E. Antigen-presenting properties of human vascular endothelial cells. *J Exp Med*. 1980;152:249s-55s.
- [12] Sundstrom JB, Ansari AA. Comparative study of the role of professional versus semiprofessional or nonprofessional antigen presenting cells in the rejection of vascularized organ allografts. *Transpl Immunol*. 1995;3:273-89.
- [13] Le Bouteiller P. HLA class I chromosomal region, genes, and products: facts and questions. *Crit Rev Immunol*. 1994;14:89-129.
- [14] Paul P, Rouas-Freiss N, Moreau P, Cabestre FA, Menier C, Khalil-Daher I, et al. HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. *Hum Immunol*. 2000;61:1177-95.
- [15] Barnstable CJ, Bodmer WF, Brown G, Galfre G, Milstein C, Williams AF, et al. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens-new tools for genetic analysis. *Cell*. 1978;14:9-20.
- [16] Ladasky JJ, Shum BP, Canavez F, Seunanez HN, Parham P. Residue 3 of beta2-microglobulin affects binding of class I MHC molecules by the W6/32 antibody. *Immunogenetics*. 1999;49:312-20.
- [17] Bian H, Reed EF. Alloantibody-mediated class I signal transduction in endothelial cells and smooth muscle cells: enhancement by IFN-gamma and TNF-alpha. *J Immunol*. 1999;163:1010-8.

- [18] Galvani S, Auge N, Calise D, Thiers JC, Canivet C, Kamar N, et al. HLA class I antibodies provoke graft arteriosclerosis in human arteries transplanted into SCID/beige mice. *Am J Transplant*. 2009;9:2607-14.
- [19] Bian H, Reed EF. Anti-HLA antibodies transduce proliferative signals in endothelial cells and smooth muscle cells. *Transplant Proc*. 1999;31:1924.
- [20] Hirohashi T, Uehara S, Chase CM, DellaPelle P, Madsen JC, Russell PS, et al. Complement independent antibody-mediated endarteritis and transplant arteriopathy in mice. *Am J Transplant*. 2010;10:510-7.
- [21] Zhang X, Rozengurt E, Reed EF. HLA class I molecules partner with integrin beta4 to stimulate endothelial cell proliferation and migration. *Science signaling*. 2010;3:ra85.
- [22] Cox ST, Stephens HA, Fernando R, Karasu A, Harber M, Howie AJ, et al. Major histocompatibility complex class I-related chain A allele mismatching, antibodies, and rejection in renal transplantation. *Hum Immunol*. 2011;72:827-34.
- [23] Jin YP, Fishbein MC, Said JW, Jindra PT, Rajalingam R, Rozengurt E, et al. Anti-HLA class I antibody-mediated activation of the PI3K/Akt signaling pathway and induction of Bcl-2 and Bcl-xL expression in endothelial cells. *Hum Immunol*. 2004;65:291-302.
- [24] Iwasaki K, Miwa Y, Haneda M, Uchida K, Nakao A, Kobayashi T. Significance of HLA class I antibody-induced antioxidant gene expression for endothelial cell protection against complement attack. *Biochemical and biophysical research communications*. 2010;391:1210-5.
- [25] Jindra PT, Jin YP, Rozengurt E, Reed EF. HLA class I antibody-mediated endothelial cell proliferation via the mTOR pathway. *J Immunol*. 2008;180:2357-66.
- [26] Jindra PT, Jin YP, Jacamo R, Rozengurt E, Reed EF. MHC class I and integrin ligation induce ERK activation via an mTORC2-dependent pathway. *Biochemical and biophysical research communications*. 2008;369:781-7.
- [27] Ziegler ME, Souda P, Jin YP, Whitelegge JP, Reed EF. Characterization of the endothelial cell cytoskeleton following HLA class I ligation. *PLoS One*. 2012;7:e29472.
- [28] Bieri M, Oroszlan M, Farkas A, Ligeti N, Bieri J, Mohacsi P. Anti-HLA I antibodies induce VEGF production by endothelial cells, which increases proliferation and paracellular permeability. *The international journal of biochemistry & cell biology*. 2009;41:2422-30.
- [29] Reyes-Vargas E, Pavlov IY, Martins TB, Schwartz JJ, Hill HR, Delgado JC. Binding of anti-HLA class I antibody to endothelial cells produce an inflammatory cytokine secretory pattern. *J Clin Lab Anal*. 2009;23:157-60.
- [30] Li F, Zhang X, Jin YP, Mulder A, Reed EF. Antibody ligation of human leukocyte antigen class I molecules stimulates migration and proliferation of smooth muscle cells in a focal adhesion kinase-dependent manner. *Hum Immunol*. 2011;72:1150-9.
- [31] Galvani S, Trayssac M, Auge N, Thiers JC, Calise D, Krell HW, et al. A key role for matrix metalloproteinases and neutral sphingomyelinase-2 in transplant vasculopathy triggered by anti-HLA antibody. *Circulation*. 2011;124:2725-34.
- [32] Bian H, Harris PE, Reed EF. Ligation of HLA class I molecules on smooth muscle cells with anti-HLA antibodies induces tyrosine phosphorylation, fibroblast growth factor receptor expression and cell proliferation. *Int Immunol*. 1998;10:1315-23.
- [33] Levade T, Auge N, Veldman RJ, Cuvillier O, Negre-Salvayre A, Salvayre R. Sphingolipid mediators in cardiovascular cell biology and pathology. *Circulation research*. 2001;89:957-68.
- [34] Kolesnick R. Signal transduction through the sphingomyelin pathway. *Molecular and chemical neuropathology / sponsored by the International Society for Neurochemistry and the World Federation of Neurology and research groups on neurochemistry and cerebrospinal fluid*. 1994;21:287-97.

- [35] Strub GM, Maceyka M, Hait NC, Milstien S, Spiegel S. Extracellular and intracellular actions of sphingosine-1-phosphate. *Advances in experimental medicine and biology*. 2010;688:141-55.
- [36] Spiegel S, Merrill AH, Jr. Sphingolipid metabolism and cell growth regulation. *FASEB J*. 1996;10:1388-97.
- [37] Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat Rev Mol Cell Biol*. 2003;4:397-407.
- [38] Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential role for sphingosine kinases in neural and vascular development. *Mol Cell Biol*. 2005;25:11113-21.
- [39] Maceyka M, Payne SG, Milstien S, Spiegel S. Sphingosine kinase, sphingosine-1-phosphate, and apoptosis. *Biochimica et biophysica acta*. 2002;1585:193-201.
- [40] Ponnusamy S, Meyers-Needham M, Senkal CE, Saddoughi SA, Sentelle D, Selvam SP, et al. Sphingolipids and cancer: ceramide and sphingosine-1-phosphate in the regulation of cell death and drug resistance. *Future Oncol*. 2010;6:1603-24.
- [41] Samad F, Badeanlou L, Shah C, Yang G. Adipose tissue and ceramide biosynthesis in the pathogenesis of obesity. *Advances in experimental medicine and biology*. 2011;721:67-86.
- [42] Levade T, Jaffrezou JP. Signalling sphingomyelinases: which, where, how and why? *Biochimica et biophysica acta*. 1999;1438:1-17.
- [43] Pavoine C, Pecker F. Sphingomyelinases: their regulation and roles in cardiovascular pathophysiology. *Cardiovascular research*. 2009;82:175-83.
- [44] Marchesini N, Luberto C, Hannun YA. Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. *J Biol Chem*. 2003;278:13775-83.
- [45] Goswami R, Ahmed M, Kilkus J, Han T, Dawson SA, Dawson G. Differential regulation of ceramide in lipid-rich microdomains (rafts): antagonistic role of palmitoyl:protein thioesterase and neutral sphingomyelinase 2. *J Neurosci Res*. 2005;81:208-17.
- [46] Silva LC, de Almeida RF, Castro BM, Fedorov A, Prieto M. Ceramide-domain formation and collapse in lipid rafts: membrane reorganization by an apoptotic lipid. *Biophys J*. 2007;92:502-16.
- [47] Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol*. 2008;9:139-50.
- [48] Clarke CJ, Truong TG, Hannun YA. Role for neutral sphingomyelinase-2 in tumor necrosis factor alpha-stimulated expression of vascular cell adhesion molecule-1 (VCAM) and intercellular adhesion molecule-1 (ICAM) in lung epithelial cells: p38 MAPK is an upstream regulator of nSMase2. *J Biol Chem*. 2007;282:1384-96.
- [49] Levy M, Castillo SS, Goldkorn T. nSMase2 activation and trafficking are modulated by oxidative stress to induce apoptosis. *Biochemical and biophysical research communications*. 2006;344:900-5.
- [50] Clarke CJ, Guthrie JM, Hannun YA. Regulation of neutral sphingomyelinase-2 (nSMase2) by tumor necrosis factor-alpha involves protein kinase C-delta in lung epithelial cells. *Mol Pharmacol*. 2008;74:1022-32.
- [51] Coatrieux C, Sanson M, Negre-Salvayre A, Parini A, Hannun Y, Itohara S, et al. MAO-A-induced mitogenic signaling is mediated by reactive oxygen species, MMP-2, and the sphingolipid pathway. *Free Radic Biol Med*. 2007;43:80-9.
- [52] Devillard R, Galvani S, Thiers JC, Guenet JL, Hannun Y, Bielawski J, et al. Stress-induced sphingolipid signaling: role of type-2 neutral sphingomyelinase in murine cell apoptosis and proliferation. *PLoS One*. 2010;5:e9826.
- [53] Filanti C, Dickson GR, Di Martino D, Ulivi V, Sanguineti C, Romano P, et al. The expression of metalloproteinase-2, -9, and -14 and of tissue inhibitors-1 and -2 is

- developmentally modulated during osteogenesis in vitro, the mature osteoblastic phenotype expressing metalloproteinase-14. *J Bone Miner Res.* 2000;15:2154-68.
- [54] Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circulation research.* 2003;92:827-39.
- [55] Thomsen M, Yacoub-Youssef H, Marcheix B. Reconstitution of a human immune system in immunodeficient mice: models of human alloreaction in vivo. *Tissue Antigens.* 2005;66:73-82.
- [56] Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. *Nature.* 1983;301:527-30.
- [57] Roder J, Duwe A. The beige mutation in the mouse selectively impairs natural killer cell function. *Nature.* 1979;278:451-3.
- [58] Lorber MI, Wilson JH, Robert ME, Schechner JS, Kirkiles N, Qian HY, et al. Human allogeneic vascular rejection after arterial transplantation and peripheral lymphoid reconstitution in severe combined immunodeficient mice. *Transplantation.* 1999;67:897-903.
- [59] Marcheix B, Yacoub-Youssef H, Calise D, Thiers JC, Therville N, Benoist H, et al. Multiple human mesenteric arterial grafts from the same donor to study human chronic vascular rejection in humanized SCID/beige mice. *J Heart Lung Transplant.* 2006;25:675-82.
- [60] Yacoub-Youssef H, Marcheix B, Calise D, Thiers JC, Therville N, Benoist H, et al. Engraftment of human T, B and NK cells in CB.17 SCID/beige mice by transfer of human spleen cells. *Transpl Immunol.* 2005;15:157-64.
- [61] Zhao B, Luo X, Shi H, Ma D. Tissue factor pathway inhibitor-2 is downregulated by ox-LDL and inhibits ox-LDL induced vascular smooth muscle cells proliferation and migration. *Thromb Res.* 2011;128:179-85.
- [62] Varadarajulu J, Laser M, Hupp M, Wu R, Hauck CR. Targeting of alpha(v) integrins interferes with FAK activation and smooth muscle cell migration and invasion. *Biochemical and biophysical research communications.* 2005;331:404-12.
- [63] Shofuda T, Shofuda K, Ferri N, Kenagy RD, Raines EW, Clowes AW. Cleavage of focal adhesion kinase in vascular smooth muscle cells overexpressing membrane-type matrix metalloproteinases. *Arterioscler Thromb Vasc Biol.* 2004;24:839-44.
- [64] Lee OH, Lee DJ, Kim YM, Kim YS, Kwon HJ, Kim KW, et al. Sphingosine 1-phosphate stimulates tyrosine phosphorylation of focal adhesion kinase and chemotactic motility of endothelial cells via the G(i) protein-linked phospholipase C pathway. *Biochemical and biophysical research communications.* 2000;268:47-53.
- [65] Alkhatib B, Freguin-Bouilland C, Litzler PY, Jacquot S, Lallemand F, Henry JP, et al. Antidonor humoral transfer induces transplant arteriosclerosis in aortic and cardiac graft models in rats. *The Journal of thoracic and cardiovascular surgery.* 2007;133:791-7.
- [66] Yopp AC, Ledgerwood LG, Ochando JC, Bromberg JS. Sphingosine 1-phosphate receptor modulators: a new class of immunosuppressants. *Clinical transplantation.* 2006;20:788-95.
- [67] Habicht A, Clarkson MR, Yang J, Henderson J, Brinkmann V, Fernandes S, et al. Novel insights into the mechanism of action of FTY720 in a transgenic model of allograft rejection: implications for therapy of chronic rejection. *J Immunol.* 2006;176:36-42.
- [68] Shimizu H, Takahashi M, Kaneko T, Murakami T, Hakamata Y, Kudou S, et al. KRP-203, a novel synthetic immunosuppressant, prolongs graft survival and attenuates chronic rejection in rat skin and heart allografts. *Circulation.* 2005;111:222-9.
- [69] Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nature reviews Immunology.* 2011;11:403-15.

- [70] Hillebrands JL, Klatter FA, Rozing J. Origin of vascular smooth muscle cells and the role of circulating stem cells in transplant arteriosclerosis. *Arterioscler Thromb Vasc Biol.* 2003;23:380-7.
- [71] Autieri MV. Allograft-induced proliferation of vascular smooth muscle cells: potential targets for treating transplant vasculopathy. *Curr Vasc Pharmacol.* 2003;1:1-9.
- [72] Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, et al. UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of computational chemistry.* 2004;25:1605-12.

Figure Legends

Fig. 1. Ribbon model of HLA class I showing residues recognized by W6/32.

The structure is based on protein data bank entry 2bvp. The three extracellular domains of the heavy chain are colored blue and the beta-2 microglobulin is grey. The two arrows show the position of residue 121 of the α chain and residue 3 of beta-2 microglobulin, which according to Ladasky et al are part of a discontinuous epitope recognized by the W6/32 antibody [16]. The figure was prepared using USCF Chimera [72].

Fig. 2 Expression of HLA molecules in human SMC and human arteries

The microphotographs represent immunofluorescence experiments on human SMC cultured from mesenteric arteries (x1000) and sections of human arteries (x400). A: SMC incubated with irrelevant antibody of same isotype as W6/32 and B: with W6/32 antibody. C: human mesenteric artery sections incubated with irrelevant antibody and D: with W6/32 antibody. Arrows show HLA class I molecules at the surface of SMC and in the endothelium and the media of the arteries.

Fig. 3. Schematic representation of sphingolipid metabolism

This scheme represents sphingomyelin metabolites and enzymes that are involved in the production and degradation of the different sphingolipids. This pathway is activated by stress-inducing agents such as anti-HLA antibodies, leading to e.g. cell proliferation. Sphingomyelin metabolites have numerous targets such as kinases, phosphatases, transcription factors or receptors.

Fig. 4. Different steps of surgery and microsurgery in the animal model

A: Radiography of human mesentery injected with contrast to show the presence of multiple small arteries that are present (from Gray's anatomy). B: Peroperative view of mesentery before removal. C: Piece of mesentery after removal. A microdissection allows identifying a great number of small arteries of same diameter as the mouse aorta. D: A fragment of a mesenteric artery after dissection, the diameter is about 1 mm and the length about 1.5 cm. E, F, G: Operative procedure: The infrarenal aorta of the mouse is dissected and cut in the middle part after ligation. The human artery is grafted with point to point sutures for the distal anastomosis and with a sleeve anastomosis proximally. The two ligatures are finally removed and the blood flow restored. The patency of the graft is secured before the wound is closed.

Fig. 5. Sections of human arteries grafted into SCID/beige mice

The microphotographs represent transversal sections of human arteries grafted into SCID/beige mice and removed 6 weeks after transplantation. Intimal hyperplasia is shown by bars. A: Hematoxylin/Eosin staining of sections from mice treated with irrelevant antibodies or B: with anti-HLA antibodies (x100). C: Hematoxylin/Eosin staining of sections from mice treated with irrelevant antibodies or D: with anti-HLA antibodies (x400). E: α -SMA expression in sections from mice treated with irrelevant antibodies or F: with anti-HLA antibodies (x400). G: PCNA expression in sections of arteries from mice treated with irrelevant antibodies or H: with anti-HLA antibodies. Arrows show some PCNA-positive cells in the media and in the intimal hyperplasia.

Fig. 6. Summary scheme of the signaling pathways activated by anti-HLA antibodies in human SMC

Two major signaling pathways are involved in the proliferative effect of anti-HLA antibodies: one implicating FAK [30] and the other MMP and nSMase [29]. FAK is also implicated in the

migratory effect of anti-HLA antibodies on SMC. The anti-HLA class I antibodies (W6/32) probably induce a crosslinking of HLA molecules. Adaptive proteins may then be recruited in order to transduce signals intracellularly.