

# Ischemia-Reperfusion Injury Accelerates Human Antibody-Mediated Transplant Vasculopathy

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**Background.** The pathogenesis of transplant vasculopathy (TV) is a multifactorial process. We hypothesized that ischemia-reperfusion injury and antibody-mediated damage contribute to the development of TV.

**Methods.** Human vessels were procured from nine separate donors undergoing cardiac surgery and stored in saline solution on ice until transplantation. BALB/c *Rag2<sup>-/-</sup>IL-2R $\gamma$ <sup>-/-</sup>* mice were transplanted with a human vessel graft on day 0. Purified anti-human leukocyte antigen class I antibody (W6/32), isotype control antibody, or saline was injected into recipient mice weekly until day 42, at which point the degree of intimal expansion (IE) of vessels was assessed by histologic analysis.

**Results.** We found that a prolonged cold ischemia time (6–12 hr) alone did not induce IE. In mice that received antibody where vessels were transplanted within 6 hr of procurement, no IE was observed. By contrast, in vessels exposed to more than 6 hr cold ischemia, both W6/32 antibody (30.4%±6.9%) and isotype control antibody (39.5%±6.0%) promoted significant IE ( $P<0.05$  vs. saline [12.4%±1.7%]). Importantly, the isotype control antibody did not cross-react with human tissue. Interestingly, the number of mouse Fc-receptor-positive cells was significantly increased in human vessels exposed to more than 6 hr cold ischemia but only in the presence of antibody ( $P<0.05$ ).

**Conclusions.** Antibody, regardless of its specificity, may promote IE in human vessels that are injured through cold ischemia via interaction with Fc-receptor-positive cells. This highlights the importance of controlling the degree of cold ischemia in clinical transplantation in an effort to reduce the risk of TV development.

**Keywords:** Donor-specific antibody, Chronic allograft dysfunction, Humanized mouse model, Cold ischemia, Transplant arteriosclerosis.

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Transplant vasculopathy (TV) is the hallmark lesion of chronic allograft dysfunction, which is in turn the principal cause of late allograft loss (1, 2). The development of TV is a multifactorial process mediated by both immune-dependent and immune-independent factors and is characterized by intimal expansion (IE) of the transplant feeding vessels (3–5). Donor-specific antibody (DSA)-directed against human leukocyte antigen (HLA) class I and II,  $\alpha$ -major histocompatibility complex (MHC) class I-related chain A antibody, and  $\alpha$ -vimentin antibody are all significantly involved in inducing IE (6–10). Importantly, patients who

develop DSA have a 4-year graft survival rate of 58% compared with 81% for those without DSA (11). In rodent models, the passive transfer of  $\alpha$ -MHC class I antibodies induces vasculopathy of mouse heart and rat aorta allografts (12–15). Moreover, in a mouse model where immunodeficient mice are engrafted with human mesenteric artery segments, treatment with the  $\alpha$ -HLA class I monoclonal monomorphic antibody W6/32 alone induces IE within the transplanted vessels (16). Furthermore, experimental evidence in rat models suggests that allograft ischemia-reperfusion injury and trauma may promote the development of IE (17–19).

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In this study, using a mouse model of human allograft vasculopathy (2, 20–22), we show that ischemia-reperfusion injury may induce IE of human vessels *in vivo* in the absence of a T-cell response but only in the presence of antibody, regardless of its specificity.

## RESULTS

### A Cold Ischemia Time of Less Than 12 hr Does Not Induce IE of Human Vessels

Human vessels were retrieved from patients undergoing cardiac surgery and used within 12 hr of procurement (see Materials and Methods). Cold ischemia time (CIT) is known to be an important risk factor for allograft rejection (23, 24). We therefore analyzed the impact of the time between procurement of the human vessels and implantation into mice on the level of IE 42 days after transplantation. Vessels were subjected to less than 6 hr or between 6 and 12 hr of cold ischemia before transplantation into T and B cell-deficient BALB/c *Rag2<sup>-/-</sup>IL-2R $\gamma$ <sup>-/-</sup>* mice. At Day 42, only a small degree of IE was observed in vessels exposed to less than 6 hr of cold ischemia ( $n=4$  mice, vessels from three separate donors) (Fig. 1A, left, and B). Interestingly, similar levels of IE were also present in vessels exposed to more than 6 hr cold ischemia ( $n=5$  mice, vessels from three separate donors) (Fig. 1A, right, and B). This suggests that a CIT of less than 12 hr is not an important factor in the development of IE in human vessel transplants in the absence of an adaptive immune response.

### Anti-HLA Antibody Does Not Promote IE in Vessels Subjected to Less Than 6 hr of CIT

Next, we investigated the effect of the monomorphic  $\alpha$ -HLA class I antibody W6/32 on the development of vasculopathy. Mice were transplanted with human vessels and subsequently treated intravenously with W6/32, isotype control antibody to W6/32 (mouse IgG2a), or saline, weekly from days 7 to 42. All mice received the vessel transplant within 6 hr of procurement. At day 42 after transplantation, no significant IE developed in any of the three groups

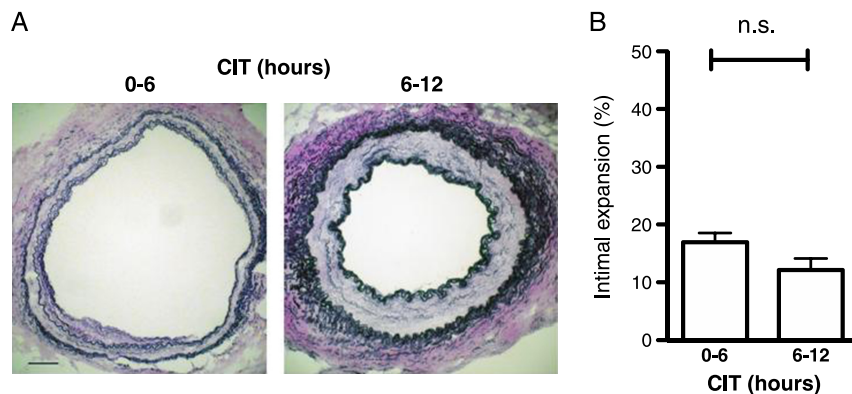
( $n=3-4$  mice per group, vessels from three to four separate donors) (Fig. 2A,B), indicating that anti-HLA antibody alone does not promote IE in human vessels subjected to less than 6 hr of cold ischemia.

### Antibody Together With a CIT of Between 6 and 12 hr Promotes the Development of IE Irrespective of the Antibody Specificity

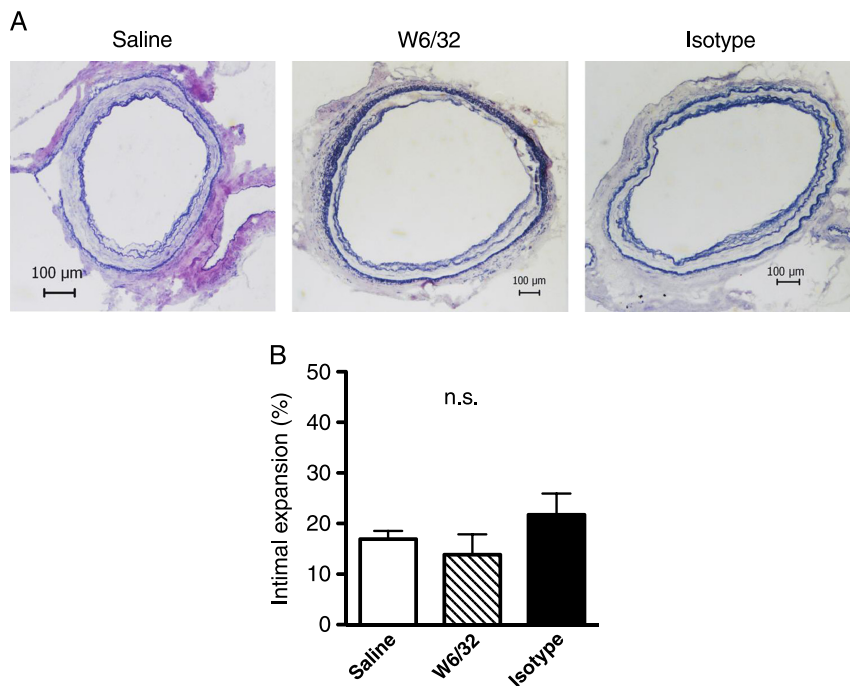
We next investigated whether anti-HLA antibody contributes to the development of IE in vessels subjected to more than 6 hr cold ischemia. To test this, we transplanted human vessels exposed to between 6 and 12 hr cold ischemia into mice and then injected W6/32 antibody, isotype control antibody, or saline, as above. At day 42 after transplantation, vessels from mice receiving saline did not display increased IE ( $n=6$ ) (Fig. 3A, left, and B). By contrast, W6/32 induced significant IE ( $n=5$ ;  $*P<0.05$ ) (Fig. 3A, middle, and B). Interestingly, vessels from mice receiving isotype control antibody also developed IE ( $n=4$ ;  $**P<0.005$ ) (Fig. 3A, right, and B). We observed very few smooth muscle cells (SMCs) in the media of vessels from the short CIT group (see **Figure S1a** and **S1b**, **SDC**, <http://links.lww.com/TP/A820>), whereas SMCs were readily detected in the media of vessels in the extended CIT group (see **Figure S1b**, **SDC**, <http://links.lww.com/TP/A820>). Of note, no IE developed in the native mouse vessel of mice receiving either W6/32 or isotype control antibody (data not shown). This indicated that antibody together with a prolonged CIT exposure of more than 6 hr can promote the development of IE irrespective of the specificity of the antibody.

### Isotype Control Antibody Does Not Cross-React With the Human Vessel Intima

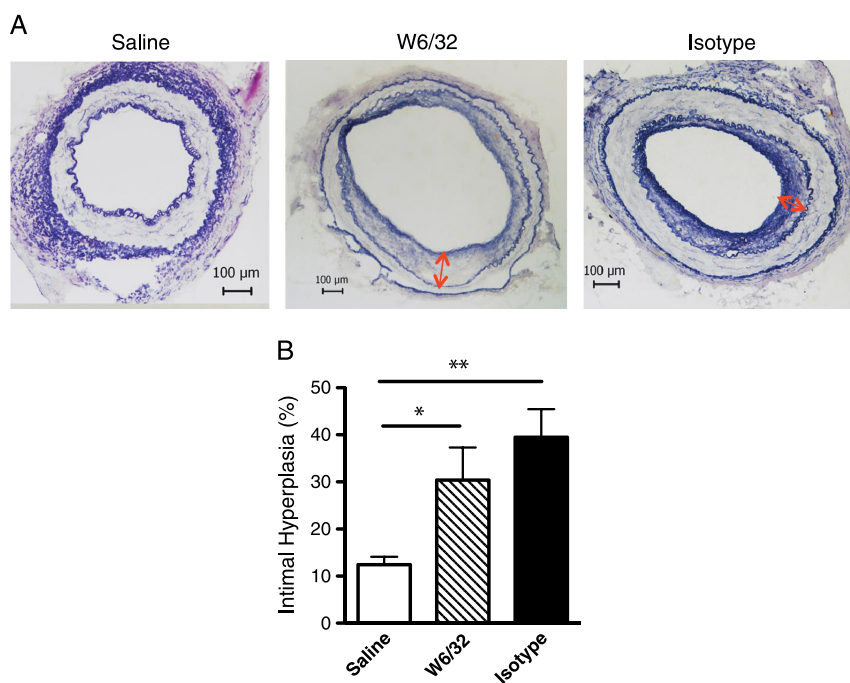
The mouse IgG2a isotype control antibody used in the assays described above was shown not to induce IE in a similar humanized mouse model (16). However, because the isotype control antibody contributed to the development of IE in our model, we assessed whether the antibody was cross-reacting with the human vessel tissue. W6/32, isotype control antibody, or saline was applied onto sections of human vessels and antibody binding was examined by



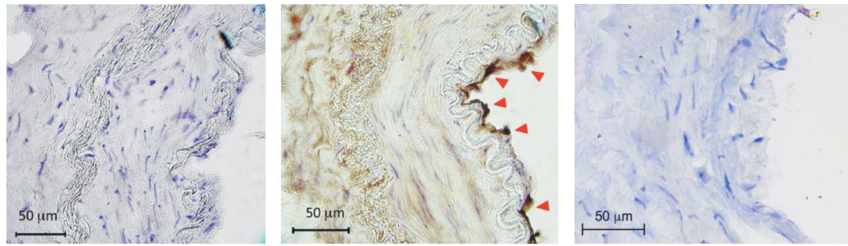
**FIGURE 1.** Impact of prolonged cold ischemia on IE in human vessel transplants. A and B, human vessel grafts were retrieved from recipient mice 42 days after transplantation. Vessels were divided into two groups according to the total period of cold ischemia before transplantation (0–6 or 6–12 hr). A, representative histomicrograph of vessel sections stained with EvG ( $\times 40$ ). B, degree of IE was calculated from data in three separate ( $200 \mu\text{m}$ ) levels as described in the Materials and Methods. Data are represented as mean  $\pm$  SEM for four to five experiments from three independent experiments using three distinct donors.



**FIGURE 2.** Impact of anti-HLA antibody on the development of vasculopathy in human vessel transplants. A and B, 42 days after transplantation, human vessel grafts were retrieved from recipient mice that had received intravenous injections of saline, W6/32 antibody (10  $\mu$ g/g), or isotype control antibody. All vessels were transplanted within 6 hr of procurement. A, representative histomicrographs of vessel sections stained with EvG ( $\times 40$ ). B, degree of IE is represented as mean $\pm$ SEM for four to five experiments from three independent assays using three distinct donors.



**FIGURE 3.** Impact of anti-HLA antibody on IE in human vessels subjected to more than 6 hr of pretransplantation cold ischemia. A and B, human vessels were subjected to 6 to 12 hr of cold ischemia before transplantation. Forty-two days after transplantation, vessel grafts were retrieved from recipient mice that had received saline or antibodies as per previous assays. A, representative histomicrographs of vessel sections stained with EvG. Red arrows indicate the area of IE demarcated by the internal elastic lamina. B, percentage IE in each group. Data are represented as mean $\pm$ SEM for four to five experiments from three independent experiments using three distinct donors. \* $P < 0.05$ , \*\* $P < 0.001$  vs. saline control group.



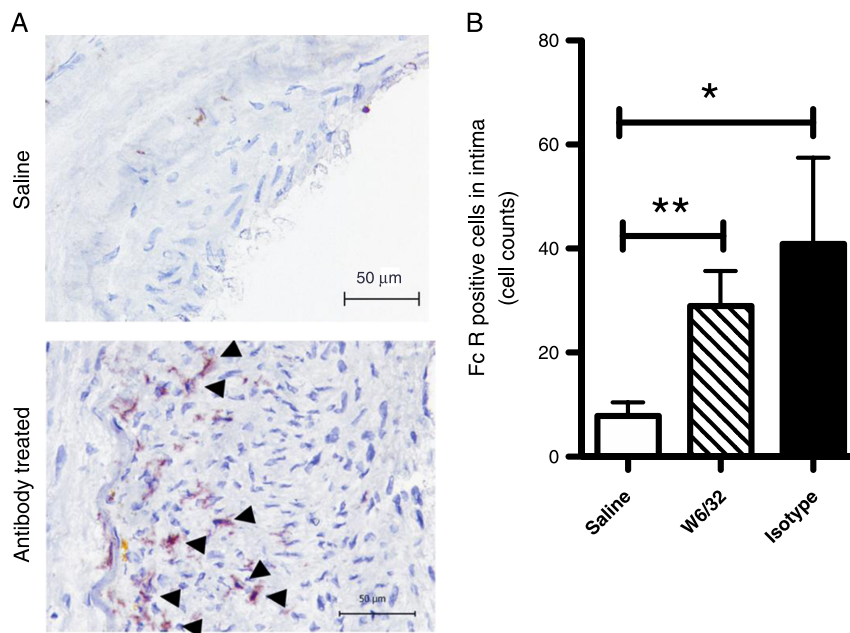
**FIGURE 4.** Binding of antibody to human vessels. Sections of human vessel were stained with W6/32 antibody, isotype control antibody to W6/32, or saline. Antibody binding was visualized using horseradish peroxidase–conjugated anti-mouse rabbit antibody. Representative images of four independent experiments are shown ( $\times 400$  magnification).

immunohistochemistry. As expected, sections stained using W6/32 antibody displayed positivity on the endothelial cell luminal surface (Fig. 4, middle). By contrast, sections stained using the isotype control antibody did not demonstrate positive staining (Fig. 4, middle), suggesting that the isotype control antibody does not induce IE in vivo through cross-reactivity with human vessels.

#### Mouse Fc-Receptor–Expressing Cells Accumulate in the Vessel Intima

To clarify the mechanisms in operation, we investigated whether mouse Fc-receptor (FcR)–positive cells were present in vessels exposed to more than 6 hr of CIT. Such cells may bind the Fc portion of W6/32 or the isotype control antibody, promoting an inflammatory response. Interestingly, in vessels exposed to prolonged CIT, there was

a significant increase in the number of FcR-expressing cells in the intima of the vessel grafts 42 days after transplantation irrespective of whether mice were treated with W6/32 antibody or isotype control antibody (Fig. 5A,B). FcR-positive cells in the mice were principally composed of CD11b<sup>+</sup>Gr-1<sup>+</sup> and CD11b<sup>+</sup>F4/80<sup>+</sup> cells (data not shown). In addition, a significantly larger number of Gr-1<sup>+</sup> cells was detected in the intima of antibody-treated vessel grafts compared with saline-treated controls (see Figure S2c, SDC, <http://links.lww.com/TP/A820>), although no significant differences were observed between the groups on analysis of the blood and spleen (see Figure S2a and S2b, SDC, <http://links.lww.com/TP/A820>). Taken together, this suggests that in the presence of prolonged periods of cold ischemia these cells play a role in the promotion of IE mediated by both W6/32 and isotype control antibody.



**FIGURE 5.** Mouse FcR-positive cells are present in the intima of human vessels subjected to a prolonged pretransplantation CIT. A and B, Human vessel grafts with exposure to more than 6 hr of cold ischemia were examined 42 days after transplantation. Vessels were stained with anti-mouse CD16/32 antibody. A, representative histomicrographs of human vessel sections ( $\times 400$  magnification). B, mouse FcR-positive cells in the intima were enumerated at three separate levels as described previously (20). Data are represented as mean $\pm$ SEM for four to five experiments from three independent assays and three separate donors. \* $P < 0.05$ ; \*\* $P < 0.001$  vs. saline group.

## DISCUSSION

In this study, we demonstrate that a prolonged CIT plays an important role in association with antibody in the development of IE in transplanted human vessels. We and others have observed that a graft CIT of more than 24 hr elicits TV in both mouse and rat aortic syngeneic transplants (data not shown) (25, 26). Importantly, alloreactivity is not necessary for the injury of grafts that have been subjected to prolonged cold ischemia (27, 28), yet transplantation of syngeneic mouse aortic grafts into T- and B-cell-deficient mice does not lead to significant IE, even if grafts are subjected to prolonged periods of cold ischemia of more than 24 hr (unpublished data). Our data together with others indicate that IE does not develop in conditions where there is neither alloreactivity nor a prolonged graft CIT (29). Together, these data suggest that two factors must be present for the development of IE in situations of absent or reduced alloreactivity: the presence of nonspecific antibody responses and cold ischemia. This finding is interesting, as the role of antibody in grafts subjected to ischemic injury has not previously been reported and virtually all previous experimental studies examining the passive transfer of antibody have focused on allospecific antibody or complement (12–15, 30).

Accumulating evidence suggests that cold ischemia induces the expression of cell adhesion molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in endothelial cells (27, 31). Interestingly, neutralizing intercellular adhesion molecule-1 reduces ischemic injury in rat kidneys (32). Up-regulation of adhesion molecules may therefore lead to the accumulation of FcR-positive cells such as macrophages, monocytes, and neutrophils at the site of injury. We found that significant numbers of mouse FcR-expressing cells accumulated in human vessels exposed to a prolonged CIT (Fig. 5). Macrophages may contribute to the development of cardiac allograft vasculopathy (33) and the proliferation of SMCs (34–36). Furthermore, glomerular and capillary CD68<sup>+</sup> monocytes may promote antibody-mediated rejection (37). Glomerular and interstitial infiltration of CD68<sup>+</sup> monocytes is also associated with C4d deposition in clinical kidney transplantation (38, 39). Neutrophils play a pivotal role in the inflammation of vessel transplants. Activated neutrophils release cytokines, such as interleukin-8, interleukin-6, tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , and transforming growth factor- $\beta$ , which promote SMC migration and proliferation (37). Thus, innate immunity may play an important role by exaggerating specific and nonspecific antibody-mediated TV under conditions of cold ischemia.

Evidence is accumulating that anti-HLA antibody is an important risk factor for allograft vasculopathy (5, 11, 16). MHC class I-related chain A antibody has also been reported to be a risk factor for vasculopathy (40). Interestingly, non-donor-specific anti-HLA antibody that emerges after transplantation may also be involved in promoting graft failure (40). The development of autoantibody against vimentin and cardiac myosin may also contribute to chronic graft dysfunction (8, 9, 41). Together with our data, it is likely that the amount of antibody per se may promote TV irrespective of the antibody's specificity.

Our results appear at odds to those from Galvani et al. (16), where isotype control antibody did not promote IE in

a humanized mouse model. This may be related to multiple factors, including the mouse strain used. Multiple types of immunodeficient mice are currently available. Galvani et al. used the same mouse strain as that used by Pober et al. (22) where the model was originally reported (i.e., the C.B-17-SCID beige mouse) (16). Mice used in our study lacked the *Rag2* gene and the IL-2R $\gamma$  chain, completely preventing natural killer (NK)-cell development. In contrast, C.B-17-SCID-beige mice have a NK-cell population with reduced cell-mediated lysis activity (42). A recent study has shown that NK cells may trigger vascular changes of the allograft in a mouse cardiac transplant model (3). Moreover, there is NK-cell involvement in clinical antibody-mediated rejection (43). The passive transfer of antibody to mouse MHC class I (anti-H2<sup>k</sup>) induces chronic allograft vasculopathy in cardiac allografts (H2<sup>k</sup>) in B6. *Rag1*<sup>-/-</sup> and B6. *Rag1*<sup>-/-</sup>C3<sup>-/-</sup> mice. In this model, treatment with the NK-depleting antibody anti-NK1.1 leads to a reduced frequency of vasculopathy. NK cells may possibly interact with the Fc part of the anti-MHC class I antibody resulting in the development of vasculopathy (29).

Although useful, humanized mouse models have limitations and data must be extrapolated with care. For example, anti-HLA antibody activates human endothelial cell exocytosis and leukocyte trafficking (44). In humanized mouse models, we must consider the replacement of human endothelial cells with mouse endothelial cells that may not react with human anti-HLA antibodies.

The role of cold ischemia injury has been well documented in transplantation. Here, we demonstrate another facet of the damage cold ischemia may cause in the presence of specific or nonspecific antibody. Importantly, we show that cold ischemia injury does not induce vascular changes in the absence of antibody or a T- or B-cell response. The elimination of immunoglobulin may therefore prove a promising strategy for the prevention of chronic vascular changes after transplantation of organs subjected to prolonged periods of cold ischemia.

## MATERIALS AND METHODS

### Ethics Statement

Experiments were performed using protocols approved by the Committee on Animal Care and Ethical Review at the University of Oxford and in accordance with the UK Animals (Scientific Procedures) Act 1986. For the collection of human tissue samples, this was performed with informed written consent and ethical approval from the Oxfordshire Research Ethics Committee (REC B) study number 07/H0605/130.

### Mice

BALB/c *Rag2*<sup>-/-</sup>IL-2R $\gamma$ <sup>-/-</sup> (H2<sup>d</sup>) male and female mice were bred and housed in the Biomedical Services Unit at the John Radcliffe Hospital (Oxford, UK).

### Procurement of Human Vessels

Human vessels for transplantation were procured from nine patients undergoing coronary artery bypass graft procedures by Prof. David Taggart and his surgical team. Side branches of internal mammary arteries were detached by the surgeon during the procedure. Vessels were strictly selected by size, with only those of approximately 800  $\mu$ m in diameter and 5 mm in length used for transplantation. We excluded human vessels with branches from use as vessel grafts. Procured vessels were stored in 4.0% phenoxybenzamine saline solution on ice until transplantation.

## Transplantation

Human vessels were transplanted into mouse abdominal aortae as described previously (2). Briefly, animals received 20 µg Domitor (Janssen-Cilag, High Wycombe, UK), 1.5 µg Ketaset (Fort Dodge Animal Health, Southampton, UK), and 2 µg Vetergesic (Alstoe Animal Health, York, UK). The anastomoses between the interpositioned vessel graft and naïve mouse abdominal aorta were performed by end-to-end interrupted suture ligation using 10-0 nylon monofilament (Bear Medic, Ibaraki, Japan). Postoperatively, 20 µg Antisedan (Janssen-Cilag) was given subcutaneously. The surgical success rate was over 90%. The total anastomotic time was less than 30 min in all groups. All procedures were performed by one experienced microsurgeon.

## Antibody Treatment Protocol

The antibody treatment protocol followed the regimen reported by Galvani et al. (16) in a humanized mouse model. One µg per 10 g (mouse body weight) of purified anti-HLA antibodies (W6/32; Daclone, Besançon, France) and isotype control antibodies (mouse IgG2a; B-Z2, Daclone), which were generated from the same type of hybridoma (myeloma X63/AG. 8653×BALB/c spleen cells), were given intravenously weekly from Days 7 to 42 after transplantation. As an additional control group, we gave the same volume of saline intravenously. Each treatment group included tissue from each patient.

## Flow Cytometry

Cell suspensions were prepared from splenocytes and blood after red cell lysis using BD Pharmlyse (BD Biosciences, San Jose, CA). Cells were stained with biotinylated anti-mouse CD16/CD32 (FcR) antibody, fluorescein isothiocyanate-streptavidin, APC-conjugated anti-mouse Gr-1 (RB6-8C5), and PE-Cy7 conjugated anti-mouse CD11b (M1/70) (all BD Biosciences). Dead cells were excluded by 7-aminoactinomycin D staining. Fluorescence-activated cell sorting analysis was carried out using a FACS Canto II (BD Biosciences) and BD FACSDiva Software version 6.1.3.

## Morphometric Analysis

Vessel grafts were collected from recipient mice 42 days after transplantation (2) and embedded in OCT compound (Tissue-Tek, Sakura, The Netherlands) before being sectioned at a thickness of 6 µm by using a Bright 5040 Cryostat. These sections were stained with elastin (VMR International, Lutterworth, UK) and van Gieson (Raymond A. Lamb, East Sussex, UK) stain (EvG) and photographed using light microscopy (Nikon, Tokyo, Japan) and a Coolpix digital camera (Nikon). The rate of IE (current open area/previous open area) was calculated by using Adobe Photoshop CS3 extended (version 10.0.1). The IE rate was calculated from the average of three histologic levels separated by 200 µm. We excluded IE measurements taken from areas adjacent to anastomoses to account for nonspecific changes. The IE rate for each level was calculated from more than three sections.

## Immunohistochemistry

Frozen sections (10 µm) of a vessel sample were defrosted and fixed by cold acetone for 15 min. Samples were neutralized by 0.3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) for 2 min and washed in phosphate-buffered saline. One percent mouse serum was added for 30 min to block any nonspecific binding sites. After washing in phosphate-buffered saline, samples were incubated with primary antibodies for 1 hr at room temperature. For staining with a biotinylated anti-mouse CD16/CD32 antibody and anti-mouse Gr-1 antibody (RB6-8C5) (BD Pharmingen, Franklin Lakes, NJ), avidin/biotin block (Vector Laboratories, Burlingame, CA), and ABC elite (Vector Laboratories) were applied before and after incubation with antibodies.

For purified antibodies, Universal Immuno-enzyme Polymer Method (Histofine Simple Stain MAX PO [MULTI], Nichirei Biosciences, Tokyo, Japan) was used according to the manufacturer's instructions. Samples were developed with 3,3'-Diaminobenzidine (Sigma-Aldrich) and counterstained with Harris hematoxylin (Anatomical Pathology International, Runcorn, UK) for 10 s. Positive cells were identified using high magnification (×400) light microscopy as described previously (20).

## Statistical Analysis

Statistical tests were performed with GraphPad Prism software (GraphPad Software, La Jolla, CA). One-way ANOVA and Student's *t* tests were applied on grouped data. *P* values < 0.05 were taken as significant.

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