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Citation: Methe, Heiko et al. "T-helper 2 Cells Are Essential for Modulation of Vascular Repair by Allogeneic Endothelial Cells." The Journal of Heart and Lung Transplantation 29.4 (2010): 479–486.

As Published: http://dx.doi.org/10.1016/j.healun.2009.11.006

Publisher: Elsevier

Persistent URL: http://hdl.handle.net/1721.1/75441

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Published in final edited form as:
J Heart Lung Transplant. 2010 April ; 29(4): 479–486. doi:10.1016/j.healun.2009.11.006.

Th2 cells are essential for modulation of vascular repair by allogeneic endothelial cells

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Abstract

Background—Endothelial cells (EC) embedded within three-dimensional matrices (MEEC) when placed in the vascular adventitia control lumenal inflammation and intimal hyperplasia. Matrix-embedding alters endothelial immunogenicity *in vitro*. T helper (Th) driven host immunity is a major impediment for of allogeneic grafts. We therefore aimed to identify if modulation of T helper balance would affect immune compatibility and endothelial regulation of vascular repair in vivo.

Methods—Pigs (n=4/group) underwent balloon injury of both carotid arteries and were left alone (group 1) or received perivascular implants of porcine MEEC (group 2), a 12 days course of cyclosporine A (CsA) (group 3), or a combination of MEEC and CsA (group 4). Host immune reactivity (EC-specific antibodies, activation of splenocytes) was analyzed after 28 and 90 days in 2 pigs/group respectively.

Results—MEEC treatment alone induced formation of EC-specific IgG_1-antibodies (41±6 mean fluorescence intensity (MFI)) and differentiation of host splenocytes into Th2, but not Th1, cytokine-producing cells (IL-4: 242±102, IL-10: 273±114 number of spots). Concomitant CsA-therapy reduced the frequency of IgG_1-antibodies (25±2 MFI; $p<0.02$) and Th2-cytokine producing splenocytes upon MEEC treatment (IL-4: 157±19, IL-10: 124±26 number of spots; $p< 0.05$). MEEC significantly inhibited luminal occlusion 28 and 90 days after balloon injury compared to untreated controls (12±7 vs. 68±14%; $p<0.001$) but to a lesser extent in the face of immunomodulation with concomitant CsA-treatment (34±13%; $p<0.02$ vs. group 2).

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Disclosures: Elazer Edelman is founder of Pervasis Technologies which has licensed patents from MIT on matrix-embedded endothelial cells. Heiko Methe is a paid consultant to Pervasis Technologies.

Conclusions—MEEC do not induce a significant Th1-driven immune response expected from alloimplants, but do enhance differentiation of splenocytes into Th2-cytokine producing cells. Reduction in this Th2 response reduces the vasoregulatory effects of allogeneic EC after injury.

INTRODUCTION

The confluent endothelium, comprised of a confluent monolayer of endothelial cells (EC) regulates every phase of the vascular response to injury (1). Isolated EC however do not represent the endothelium and in fact promote, rather than inhibit, thrombosis, inflammation and proliferation of other vascular cells. The embedding of EC within three-dimensional matrices (MEEC) preserves the confluent regulatory phenotype of EC (2–4) and allows for the examination of endothelial regulation of vascular repair in controlled experimental settings.

MEEC are especially helpful in understanding the interaction between EC and immune activation. Different forms of EC can be embedded within matrices and implanted *in vivo* without direct contact with host immune cells or eliciting an immune response (2,5). In this manner one can begin to examine the effects of EC on vascular repair with independent modification of the immune state. We accomplished this by using the well-established immunosuppressant (cyclosporine A, CsA) in an allogeneic vascular injury pig model (3,4). CsA is not only an immune modulator in tissue and organ transplantation but its long-term use is limited by side effects such as development of endothelial dysfunction and transplant vasculopathy. The choice of CsA as an immunosuppressant is further amplified by the conflicting results on direct CsA effects on EC immunogenicity *in vitro* (6–11) as these influences might have a direct effect on the therapeutic use of allogeneic MEEC.

METHODS

Isolation and matrix-embedding of endothelial cells

Porcine aortic EC (PAE) were isolated from LargeWhite adult swine aortae by collagenase treatment. PAE were grown to confluence either embedded within Gelfoam blocks (Pfizer, NY) as previously described (2) or on polystyrene tissue culture plates (TCPS) in DMEM supplemented with 2 mM L-glutamine, 10% FBS (HyClone, UT), 100 U/ml Penicillin G, and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY). EC surface attachment and confluence on Gelfoam matrices were demonstrated by confocal microscopy (data not shown). Cell viability was determined by trypan blue exclusion and a LIVE/DEAD viability/ cytotoxicity kit (Molecular Probes, OR). For cell counting, blocks were washed with HBSS (Life Technologies, Inc), digested with collagenase (1 mg/mL, type I, Worthington Biochemical Corp) and density determined with a Neubauer's counting chamber.

Animals, surgical procedure and tissue processing

This study conformed to the US Department of Agriculture regulations and National Research Council guidelines and to the guidelines specified in the National Institutes of Health *Guide for Care and Use of Laboratory Animals*. All animal procedures were reviewed and approved by the local ethics committee on animal care. The study used 16 domestic LargeWhite pigs weighing 47.4±1.2 kg and 12 to 14 weeks of age. Anesthesia was induced with Telazol (4.4 mg/kg; Wyeth, Madison, NJ), ketamine (2.2 mg/kg), glycopyrrolate (0.2 mg/mL), acepromazine maleate (10 mg/mL), and thiopental 2.5% (0.22 mL/kg). The pigs were intubated, anesthesia was maintained with isoflurane inhalant (0.5% to 2%) delivered through a volume-regulated ventilator. All of the pigs underwent vascular balloon injury of both carotid arteries as previously described.(3) The pigs were assigned to four different treatment groups (n=4/group): vascular injury and sham surgery (neck incision) alone (group 1), with perivascular implantation of 5×10^5 matrix-embedded PAE at day 0 alone (group 2), with 12

day immunosuppressive course of CsA (10 mg/kg/d qd, Novartis Pharmaceuticals Corp., East Hanover, NJ) begun prior to implantation and sham operation (neck incision, group 3), and a fourth group with a 12-day immunosuppressive course of CsA and implantation of 5×10^5 matrix-embedded PAE. A 12 day immunosuppressive course of CsA (10 mg/kg, i.v. q.d.) has been demonstrated effective in inducing tolerance in kidney transplantation or heart plus kidney transplant recipient pigs across MHC class I mismatched barrier (12,13). CsA aiming levels were 400–800 ng/ml and reached throughout the whole study period in all of the treated animals.

All neck incisions healed well and all animals gained weight during the postoperative period. No animal was lost during follow-up. Two animals of each group were sacrificed at 28 and 90 days respectively and angiograms quantified (baseline and at time point of euthanasia). Angiograms were reviewed by an operator unaware of the treatment groups. Calibrating with the outer diameter of the contrast-empty catheter, the diameter stenosis percentage was measured and the area stenosis percentage determined.

EC Biosecretory Function

In vitro biosecretory function of PAE in Gelfoam and on TCPS with and without CsA incubation (400 ng/ml added for the last 48 hours of culture) were compared (14,15). Total protein production was determined by Bicinchoninic Acid protein assay-kit (Pierce). Total glycosaminoglycans and heparan sulfate production were determined using a dimethylmethylene blue assay before and after cell-conditioned medium treatment with chondroitinase ABC (0.1 U/sample, Seikagaku America) for 3 hour at 37°C to eliminate chondroitin and dermatan sulfate (16,17). Prostacyclin concentrations were determined by a 6-ketoprostaglandin F1α enzyme immunoassay system (Amersham Biosciences). Transforming growth factor-β production was determined using standard ELISA assays (Amersham).

Expression of proinflammatory molecules

Expression-levels of costimulatory and adhesion molecules on cultured PAE were quantified by flow cytometry as previously described (2). In short PAE monolayers or PAE embedded in Gelfoam were harvested after culture with or without CsA (400 ng/ml added for the last 48 hours of culture) stimulated with 100 U/mL TNF((CD54, CD80, CD86, CD106, E-selectin, P-selectin) for 48 hours. 104 cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson).

Host immune surveillance—Sera were collected serially from 0 to 90 days after vascular injury and stored at −70°C. Splenocytes were isolated 28 and 90 days after vascular injury from two animals/group respectively. Spleens were harvested and cut in several pieces under sterile conditions. Clumps were immersed in solution and further dispersed by drawing and expelling the suspension several times through a sterile syringe with a 19-G needle. The suspension was filtered through a 200 (m mesh nylon screen to remove debris. Erythrocytes were lysed by treatment with ACK buffer (Cambrex, Walkersville, MD) for 5 minutes at room temperature. Remaining cells were washed twice with RPMI (containing 2 mM L-glutamine, 0.1 M HEPES, 200 U/ml Penicillin G, 200 μg/ml streptomycin, 5% heat-inactivated calf serum, Life Technologies) and immediately used.

EC-specific antibodies

For determination of reactive antibodies specific for the implanted PAE serum was isolated at days 0, 5, 12, 28, 56 and 90. 2×10^5 PAE, from the same strain as the implanted cells, were detached from cell culture plates with 0.25% trypsin/0.04% EDTA, pelleted, washed, and resuspended in FACS buffer (PBS, 1% FBS, 0.1% sodium azide, Sigma Chemicals, MO).

These cells were then incubated with porcine serum from the four treatment groups for 60 min at 4°C (diluted 1:10 in FACS buffer). After washing three times with FACS buffer, cells were incubated with mouse anti-porcine immunoglobulin (Ig)M (clone K52 1C3), IgG_1 (clone K139 3C8), or IgG_2 (clone K68 Ig2; MorphoSys US Inc., NC) respectively. Following 30 min incubation at 4°C, samples were again washed twice with cold FACS buffer, and incubated with FITC-conjugated rabbit anti-mouse IgG (MorphoSys US Inc.) for another 30 min at 4°C. Following two washing steps with FACS buffer, cells were fixed in 0.25 ml 1% paraformaldehyde, and 10^4 cells were analyzed by flow cytometry. Control samples included sera from naïve pigs and antibody incubation of PAE without serum to account for non-specific binding of the secondary antibodies (background). Data are presented as mean fluorescence intensity per PAE with background subtraction for all cells analyzed.

Enzyme-linked immunosorbent spot (ELISPOT) assay

ELISPOT assays were conducted as previously described (5,18). In short, immunospot plates (Millipore, MA) were coated with 5 μg/ml of anti-porcine (Biosource, CA) interferon (IFN)-γ, interleukin (IL)-2, IL-4, or IL-10 monoclonal antibodies overnight. 5×10^5 PAE from the same strain as the implanted cells were placed in immunospot wells with 5×10^5 splenocytes and cultured for 48 hours at 37°C/5% CO_2. After washing with deionized water immunospot wells were incubated overnight with porcine IFN-γ, IL-2, IL-4, or IL-10 monoclonal antibodies (Biosource) followed by incubation with horseradish peroxidase–conjugated streptavidin (BD Pharmingen, CA) for one hour. Plates were developed using 3-amino-9-ethyl-carbazole (BD Pharmingen) and resulting spots counted on a computer-assisted enzyme-linked immunospot image analyzer (Cellular Technology, CA). To account for background in data analysis the number of spots in negative control wells (medium, splenocytes, or PAE alone) were subtracted from those in responsive wells.

Calcein-Acetyoxymethyl release assay

2×10^4 TCPS-cultured PAE (same strain as the implanted cells) were incubated with 15 μM calcein-acetyoxymethyl (Calcein-AM, Molecular Probes) for 40 min at 37°C with occasional agitation. After two washes with complete medium, porcine splenocytes were added for 3 hours at 37°C/5% CO_2 at effector:target cell ratios of 50:1 to 1:1. Calcein-AM release was measured using a Fluoroskan Ascent FL dual-scanning microplate luminofluorimeter (Thermo Electron Corporation, TX). Specific lysis was calculated according to the formula [(test release - spontaneous release)/(maximum release - spontaneous release)] × 100. Spontaneous release represents calcein-AM release from target cells in medium alone, and maximum release is the calcein-AM release from target cells lysed in medium plus 2% Triton X-100, each measured in at least six replicate wells. Data presented within this manuscript represents effector:target ratios of 25:1 as calcein-AM release reached a plateau at this ratio (5).

Statistical analysis

All statistical analyses were performed with JMP software (SAS Institute, USA 2002). Data were normally distributed and expressed as mean±SD. Comparisons between 2 groups were analyzed by Student's *t* test, and comparisons between more than 2 groups were analyzed by ANOVA. A value of $p<0.05$ was considered statistically significant.

RESULTS

In vitro biosecretory endothelial cell function and expression of proinflammatory molecules are not influenced by Cyclosporine A

PAE cultured in three-dimensional Gelfoam blocks produce similar amounts of glycosaminoglycan (2.8±0.15 versus 2.8±0.12 $\mu g/10^6$ cells), heparan sulfate (1.5±0.04 versus

1.6±0.06 μg/10^6 cells), prostacyclin (74±5.1 versus 78±3.4 pg/10^6 cells), and transforming growth factor-β (819±99 versus 864±103 pg/10^6 cells) as PAE grown in TCPS. Incubation of PAE with 400 ng/ml CsA for the last 48 hours had no effect on biosecretory endothelial function (Table).

In addition, coincubation of PAE with 400 ng/ml CsA for the last 48 hours was without effect on basal and cytokine-induced expression of adhesion (ICAM-1, VCAM-1, E-selectin, P-selectin), costimulatory (CD80, CD86) and MHC II molecules with lower expression levels in MEEC throughout (Fig. 1).

Cyclosporine A limits the induction of PAE-specific IgG_1 antibodies *in vivo* after implantation of allogeneic matrix-embedded PAE

Circulating anti-PAE specific IgM and IgG_{2a} levels were similar in pigs among the four treatment groups (Fig. 2A, B). MEEC implantation induced a significant rise in PAE-specific IgG_1 antibodies from day 12 until day 56 post implantation compared to control animals (group1, $p<0.005$) and animals that received a 12 day immunosuppressive course of CsA alone (group 3, $p<0.005$; Fig. 2C). Concomitant CsA administration (group 4) limited the induction of PAE-specific IgG_1 antibodies ($p<0.05$ vs. group 2). Levels of PAE-specific IgM and IgG_{2a} antibodies were not affected by CsA treatment.

Cyclosporine A limits the induction of Th2 expressing splenocytes in animals treated with perivascular implants of matrix-embedded PAE

Differentiation of splenocytes isolated form pigs from the four different treatment groups upon *in vitro* stimulation with the same batch of PAE used for implantation was analyzed via ELISPOT assay 28 and 90 days after surgery. As expected splenocytes from pigs that did not receive PAE (group 1 and group 3) did not differentiate into T-helper cell 1 (Th1) cytokine (IFN-γ and IL-2) or Th2 cytokine (IL-4 and IL-10) producing splenocytes.

Perivascular implantation of MEEC elicited a weak cellular immune response. Splenocytes from pigs that were treated with allogeneic MEEC (group 2) revealed a weak non-significant differentiation into Th1 cytokine producing splenocytes 28 days after surgery (IFN-γ: 15±4, IL-2: 17±8 number of spots, $p=0.52$ vs. group 1 and group 3). Concomitant CsA treatment (group 4) had a non-significant effect on the differentiation of splenocytes into Th1 cytokine producing cells (IFN-γ: 12±3, IL-2: 11±1 number of spots, $p=0.6$ vs. group 1 and group 3). As observed earlier, implantation of allogeneic MEEC induced a significant induction of Th2 cytokine producing splenocytes (IL-4: 237±109, IL-10: 272±119 number of spots, $p<0.002$ vs. group 1 and group 3) (2). Interestingly, concomitant CsA-treatment (group 4) limited the induction of Th2 cytokine producing splenocytes by allogeneic MEEC (IL-4: 156±21, IL-10: 121±38 number of spots, $p<0.05$ vs. group 2; Fig. 3A).

90 days after surgery splenocytes from treatment groups 1 and 3 lacked differentiation into Th1 or Th2 cytokine producing splenocytes. The Th1-inducing effect of matrix-embedded PAE observed after 28 days was not detectable 90 days after surgery (group 2: IFN-γ: 3(1, IL-2: 4 (2 number of spots; group 4: IFN-(: 2(1, IL-2: 3(2 number of spots). A weak induction of Th2 cytokine producing splenocytes was still present 90 days after surgery again with higher levels of Th2 cytokines in the pigs that received only matrix-embedded PAE (IL-4: 78(23, IL-10: 69 (17 number of spots, $p<0.02$ vs. group 1 and group 3) when compared to pigs that received matrix-embedded PAE and a concomitant 12 day course of CsA (IL-4: 47(4, IL-10: 32(7 number of spots, $p<0.05$ vs. group 2; Fig. 3B).

Cyclosporine A has no effect on in vitro PAE lysis by splenocytes—The ability of host splenocytes to damage allogeneic PAE was characterized 90 days after surgery.

Splenocytes isolated from the four treatment groups demonstrated only a weak lytic activity to damage PAE (the same batch as used for the implantation) *in vitro*. Lytic activity of splenocytes from pigs that had received matrix-embedded PAE (group 2) was 1.5-fold higher than from sham-operated pigs (group 1) but the difference did not reach statistical significance. CsA treatment of the pigs had no significant impact on the lytic activity of isolated splenocytes (Fig. 4).

Cyclosporine A affects the vascular reparative effects of allogeneic matrix-embedded endothelial cells

Analysis of carotid angiograms obtained at baseline and euthanasia revealed significant stenosis of the arteries in control pigs without concomitant treatment (group 1, 68±14%). A 12-days course of CsA had no significant impact on the severity of luminal occlusion (group 3, 66±13%). In contrast, perivascular implants of allogeneic MEEC (group 2) had only minimal angiographic signs of luminal occlusion (12±7%; $p<0.001$ vs. group 1 and group 3). Periprocedural therapy with CsA with perivascular MEEC implants (group 4) had a middling effect (34±13%; $p<0.02$ vs. group 2, $p<0.005$ vs. group 1 and 3; Fig. 5). There was no significant difference between the pigs sacrificed after 28 and after 90 days.

DISCUSSION

The embedding of EC within three-dimensional collagen-based matrices not only passively shields the cells from contact with host immune cells, but actively modulates immune reactivity *in vitro* and *in vivo*. MEEC induce regulatory T cells, inhibit dendritic cell maturation, and shift the balance in Th1 and Th2 cells (2,18,21).

Questions have been raised as to whether some form of immune response is important for vascular repair and endothelial modulation of vascular injury. We used CsA as a potent and specific immunmodulator to address these issues. Though the interference by calcineurin inhibitors on T-cell proliferation is well-established (20) direct effects of CsA on EC remain controversial. While some demonstrated that calcineurin inhibitors impaired endothelial functionality - reducing prostacyclin and nitric oxide production, increasing superoxide anion, endothelin-1 and thromboxane A_2 production (10), others showed decreased expression of costimulatory and adhesion molecules by EC (6,7,11) or even unchanged expression of proinflammatory molecules (8,9).

We now show that commensurate with a moderate reduction in MEEC upregulation in IgG_1-antibodies and induction of Th2 cytokine-producing splenocytes, CsA also limits MEEC inhibition of intimal hyperplasia after endothelial denudation. CsA had no effect on vascular injury when administered alone and did no alter MEEC effects on expression of endothelial adhesion and costimulatory molecules, on *in vivo* formation of EC-specific IgM, IgG_{2a} levels, or *ex vivo* lytic ability of isolated splenocytes.

There are still conflicting data on which subset of Th cells is involved in allorejection. The polarized subsets Th1 and Th2 both develop from the same Th precursor and differentiate into the two phenotypes via a complex development process. The dosage of antigen, strength of signal through the T-cell receptor and costimulation influence the initiation of T helper cell differentiation (22). Th1 and Th2 cytokines are cross-regulatory, IL-10 being an inhibitor of the Th1 pathway and IL-12 inhibiting Th2. Evidence is accumulating that Th1 cytokines IFN-(and IL-2 are mediators of acute and chronic graft rejection whereas Th2 cytokines, such as IL-4 and IL-10 may have a protective role and correlate with allograft acceptance □ ADDIN EN.CITE □ ADDIN EN.CITE.DATA (23–26).

Our results of limited induction of Th2 cytokine producing splenocytes by allogeneic MEEC upon concomitant CsA therapy might be mirrored by the reduction in therapeutic efficacy of MEEC in conjunction with concomitant calcineurin treatment. There exists consensus in different experimental settings, that Th2 cytokines are sensitive to the effect of calcineurin antagonists. Rafiq et al. (27) as well as Dumont et al. (28) have shown decreased production of IL-4 and IL-10 upon *in vitro* calcineurin incubation of T lymphocytes. The link between qualities of host alloimmune reactivity, therapeutic efficacy of allogeneic EC to modulate vascular repair and choice of immunosuppressant might by of significance for the choice of immunosuppressive protocols in other transplantation settings. Our results indicate the importance to determine not only the immune modulating effects of a given immunosuppressant but also its effects on the function of transplanted cells and tissues.

Limitations

A series of limitations that must be appreciated in evaluating this trial are that it was performed with a small number of animals and did not evaluate other immunosuppressive agents. Further studies might examine the importance of reduced Th2 cytokines in allograft acceptance in general and for the therapeutic efficacy of allogeneic MEEC in specific.

Acknowledgments

Funding Sources: This work was supported in part by a Philip Morris External Research Program Postdoctoral Fellowship to Heiko Methe and a grant from the National Institutes of Health to Elazer R. Edelman (HL 49039).

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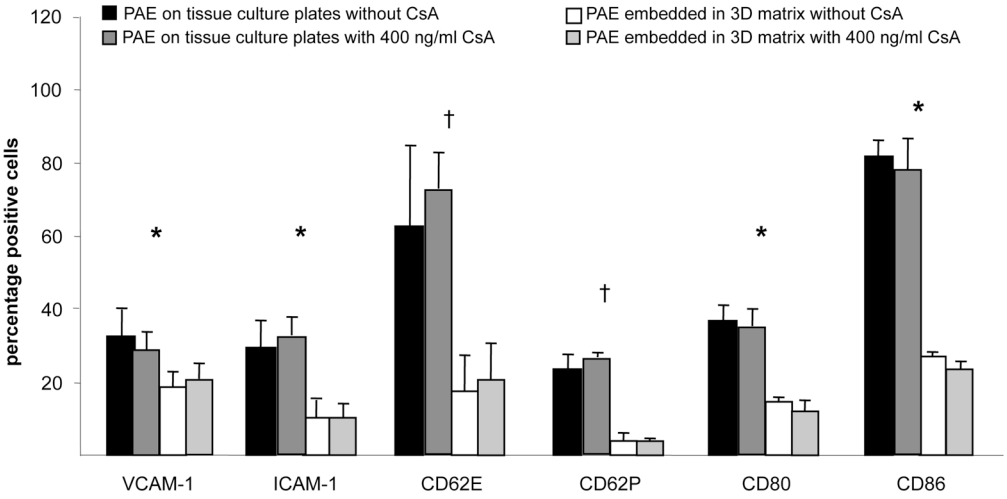
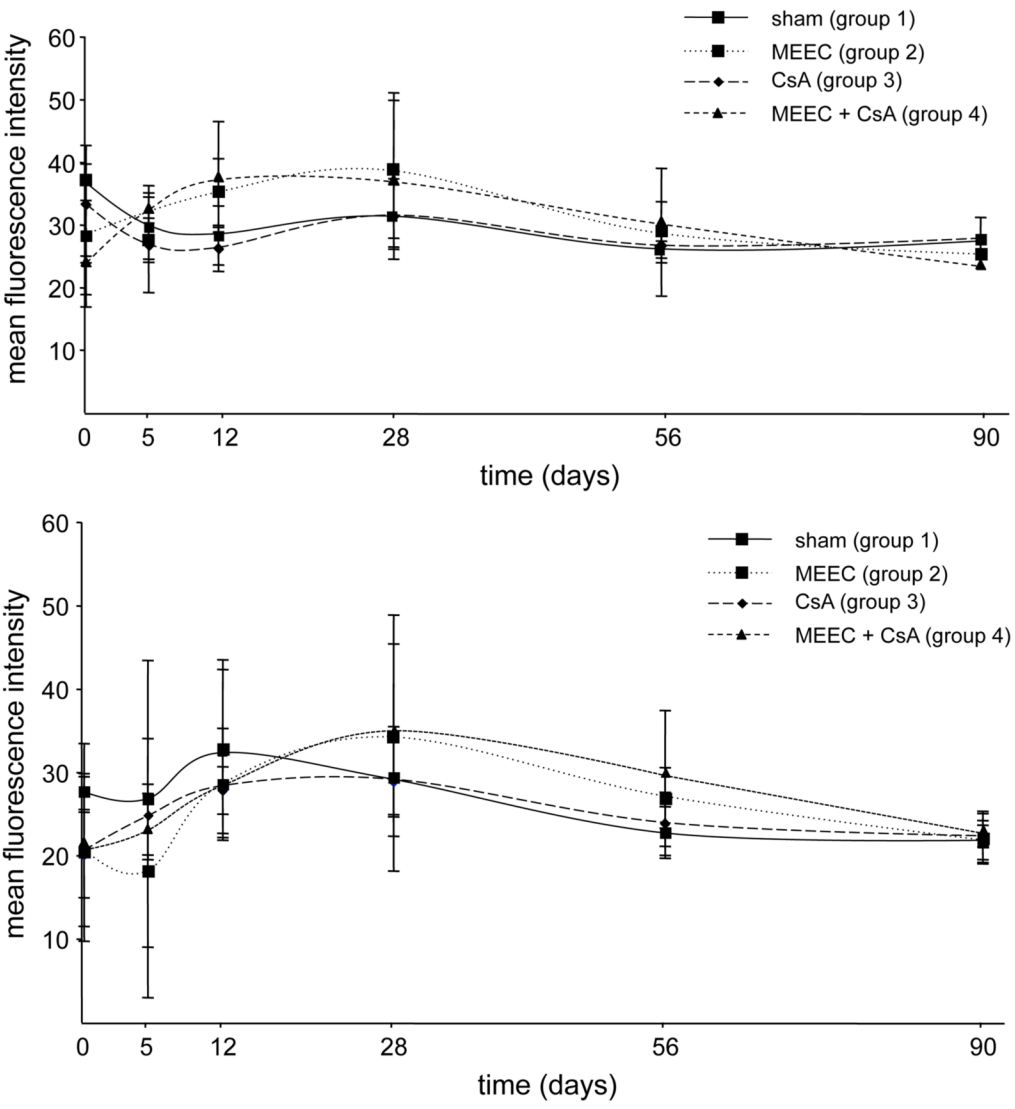


Figure 1. Cyclosporine A is without effect on cytokine-induced endothelial expression of adhesion and costimulatory molecules. Matrix-embedded EC reveal a significant reduced expression of proinflammatory molecules

*p<0.05 PAE on TCPS vs. MEEC

†p<0.02 PAE on TCPS vs. MEEC



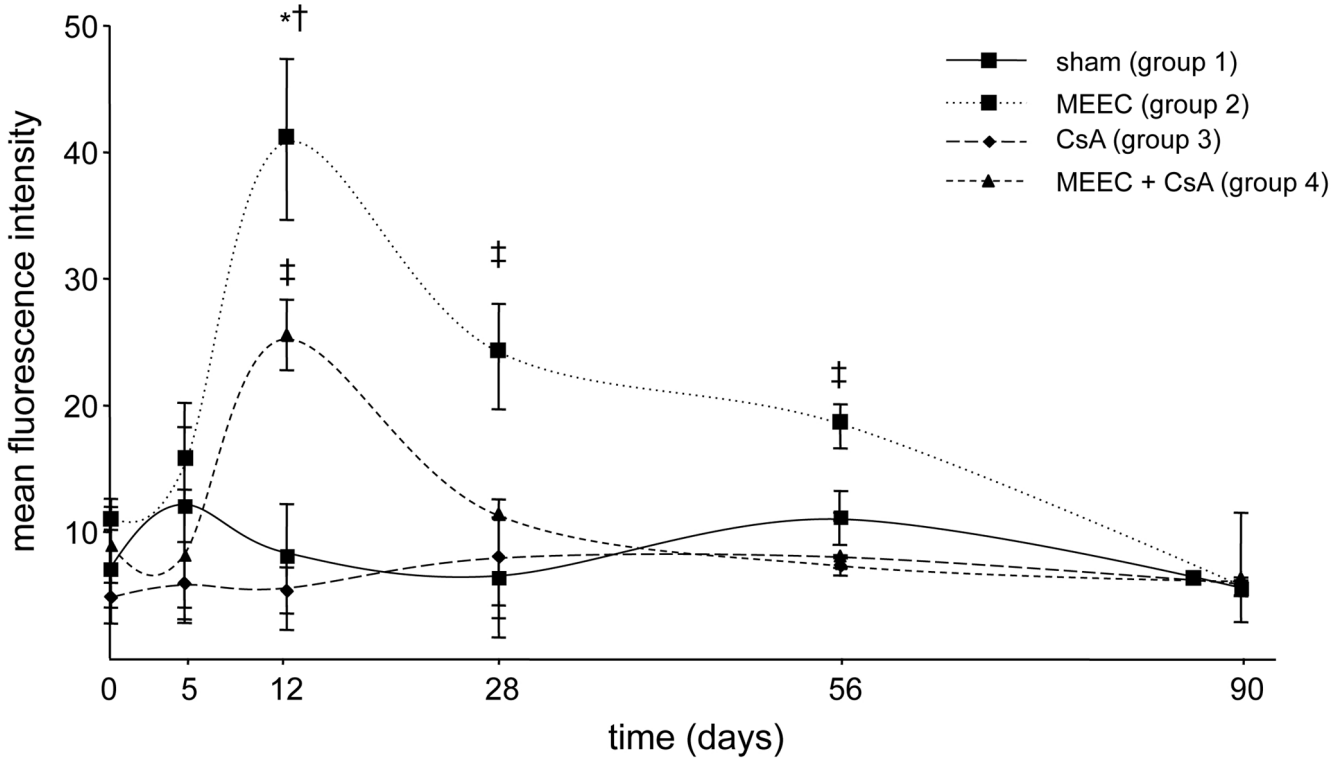


Figure 2. Circulating PAE-specific IgG in pigs from the four treatment groups (n=4/group day 0–28, n=2/group day 29–90). Formation of PAE-specific IgG_1 antibodies by perivascular implantation of allogeneic matrix-embedded PAE (MEEC) is limited by concomitant cyclosporine A treatment (CsA)

A circulating PAE-specific IgM
B circulating PAE-specific IgG_{2a}
C circulating PAE-specific IgG_1
*$p<0.005$ vs. group 1 and group 3
†$p<0.05$ vs. group 4
‡$p<0.02$ vs. group 1 and group 3

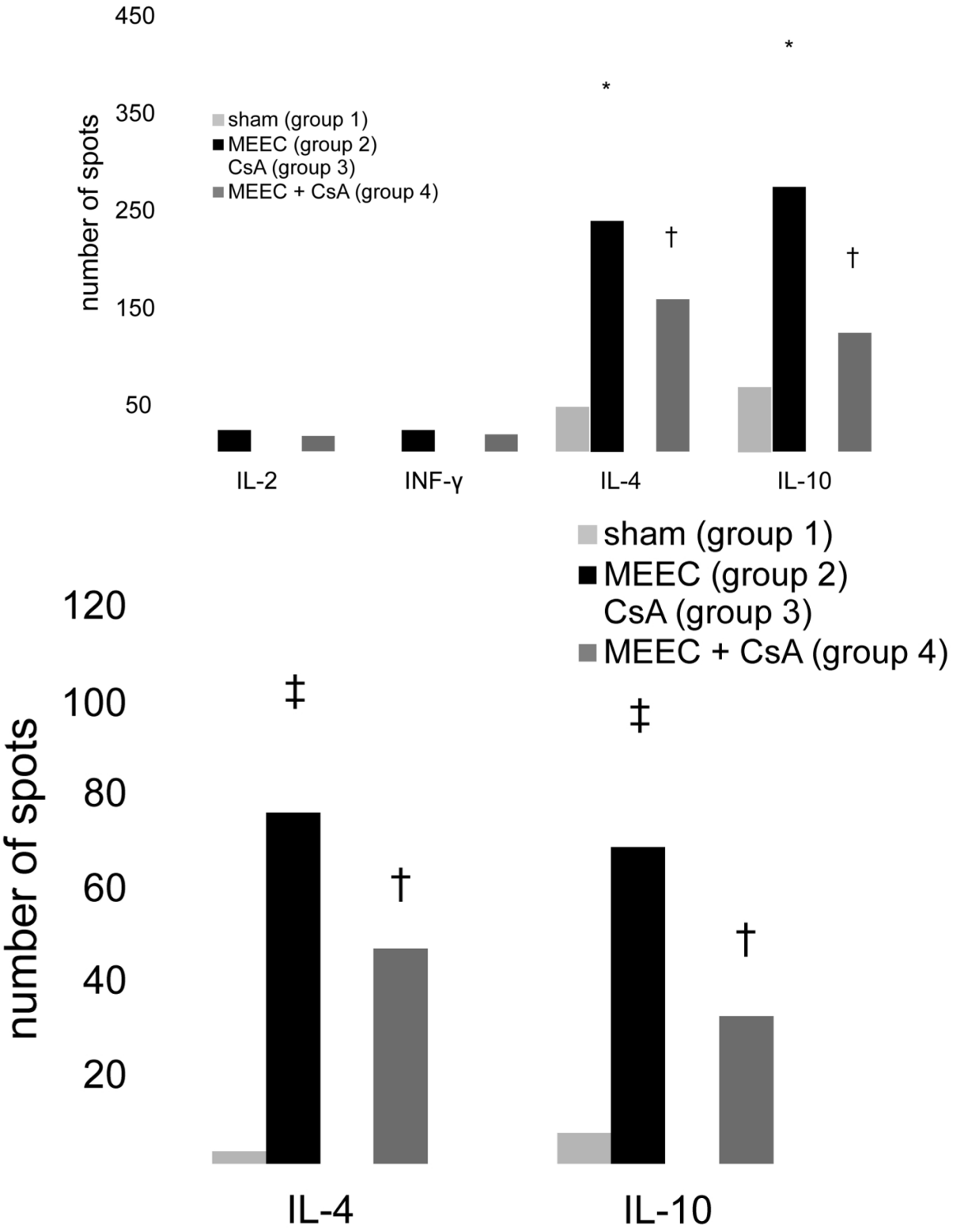


Figure 3. Induction of Th2-polarized splenocytes is reduced in pigs after vascular injury and treatment with perivascular implants of allogeneic matrix-embedded PAE (MEEC) with

concomitant cyclosporine A treatment (CsA). ELISPOT assay was performed 28 (A) and 90 days (B) after balloon injury

*p<0.002 vs. group 1 and group 3

†p<0.05 vs. group 2

‡p<0.02 vs. group 1 and group 3

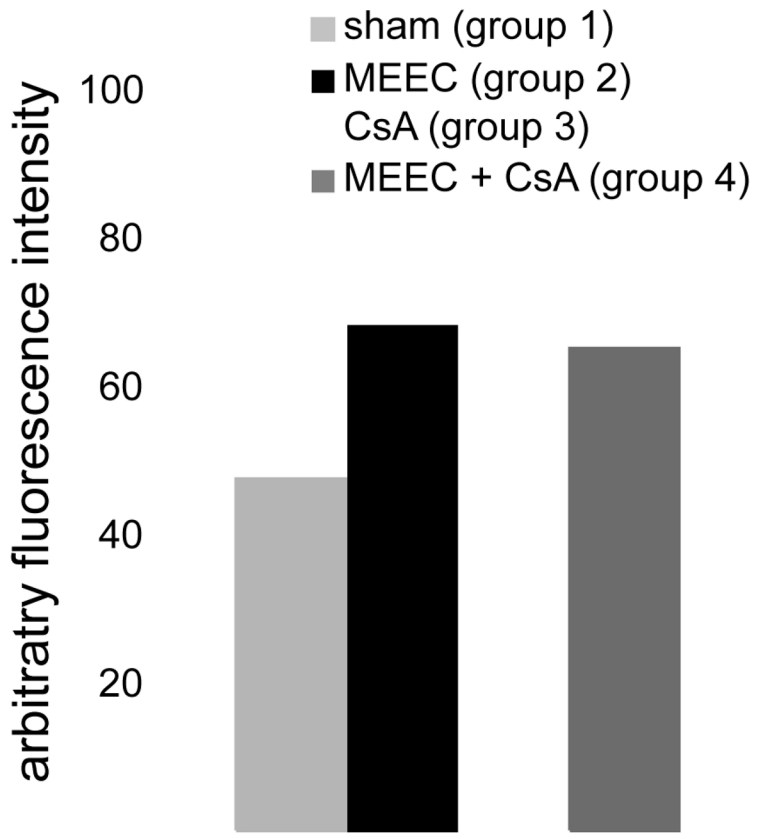
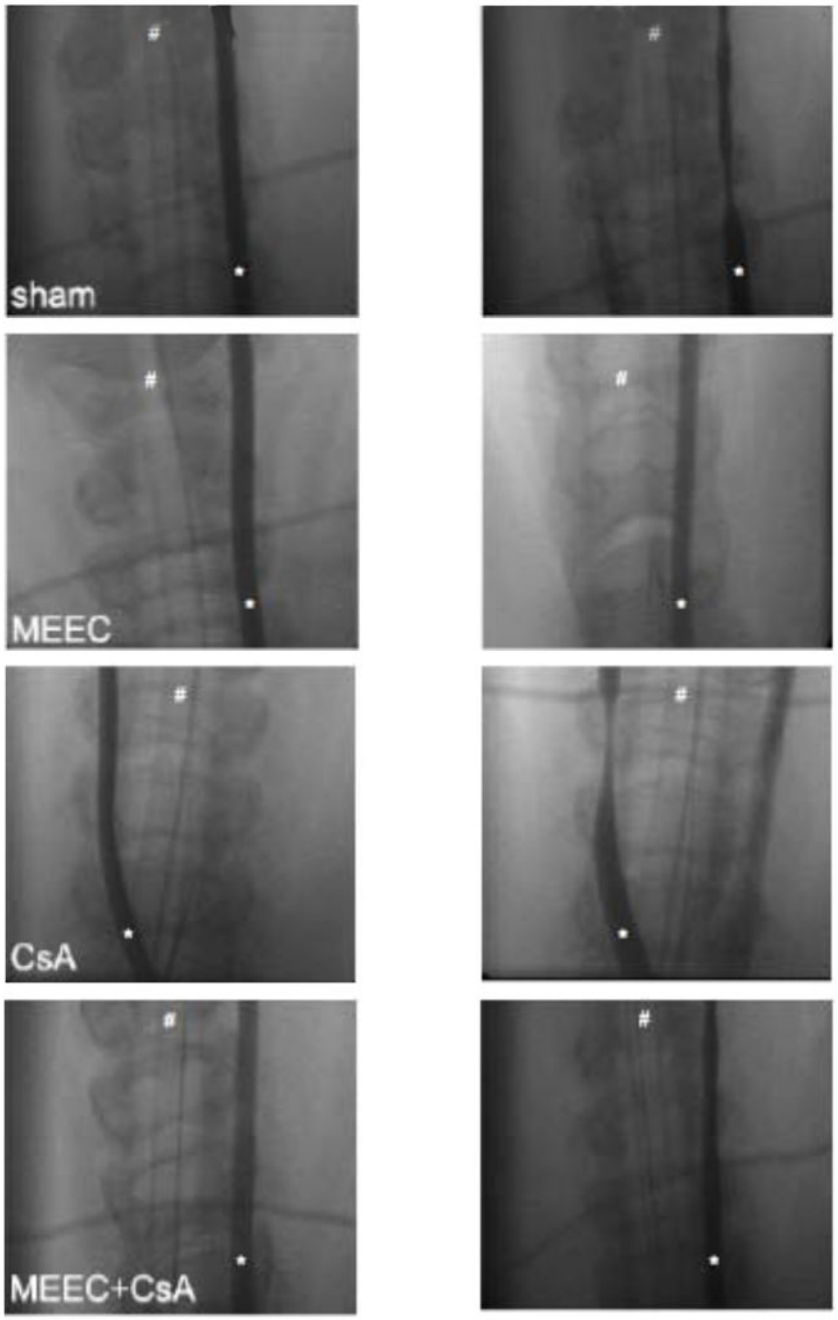


Figure 4. Splenocytes isolated from the four different treatment groups display no significant difference in *in vitro* lysis of allogeneic PAE. 10^4 PAE were labeled with calcein and incubated with 5×10^5 splenocytes isolated after 28 and 90 days, respectively

MEEC: matrix-embedded PAE, CsA: cyclosporine A



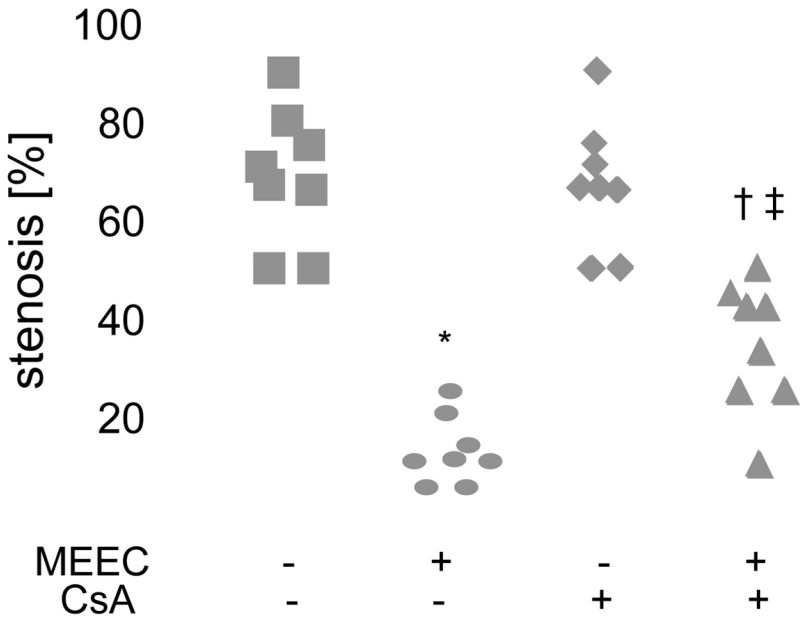


Figure 5. Concomitant cyclosporine A treatment (CsA) modulates the ability of perivascular implanted matrix embedded PAE (MEEC) to influence vascular repair after balloon injury

A Representative angiograms of carotid arteries before (left) and 90 days (right) after balloon injury in the four treatment groups (sham; matrix-embedded PAE, MEEC; cyclosporine A alone, CsA; matrix-embedded PAE with CsA, MEEC+CsA).

*carotid artery

#endotracheal tube

B Degree of stenosis (%) after balloon injury of both carotid arteries in the four pig treatment groups. Symbols: individual data, dashed lines: mean±SD

*$p<0.001$ vs. group 1 and group 3

†$p<0.02$ vs. group 2

‡$p<0.005$ vs. group 1 and group 3

TABLE

Cyclosporine A is without effect on biosecretion of porcine aortic endothelial cells *in vitro*

	PAE MEEC	PAE MEEC 400 ng/ml CsA	PAE TCPS	PAE TCPS 400 ng/ml CsA
GAG [μg/10^6 cells]	2.8±0.15	2.6±0.2	2.8±0.12	2.7±0.17
HS [μg/10^6 cells]	1.5±0.04	1.5±0.12	1.6±0.06	1.5±0.08
prostacyclin [pg/10^6 cells]	74±5.1	69±6.4	78±3.4	75±7.1
TGF-β [pg/10^6 cells]	819±99	794±131	864±103	839±167

PAE: porcine aortic endothelial cells, MEE: matrix-embedded, TCPS: tissue culture polystyrene plates, GAG: glycosaminoglycans, HS: heparan sulfate, TGF-β: transforming growth factor-β CsA: cyclosporine A