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Smooth muscle cells clonally expand in a murine carotid allograft model complicated by immune reactions to reporter transgenes

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ABSTRACT

Background and aims: Most experimental studies of allograft vasculopathy (AV) have relied on transplantation between major histocompatibility complex-mismatched inbred mouse strains, but this leads to the complete eradication of donor smooth muscle cells (SMCs) and lesions formed by recipient cells. This is unlike human AV which is thought to form mainly by donor SMCs. Here, we studied sources of neointimal cells in a minor histocompatibility antigen-mismatched AV model by combining male-to-female orthotopic carotid transplantations and lineage tracing by SMC-specific expression of fluorescent proteins.

Methods: To track SMC-derived cells in allograft vasculopathy, we used male donor mice with SMC-restricted Cre recombination of the mT/mG reporter transgene, which switches expression of membrane-bound red fluorescent protein (RFP) to green fluorescent protein (GFP), or the stochastically recombining Confetti reporter transgene, which yields a mosaic expression of four fluorescent proteins. Donor carotid segments were harvested and orthotopically allografted to female recipients that were wildtype or had non-recombined reporter transgenes. Inhibition of T cell responses by CTLA4Ig was used in some experiments. Sections of lesions harvested after 4 weeks were analyzed by fluorescence microscopy.

Results: Donor-derived SMCs survived and gave rise to part of the neointimal cells in experiments where carotid segments from recombined mT/mG male mice were transplanted into wild-type or non-recombined mT/mG female mice. Sex-mismatched transplants developed significant lesions, increasing the intimal and medial area 4.6-fold ($p = 0.038$) and 2.0-fold ($p = 0.024$) compared to sex- and fluorescence-matched controls, respectively. Interestingly, sex-matched fluorescence-positive transplants developed intimal lesions in 50% of fluorescence-naïve recipient controls. To study the clonal structure of the neointimal donor-derived SMC lineage cells, we then transplanted male carotids with heterozygous or homozygous recombined Confetti transgenes into female recipients. These transplants developed lesions with few surviving donor SMCs, indicating that expression of the Confetti reporter increased rejection and donor-specific SMC death. Some of the few remaining donor SMCs underwent clonal expansion. CTLA4Ig administration at the time of surgery did not improve SMC survival in mT/mG or Confetti transplants.

Conclusion: Male-to-female transplant models feature donor-derived SMCs, some of which undergo clonal expansion, but immune rejection to fluorescence reporters appears to bias results in lineage tracing models. Overcoming these challenges with alternative reporter transgenes or tolerant recipients is necessary to study the mechanisms by which donor SMCs contribute to allograft vasculopathy.

1. Introduction

Allograft vasculopathy (AV) is a chronic rejection response in solid-organ transplants. The graft vasculature develops concentric lumen-restricting intimal hyperplasia causing ischemia and eventually graft failure. In most experimental studies of the mechanisms of allograft vasculopathy, the immune-mediated vascular injury has been driven by major histocompatibility differences between donor and recipient animals. However, this leads to the complete eradication of donor SMCs and a repair process driven by the ingrowth of recipient cells from the

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adjoined arteries [1]. Consequently, this does not translate well to human pathology, where neointimal SMCs in transplanted organs are found to originate mainly from donor cells with a minor potential contribution from ingrowing or circulating progenitor-derived recipient cells [2,3].

Because of the lack of appropriate models, the mechanism by which local SMCs are activated and expand in allograft vasculopathy has not been studied in detail. Yet advances in the understanding of SMC-driven vascular disease have recently been made for other vascular diseases. In murine models for atherosclerosis and ligation-induced neointima, very few individual medial SMCs were found to proliferate and make up the neointimal lesions [4,5]. In contrast, a large fraction of surviving SMCs proliferated to heal arteries after severe mechanical injury [6]. Whether neointima formation in allograft vasculopathy is generated by a few clonally expanding SMCs or is a polyclonal process has not yet been explored.

2. Objective

In the present study, we aimed to develop a model to study donor SMCs in allograft vasculopathy. Previous studies indicated that minor histocompatibility mismatch models, such as the male-to-female transplantation model, feature donor-derived neointimal SMCs [7–9], but this has not yet been documented by lineage tracing of SMCs. To investigate this, we established an orthotopic male-to-female carotid transplantation model and assessed the recruitment and clonal expansion of donor SMC by lineage and clonality tracing employing dual and multi-color fluorescent reporters, respectively.

3. Materials and methods

3.1. Animals and ethics

Myh11-CreER^{T2} (B6.FVB-Tg(Myh11-Cre/ERT2)1Soff/J, strain #:019079, Jackson Laboratories, Bar Harbor, Maine, USA) [10] were intercrossed with mT/mG (B6.129(Cg)- Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, strain #:007676, Jackson Laboratories) [11] and Confetti (B6.129P2-Gt(ROSA)26Sortm1(CAG-Brainbow2.1)Cle/J, strain #:017492, Jackson Laboratories) mice [12], to obtain male donor Myh11-CreER^{T2}-mT/mG (homozygous for the mT/mG transgene) and Myh11-CreER^{T2}-Confetti mice (hemi- or homozygous for the Confetti transgene as indicated in text). Female littermates or wildtype C57BL/6J

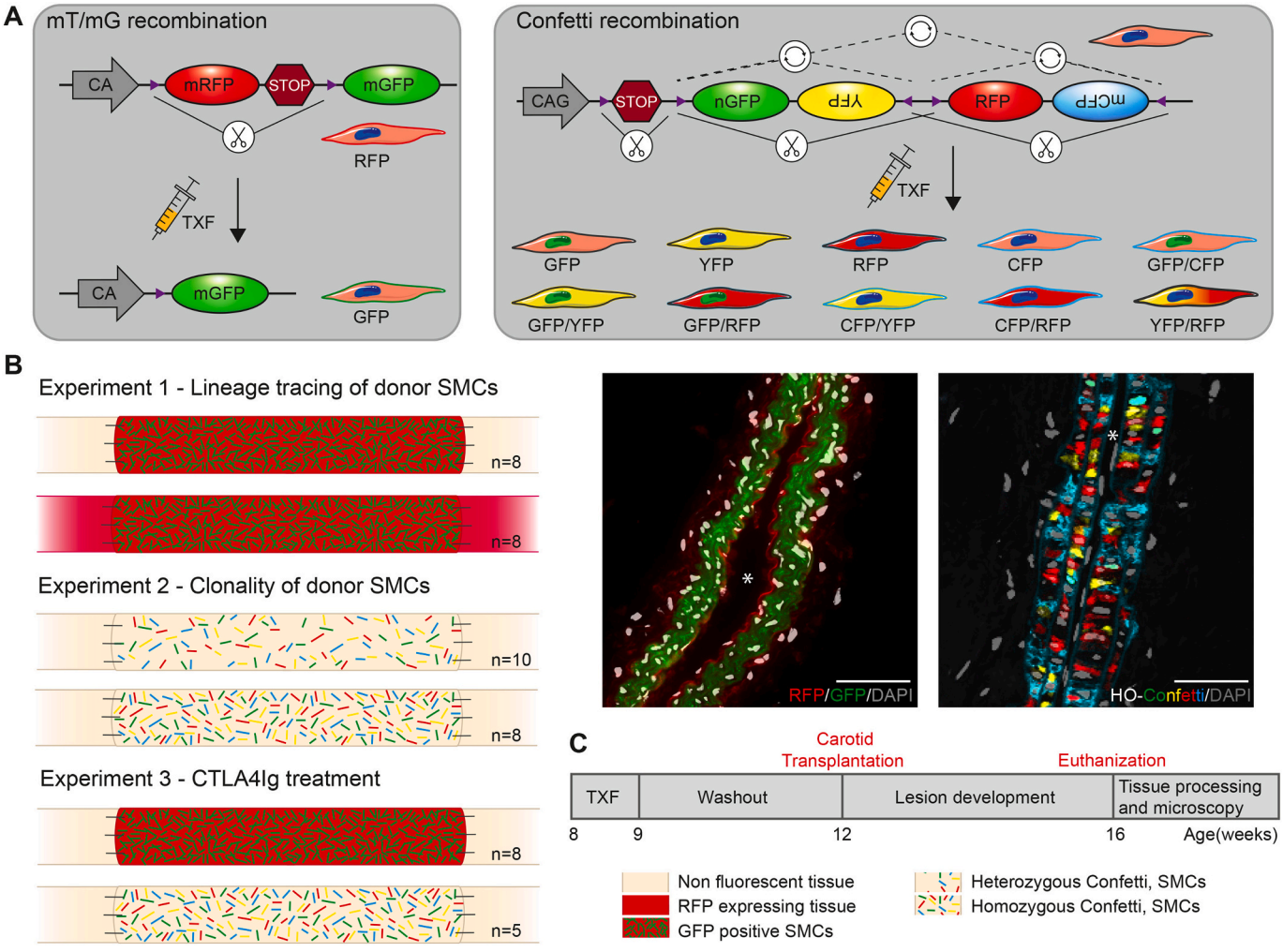

Fig. 1. Schematic representation of the study design. (A) Upon tamoxifen-dependent recombination, the mT/mG and Confetti transgenes lead to traceable fluorescent reporter expression. Hemizygous Confetti allows for four different recombination outcomes, each encoding a single fluorescent reporter phenotype, whereas homozygous Confetti allows for the creation of 6 additional double-reporter phenotypes. Fluorescence microscopy examples of healthy carotid arteries from induced male Myh11-CreER^{T2}-mT/mG and homozygous Myh11-CreER^{T2}-Confetti mice are shown. (B) Overview of the three experiments of the study and their specific aims. For simplification, only male-to-female experimental animals and no controls are included in the figure. (C) The common timeline for the three carotid transplantation experiments. TXF, tamoxifen; SMC, smooth muscle cells; RFP, red fluorescent proteins; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein. An asterisk marks the lumen. Scale bars 50 μm.

(Janvier Labs, Le Genest-Saint-Isle, France) were used as recipients. All females were CreER^{T2}-negative since the transgene resides on the Y chromosome. Animals were housed at a 12-h light/dark cycle, 22–23 °C room temperature and with ad libitum access to water and standard chow. All described animal procedures were approved by the Danish Animal Experiments Inspectorate (approval no. 2020-15-0201-00410) and conducted at the Department of Clinical Medicine, Aarhus University, in accordance with national regulatory standards.

3.2. Study design

To study donor-derived SMCs in sex-mismatched AV, we performed 3 consecutive rounds of experiments. Myh11-CreER^{T2}-mT/mG and Myh11-CreER^{T2}-Confetti male donor mice were used for lineage and clonality tracing, respectively. After recombination with intraperitoneal injection (i.p.) of 2 mg tamoxifen (#T5648, Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in corn oil (#C8267, Sigma-Aldrich) for 5 days, both strains harbor SMC-specific expression of fluorescent reporters (Fig. 1A). Donor-recipient combinations and the common timeline for the experiments can be found in Fig. 1B-C.

3.3. Carotid transplantation

The carotid transplantations were performed as described earlier [1]. Briefly, both common carotid arteries (CCA) from the donor mouse were dissected free and stored in saline at room temperature. Recipient mice were anesthetized with sevoflurane (induction 4%, maintenance 2–3%), injected with buprenorphine (0.1 mg/kg, s.c.) and heparin (200 IU/kg, i.m.), and placed in a supine position on a heating pad. After dissecting the CCA free from surrounding tissue, a double microvascular clamp was placed on the CCA and the vessel cut between the clamps. The graft was interposed in orthotopic orientation by two end-to-end-anastomoses with five evenly spaced 11–0 polyamide single sutures in each. After removing the microvascular clamps, hemostasis was controlled before closing the wound with a 6–0 suture. Postoperative analgesia consisted of a single injection of carprofen (5 mg/kg, subcutaneous injection) and drinking water containing buprenorphine (0.01 mg/mL) for 3 days. A list of the performed transplantations can be found in Supplementary Table 1.

3.4. Tissue processing

Mice were terminated and perfusion fixated 4 weeks after surgery. Briefly, mice were anesthetized by an i.p. injection of pentobarbital (250 mg/kg) and lidocaine (20 mg/kg). Perfusion fixation was performed at 100 mmHg through the left ventricle with Kardioplex (#721061, Capital Region Pharmacy, Copenhagen, Denmark) and 4% phosphate-buffered formaldehyde for 30 s and 5 min, respectively. Subsequently, mice were immersion fixated in 4% phosphate-buffered formaldehyde for 24 h at room temperature.

For analysis, carotid grafts were excised and cryoprotected in sucrose solution (25% for 24 h and 50% for 24 h). Subsequently, the grafts were cut in half, embedded in OCT compound (Sakura Finetek, Netherlands), and snap-frozen using liquid nitrogen. Five μm thick cross-sections from the center of the vessels were obtained for analysis. Sections were stained with DAPI or HE, using standard protocols, and mounted for microscopy. Additionally, sections were stained for CD3 and Acta2 using rabbit monoclonal (SP7) anti-CD3 (ab16669, 1:200, Abcam, Cambridge, Great Britain) or rabbit monoclonal anti-Acta2 (ab32575, 1:200, Abcam) primary antibody, followed by Alexa Fluor 647–conjugated donkey anti-rabbit (ab150075, 1:400, Abcam) secondary antibody.

3.5. Microscopy

Confocal images were obtained using a Zeiss Laser Scanning Confocal Microscope equipped with a C Apochromat 40×/1.2 water objective and ZEN Black 2.3 software. The entire cross-section was scanned by automated stitching of 3 × 3 images, acquired with 4 sequential captures. First, the 514 nm and 633 nm lasers were used to simultaneously acquire the yellow fluorescent protein (YFP) and far-red (autofluorescence). Second, green fluorescent protein (GFP) was imaged with a 488 nm laser. Third, the cyan fluorescent protein (CFP) and red fluorescent protein (RFP) channels were acquired with 458 nm and 543 nm lasers, respectively. Finally, DAPI was imaged with a 405 nm laser.

Images containing far-red AF647 signal were acquired on a Nikon eclipse Ti2 wide-field fluorescence microscope equipped with a pE-300^{ultra} LED light source, a Plan Apochromat 40×/0.95 air objective, and NIS-Elements AR 4.5 software. Entire carotid cross-sections were imaged by automated stitching of 3 × 3 images, acquired with Semrock Brightline filter-sets (GFP/FITCH, RFP/CY3, DAPI and far-red/CY5) in 4 sequential captures. No filter was available for detection of CFP and the YFP signal was included in the GFP channel in this setup.

All acquisition and postprocessing settings were tested with sections from fluorescent negative female controls to confirm specificity. ZEN 3.6 Blue software was used to measure areas and calculate recombination rates on confocal images. ImageJ was used for the postprocessing of wide-field fluorescence images. Recombination efficiency was determined by examining medial nucleated cells for reporter expression in a single z-layer of DAPI-stained cryosections from healthy mT/mG and Confetti carotid arteries. Brightness and contrast were adjusted to enhance the visibility of the fluorescent signal in all the publication images.

3.6. Statistical analysis

All statistical tests were performed in Prism 9.3.1 (GraphPad Software). We used Kruskal-Wallis for comparison between groups and $p < 0.05$ was considered statistically significant. All data are shown as mean ± SD.

4. Results

4.1. Recombination rates of SMC-specific reporters

To evaluate the efficiency of reporter transgene activation in SMCs of Myh11-CreER^{T2}-mT/mG and Myh11-CreER^{T2}-Confetti mice, we examined nucleated cell profiles in the arterial media of healthy carotid arteries at 4–6 weeks after tamoxifen injections (Fig. 1A). The recombination rate in homozygous Myh11-CreER^{T2}-mT/mG mice was 99.4% ± 0.7% (mean ± SD, $n = 3$). The Confetti transgene is more difficult to recombine, and we have previously reported an SMC-specific recombination rate of 56.3% in hemizygous Myh11-CreER^{T2}-Confetti males [4]. In homozygous Myh11-CreER^{T2}-Confetti mice of the present study, we found the recombination rate to be substantially higher at 82.8% ± 7.8 (mean ± SD, $n = 6$). The observed distribution of Confetti colors was consistent with previous reports [5], with RFP being the most frequently observed Confetti color and, on average, present in 33.9% of medial SMCs (SD: ± 15.1%). CFP was the second most frequently observed and present in 32.1% (SD: ± 9.1), followed by YFP in 22.9% (SD: ± 6.4%) and GFP in 10.8% (SD: ± 7.9) of medial SMCs.

4.2. Experiment 1 - Neointimal cells of donor origin in sex-mismatched transplants

Previous studies have indicated that neointimal cells in sex-mismatched murine transplantations are mainly of donor origin, but this has not yet been documented by direct lineage tracing [7,8]. To investigate donor SMC contribution to neointima in the male-to-female carotid allograft vasculopathy model, we recombined SMCs in Myh11-CreER^{T2}-mT/mG male mice ($n = 8$) and transplanted carotid arteries to either female (non-recombined) mT/mG mice (n = 8) or female wild-type mice (Fig. 1B). Both carotid arteries from donor animals were

successively transplanted to reduce animal use. Eight sex-matched female transplant procedures served as controls (4 mT/mG-to-wildtype and 4 wildtype-to-mT/mG). All experimental animals were 12 weeks old at the time of surgery, and arteries were examined after 4 weeks (Fig. 1C).

The medial SMCs of the grafts were strongly GFP$^+$ and were found to expand into the developed neointimal lesions in all 16 sex-mismatch transplants. The number of GFP$^+$ SMCs in the lesion ranged from a few cells up to covering almost the entire lesion (Fig. 2A-B). The relatively confined distribution of the GFP-positive cells indicated clonal development, even though they were interspersed with non-fluorescent cells in wildtype recipients and weakly RFP$^+$ or non-fluorescent cells in mT/mG recipients. Many of these were likely immune cells, shown before to have low expression of reporters driven by the CAG promoter [13,14]. RFP$^+$ endothelial cells from the donor survived in 8/8 sex-mismatched transplants with wildtype recipients. All 16/16 sex-mismatched samples contained RFP$^+$ cells within the neointimal lesions. Considering the high recombination rate in donor SMCs, they were unlikely to be SMC-derived and could instead derive from adventitial fibroblasts or through endothelial-to-mesenchymal transition (Endo-MT) from donor endothelial cells. However, some of the cells can potentially be explained by neovascularization or embedding of endothelial cells, due to extensive lymphocyte infiltration.

When comparing the sex-mismatch samples, medial and neointimal

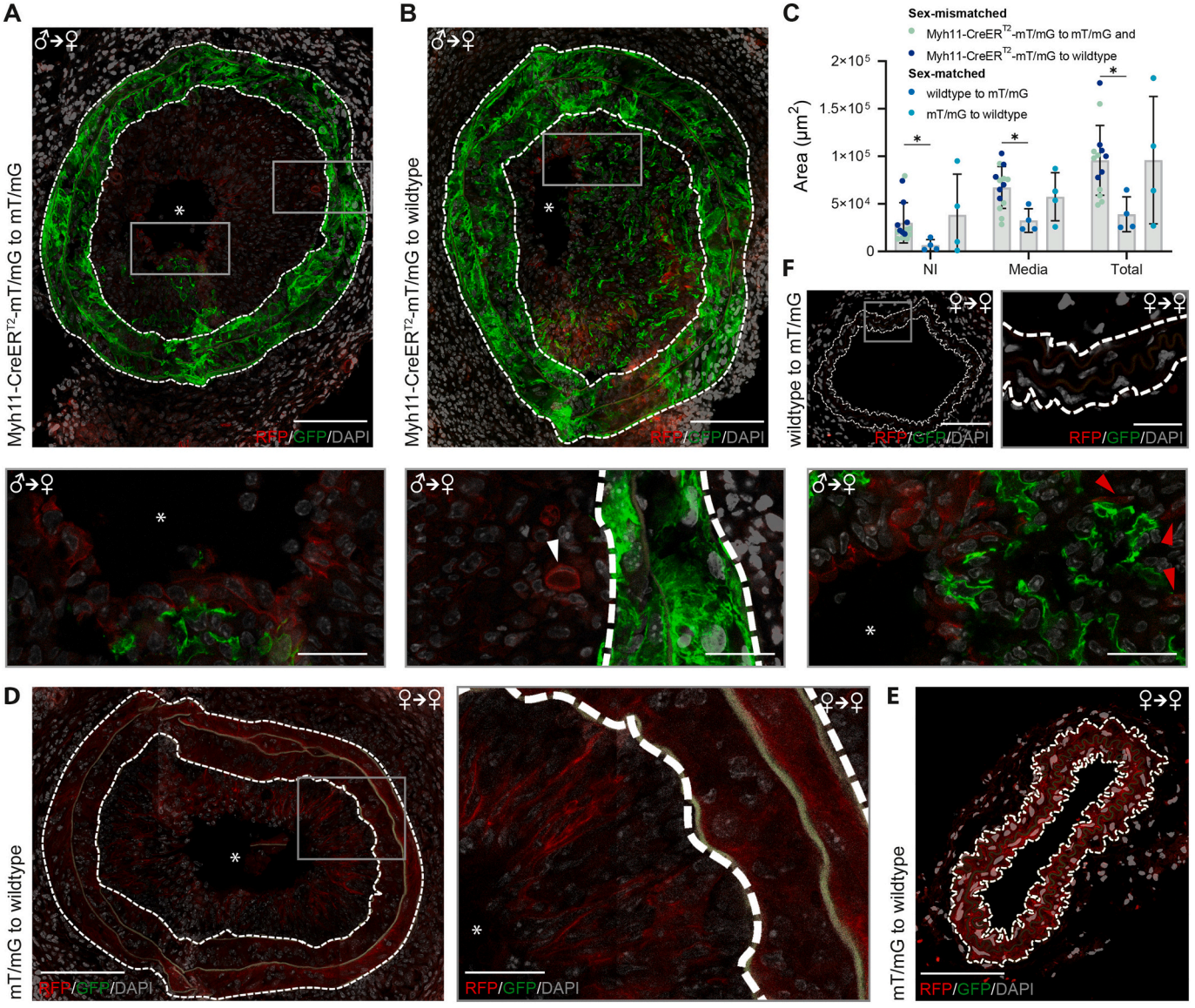

Fig. 2. Lineage tracing of SMCs in sex-mismatched carotid transplants. (A) An example of a neointimal lesion where GFP$^+$ donor SMCs are present in <30% of the lesion. We observed structures consistent with neovascularisation within the neointima (white arrow). The media largely contained surviving donor GFP$^+$ cells but appeared severely affected as it was much thicker than normal. (B) An example of a transplant where GFP$^+$ donor SMCs are present in almost the entire lesion. RFP$^+$ endothelial cells and neointimal cells (red arrows) are of donor origin. (C) Comparison of the neointimal, medial, and total area between the sex-mismatched and sex-matched control transplants. Areas were significantly larger in sex-mismatched samples compared to sex-matched controls when the recipients expressed RFP (* = P <0.05). In sex-mismatched transplants, a tendency for increased areas was observed when the recipient animals did not express any fluorescent proteins. In sex-matched controls, where RFP$^+$ carotid arteries were grafted into fluorescent negative wildtype mice, 3/4 samples developed neointimal lesions and the medial area increased (example in (D)). Only one mT/mG to wildtype control graft did not develop any lesion in media or intima (shown in (E)). If the donor was non-fluorescent and the recipient RFP$^+$, no lesions developed (example in (F)). Data are shown as mean ± SD and statistical significance between groups was analyzed by Kruskal-Wallis. RFP, red fluorescent protein; GFP, green fluorescent protein; NI, neointima. White asteriks marks the lumen. Scale bars 100 μm and 25 μm in magnified regions.

areas were slightly increased when the recipient was wildtype compared with non-recombined mT/mG (Fig. 2C). Moreover, we found substantial neointima development (2/4) and increased medial areas (3/4) in sex-matched controls, when transplanting RFP-expressing arterial segments from female mT/mG donor mice to female wildtype recipients (Fig. 2C-D). Only 1/4 did not show signs of lesion development (Fig. 2E). In arterial segments transplanted from female wildtype to female mT/mG recipients, no neointima formation was observed and only 1/4 showed some medial thickening. The remaining three controls did not develop any lesions (Fig. 2F). Combined, the findings from the sex-mismatched and sex-matched transplants indicate that the mT/mG-encoded RFP is immunogenic. GFP has previously only shown minimal immunogenic properties in the C57BL/6 background [15], and therefore, the contribution of lineage-traced GFP$^+$ SMCs in the sex-mismatched transplants is not likely to be affected. However, the expression of RFP$^+$ in the endothelium and adventitia in grafted arteries in the sex-mismatched transplants may contribute to immune rejection in addition to the Y chromosome-encoded minor histocompatibility complex.

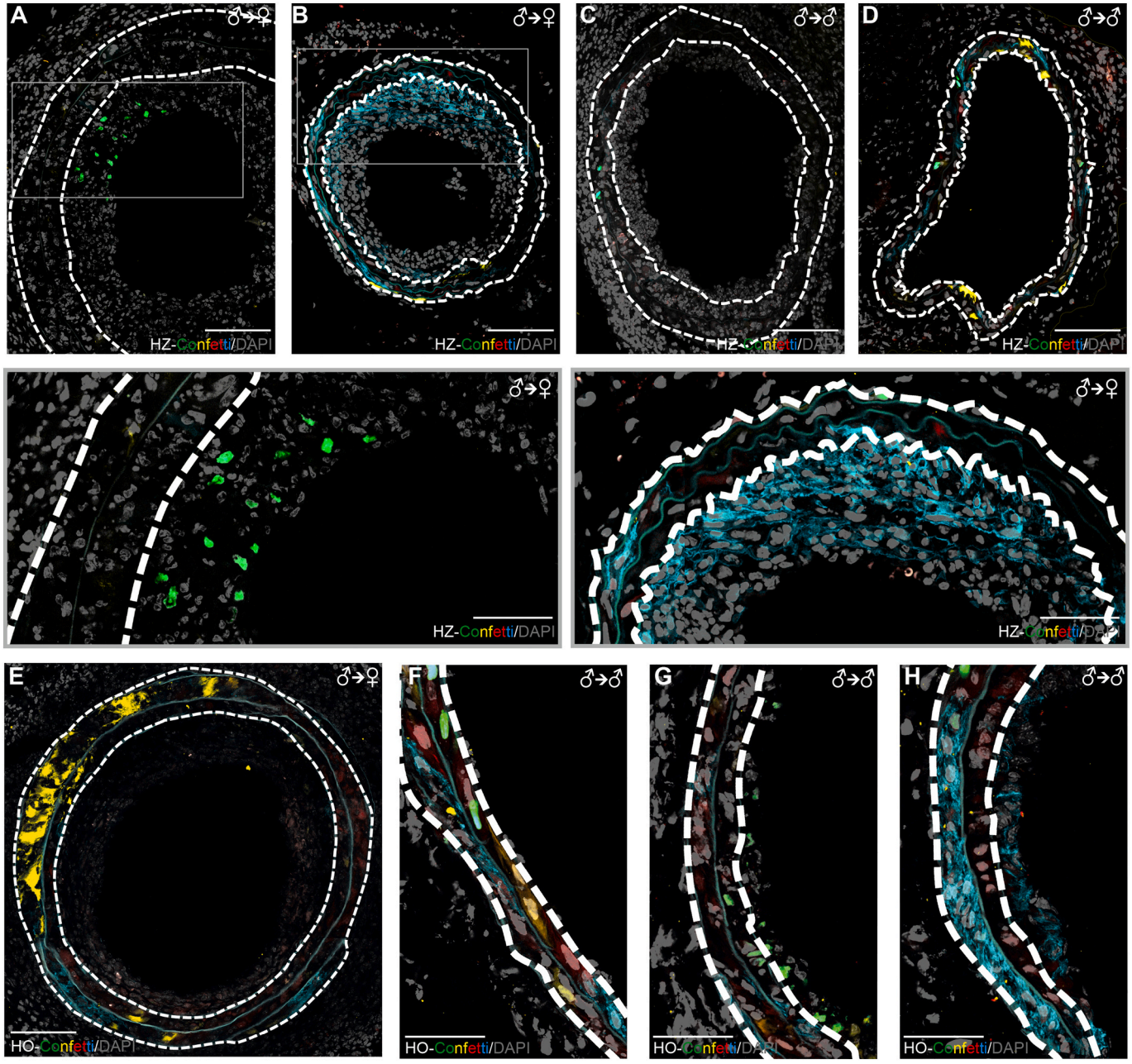

Fig. 3. Clonal architecture of SMCs in carotid transplantation. (A-B) Monoclonal expansion of a GFP$^+$ and a CFP$^+$ SMC clone was observed in sex-mismatched transplants. The indicated areas are shown below in higher magnification. (C-D) Sex-matched control experiments in which Confetti$^+$ carotids from tamoxifen-injected males were transplanted into non-recombined male recipients. Most Confetti$^+$ cells have been eradicated and the example in C shows neointima formation. E) In sex-mismatched homozygous Confetti grafts, the numbers of Confetti$^+$ cells in the media were increased compared with transplants from hemizygous Confetti mice. An example of clonal expansion of YFP (yellow fluorescent protein)$^+$ cells in the arterial media is shown. (F-H) Negative sex-matched control experiments where both donor and recipient were male, tamoxifen-injected mice with Confetti$^+$ SMCs. Most sections showed normal arterial walls with the characteristic mosaic Confetti expression, but some areas in one of the mice showed Confetti$^+$ neointima accompanied by underlying clonal expansion of medial SMCs. Scale bars 100 μm and 50 μm in magnified regions.

4.3. Experiment 2 - Clonal architecture of neointimal SMCs in allograft vasculopathy

To investigate the clonal architecture of neointimal SMCs in allograft vasculopathy, we transplanted 10 carotid arteries from male hemizygous Myh11-CreER^{T2}-Confetti mice into female non-fluorescent Confetti littermates. Confetti^{+} SMCs were found in the intimal lesions in 3 of the 10 samples. One sample harbored a GFP-positive clone in the neointima and the second had a CFP-positive clone approx. covering 50% of the neointima and a few cells spanning into the media (Fig. 3A-B). In the third Confetti^{+} sample, only 3 YFP^{+} cells were detected in the neointima. In all 10 sex-mismatched samples, only a few Confetti^{+} SMCs survived in the media. To understand if the loss of Confetti^{+} cells was driven by immune recognition of the Confetti-encoded proteins, rather than the sex-mismatching alone, we analyzed two sex-matched transplants where carotids from male Myh11-CreER^{T2}-Confetti carotids were inserted into non-induced male littermate recipients. One of the control samples developed a neointimal lesion similar to the sex-mismatched samples, containing substantial lymphocytic infiltration and almost complete eradication of the Confetti signal (Fig. 3C). The other control did not develop neointima, but only few Confetti positive cells could be detected in the media (Fig. 3D). Additionally, the Confetti signal in both control transplants appeared drastically decreased compared to our own experience and previous publications [4]. These observations indicate an immune reaction toward the foreign fluorescent proteins in the Confetti transgene.

In an attempt to increase the contribution of Confetti+ cells in the allograft vasculopathy model, we performed a similar experiment with 8 carotids from homozygous Myh11-CreER^{T2}-Confetti donor animals transplanted into female non-recombined littermates. In addition to potentially enhancing the number of Confetti^{+} cells because of the higher cellular recombination rate, these animals also feature 10 fluorescent phenotypes instead of 4, which strengthens the ability to determine clonal structures. Furthermore, we performed 4 sex-matched control transplantations with 2 syngeneic homozygous Myh11-CreER^{T2}-Confetti transplantations and 2 Myh11-CreER^{T2}-Confetti-to-mT/mG male-to-male transplantations, as negative controls. In these four controls, SMC-specific recombination was induced both in donor and recipient animals.

The use of homozygous donors did not increase the investment of Confetti^{+} cells in the neointima, which were found in 2/8 sex-mismatched samples. In both cases, they consisted of a single clonal patch. However, more Confetti^{+} cells were retained in the media compared with the hemizygous Confetti grafts, indicating clonal expansion of medial SMCs within the diseased media (Fig. 3E).

The four sex-matched controls featured normal Confetti^{+} arterial

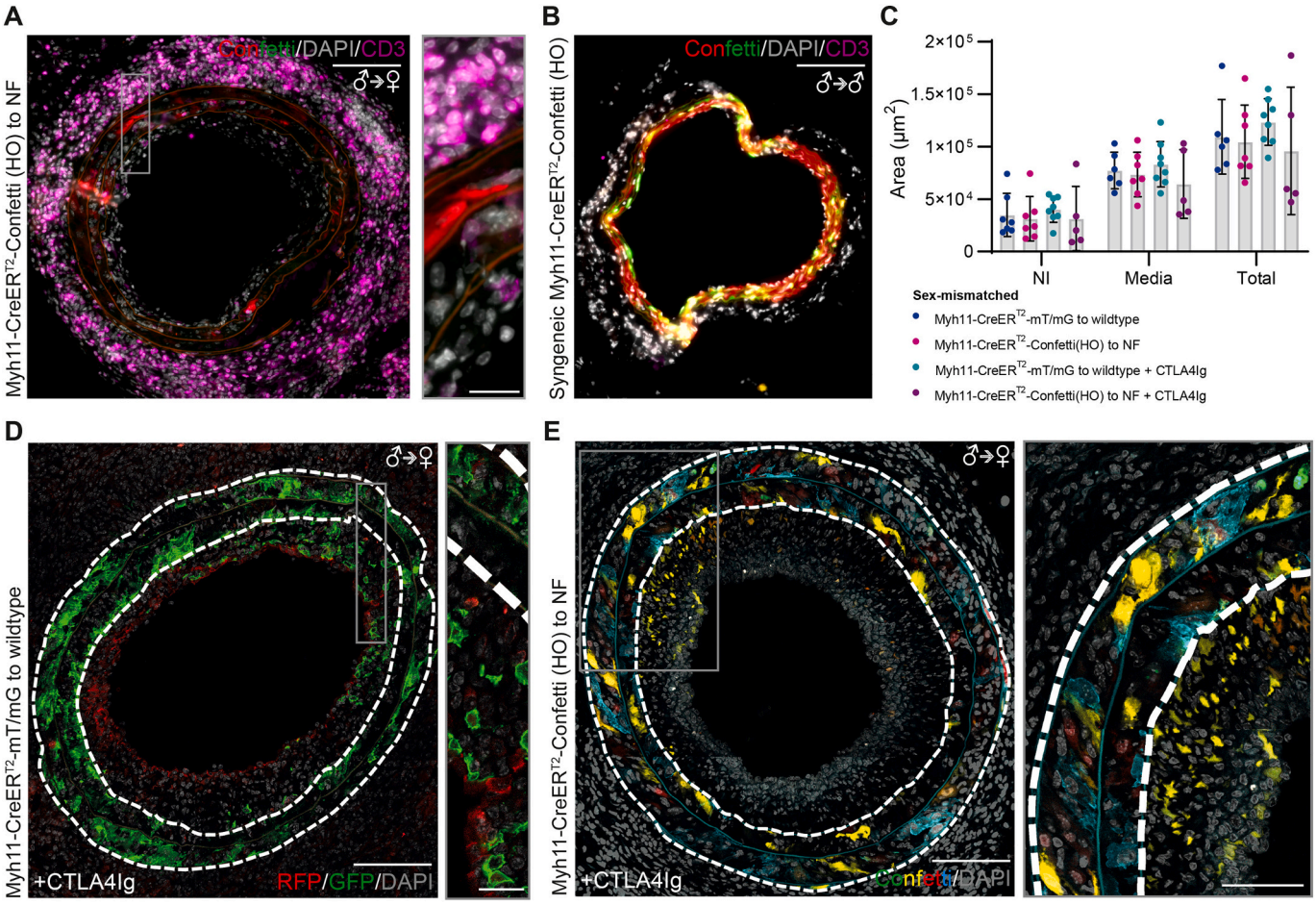

Fig. 4. T cell inhibition with CTLA4Ig in allograft vasculopathy. (A) CD3 Immunofluorescence staining visualizing substantial CD3^{+} T cell infiltration in sex-mismatched transplants. (B) Few CD3^{+} T cells were observed in sex-matched transplants. The available filters on the fluorescence microscope limited the Confetti detection to RFP and YFP+GFP, visualized in red and green, respectively. (C) Neointimal (NI), medial and total areas were compared for CTLA4Ig treated and non-treated transplants (from experiment 1 and 2). No obvious difference was observed in any of the comparisons. Data are shown as mean ± SD. (D-E) Clonal expansion of neointimal SMC was observed in Myh11-CreER^{T2}-mT/mG and Myh11-CreER^{T2}-Confetti (HO) transplants in CTLA4Ig-treated recipients. RFP, red fluorescent protein; GFP, green fluorescent protein; HO, homozygous; NF, non-fluorescent female littermates. Scale bars 100 μm and 20 μm and 50 μm for the magnified regions in A+D and E, respectively.

walls (Fig. 3F), except for two small areas with thin neointima and underlying SMC clonal expansion in one mouse (Fig. 3G-H), potentially caused by local injury during the transplantation procedure.

4.4. Experiment 3 - Medial SMC protection with CTLA4Ig treatment

Based on the first two experiments we hypothesized that the variable and often low proportion of GFP/Confetti positive signals in the neointima might be caused by an excessive T cell-mediated immune response against the male tissue. This could potentially be accelerated by an immune response targeting cells expressing the foreign fluorescent proteins. To estimate T cell involvement, we stained the homozygous Confetti grafts from Experiment 2 for the T cell marker CD3. In all 8 sex-mismatched samples, there were excessive amounts of T cells in the adventitia of the transplants (Fig. 4A). $CD3^+$ T cells were also found in the media and neointima of all samples, however, the amount varied between samples. Few $CD3^+$ T cells were observed in the sex-matched control samples (Fig. 4B).

To decrease the T cell-mediated rejection, and thus hopefully protect more fluorescent-positive donor SMCs, we repeated experiments but blocked co-stimulation of T cells with CTLA4Ig. CTLA4Ig has previously been used successfully to dampen the immune response in murine transplantation experiments [16].

We performed 8 male Myh11-CreER^{T2}-mT/mG to female wildtype and 5 male Myh11-CreER^{T2}-Confetti (homozygous) to female non-recombined Confetti transplants. Two female mT/mG-to-mT/mG sex-matched surgeries served as controls. The animals received a single i.p. injection of 0.25 mg CTLA4Ig during surgery.

Lesion and medial size in CTLA4Ig treated mice were similar to those of Experiment 1 and 2 (Fig. 4C). For the Myh11-CreER^{T2}-mT/mG transplants, we did not detect any obvious difference compared with the experiments performed without CTLA4Ig treatment. GFP-positive cells were only found in the neointimal lesion of 6/8 mT/mG samples (Fig. 4D). In the Myh11-Confetti (HO) samples, the number of medial Confetti$^+$ cells appeared higher compared to Experiment 2 and 5/5 samples harbored clonal Confetti$^+$ cells in the neointima (Fig. 4E). The 2 negative controls did not develop neointima and medial integrity was conserved (data not shown). However, we did not find any clear and substantial improvement when administering CTLA4Ig in our studies, as large areas of the neointima remained Confetti negative.

To evaluate the phenotypic modulation of the SMCs we stained the samples for Acta2. We found the medial SMCs in our samples to have reduced Acta2 expression, whereas the SMCs in controls remained in a contractile phenotype with high Acta2 expression (Fig. 5A-B). Confetti-positive cells in the neointima presented with concomitant Acta2 signal. Neointimal lesions were not necessarily Acta2 positive, probably representing areas dominated by leucocyte infiltrations. We observed Acta2 positive Confetti$^-$ cells, which might originate from Confetti negative SMCs since these account for approx. 17% of the total SMC population. However, these cells could potentially also originate from other mesenchymal cells e.g., endothelial cells or myofibroblasts.

5. Discussion

Delineating SMC origin and clonality holds important information for future treatment development to reduce graft-threatening neointimal fibrosis in allograft vasculopathy. Previous animal models for allograft vasculopathy frequently feature major histocompatibility differences resulting in acute rejection of the donor tissue. Consequently, donor SMCs become completely eradicated and replaced by recipient SMCs originating from the adjoined healthy vasculature [1]. In the present study, we aimed to use a minor mismatch model where rejection is limited to recognition of the minor histocompatibility locus on the Y chromosome [17]. This sex mismatch has previously been described as resulting in neointima formation where the donor cells are conserved [7–9], but direct lineage and clonality tracing of SMCs has not yet been performed.

In Experiment 1, we performed carotid transplantations from male Myh11-CreER^{T2}-mT/mG to female wildtype and non-recombined mT/mG recipients. Both types of experiments confirmed donor-derived SMC contribution to the lesions of sex-mismatched grafts, but large parts of lesions remained devoid of donor SMC-derived cells, including Acta2$^+$ cells, indicating that the model was only partly successful in modeling the donor SMC expansion thought to occur in human allograft vasculopathy. Interestingly, other donor-derived cells contributed to cells within the neointima as seen in male Myh11-CreER^{T2}-mT/mG carotids transplanted to non-fluorescent wildtype females. The RFP$^+$ cells in those grafts are unlikely to be of SMC origin, as we achieved almost complete recombination of the mT/mG transgene. They could be derived from endothelial-to-mesenchymal transitioning of donor ECs, which were preserved in grafted arteries, or from adventitial fibroblasts. New lineage tracing experiments with alternative Cre lines will be required to resolve this question, but both sources of neointimal cells have been described in other models with severe vascular injury [18–20].

Another central observation from Experiment 1 was that the reporter fluorescent proteins themselves appeared to be immunogenic. When female RFP$^+$ carotids were transplanted to wildtype sex-matched controls, we observed substantial lesion development in 2/4 transplants. The *Aequorea Victoria* jellyfish GFP that is expressed in mT/mG mice has previously been found minimally immunogenic in the C57BL/6 strain [15], but this may be context-dependent, and immunogenicity may be

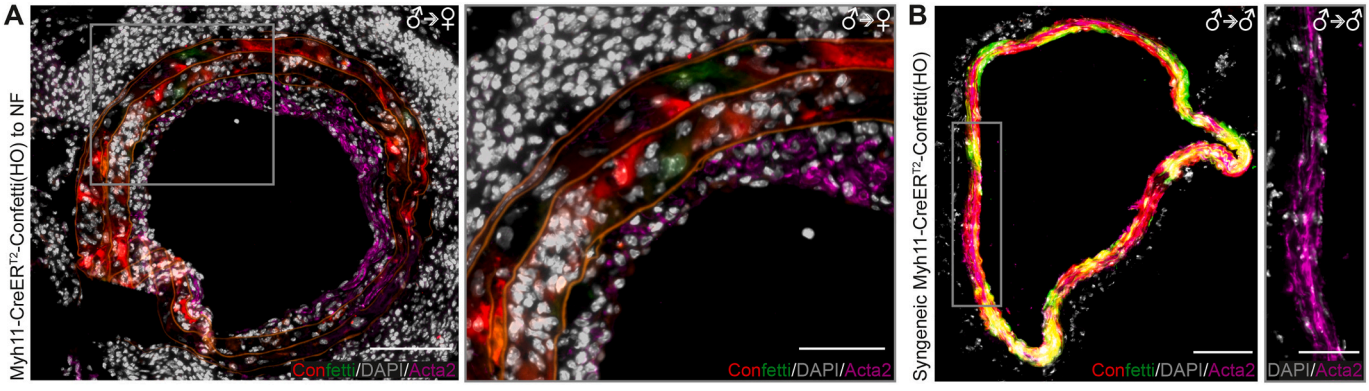

Fig. 5. Assessment of SMC phenotype with immunofluorescent Acta2 staining. (A) Example of Acta2 staining in a sex-mismatched transplantation. (B) Example of an Acta2 staining in a male sex-matched control, where both donor and recipient were recombined. The available filters on the fluorescence microscope limited the Confetti detection to RFP and YFP+GFP, visualized in red and green, respectively. HO, homozygous; NF, non-fluorescent female littermates. Scale bars 50 μm and 25 μm in magnified regions.

higher in vessels with ongoing alloreactions. RFP is only tolerated in C57BL/6 mice when modified to a monomeric form [21], and the RFP expressed in mT/mG mice is the dimeric tdTomato derived from the *Discosoma* coral [22]. The expressed reporters may thus contribute to the immune rejection leading to allograft vasculopathy and it cannot be excluded that differential immune reactions toward cells marked with different reporters can bias results.

In Experiment 2 and 3, we performed transplantations of hemizygous and homozygous Myh11-CreER^{T2}-Confetti male grafts into female littermates. Neointimal SMC patches expressing Confetti multi-color reporter were observed, indicating that the donor-derived SMC patches from the first experiment must be of mono- or oligo-clonal origin, particularly since the developed Confetti^{+} clones were of similar size to the patches of GFP^{+} cells in the grafted mT/mG arteries. However, the contribution overall of Confetti^{+} SMCs was low and presumably significantly reduced by fluorescence-specific rejection. A further indication of reporter-specific rejection was that male sex-matched control transplants developed lesions and loss of Confetti^{+} SMCs when transplanted into uninduced male littermates. On the other hand, when male recipients were induced and expressed Confetti or mT/mG proteins, no lesions developed in 3 out of 4 Confetti^{+} donor arteries. Thus, immune-mediated rejection of the fluorescent reporters, especially RFP, might be impairing reporter^{+} SMC survival in non-fluorescent recipients. Importantly, the GFP in Confetti is not the *Aeqourea victoria* jellyfish protein but codon-optimized *Renilla reniformis* GFP (hrGFPII) derived from a coral [23]. In addition to the potentially immunogenic properties of a coral-derived GFP, the hrGFPII has previously been reported as cytotoxic in mouse muscle [24]. YFP and CFP are monomers derived from the jellyfish GFP protein [25], thus presumably minimally immunogenic in mice. The RFP expressed in Confetti mice is the dimeric tdimer2 [22], derived from *Discosoma* coral, and therefore similar to the tdTomato potentially immunogenic in this model. However, further studies are needed to determine the actual immunological properties of the different mT/mG and Confetti reporters in C57BL/6 transplantation studies.

Recently, a study by Almonte et al. employed global fluorescent labeling to track cells of donor (tdTomato^{+}) and recipient (YFP^{+}) origin in a carotid AV model similar to ours [9]. In agreement with our study, the authors observed high variability in contributions of donor- and recipient-derived cells in the developed lesions. Interestingly, they found that 55% of Acta2^{+} neointimal cells were derived from the donor, but the design did allow lineage tracing specifically from SMCs [9]. It was not addressed if the potential immunogenic properties of RFP^{+} donor cells limited donor contribution, but this cannot be excluded.

The consistent negative selection of reporter^{+} cells observed in experiment 1 and 2 led to speculations on whether administered immunosuppression would preserve more donor SMCs. Effective immunosuppression is expected to reduce allograft vasculopathy overall and, therefore, lead to less total neointimal SMCs, but we considered that moderate suppression may avoid the eradication of donor cells while still producing sufficient local stimuli for their proliferation. In experiment 3, we, therefore, sought to reduce the rejection of the fluorescent grafts by blocking T cell-specific co-stimulation by administering CTLA4Ig [26]. We did not observe any clear increase in reporter-positive SMC survival nor a difference in the size of the developed lesions. This is presumably explained by the model featuring chronic and not acute rejection mechanisms which are more likely to be influenced by CTLA4Ig [16]. However, the administration at the time of surgery might also have been suboptimal, and delayed or repeated administration might have been more effective [27].

Recently a transgenic mouse model facilitating the induction of central tolerance for selected reporter proteins has been developed [28]. In this particular mouse model, a transgene allows expression of scrambled fragments of chosen reporters from a single open reading frame, while avoiding expression of functional proteins [28]. A similar construct securing constitutive expression of fragmented CreER^{T2} and Confetti proteins in the recipient mice could thus induce central tolerance for the recombinase and reporters. This could potentially be an extremely valuable tool, not only to avoid rejection in transplantation studies but all studies involving mice with inducible reporter expression.

In conclusion, our studies indicate that donor-derived SMCs contribute to allograft vasculopathy in male-to-female transplants and that the contribution is driven by the clonal expansion of a few individual SMCs. However, our studies were impaired by negative selection of fluorescence-positive SMCs, presumably caused by immune-mediated rejection of the reporters. Overcoming these technical problems will be key to obtaining a more comprehensive understanding of the clonal structure of SMC-derived cells in murine allograft vasculopathy in future studies.

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CRediT authorship contribution statement

Gro Grunnet Pløen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Charlotte Brandt Sørensen:** Writing – review & editing, Supervision. **Jacob Fog Bentzon:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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