Research Article

Inflammation-Induced Tissue Damage Mimicking GvHD in Human Skin Models as Test Platform for Immunotherapeutics

Julia Wallstabe[1#], Lydia Bussemer[1#], Florian Groeber-Becker[2,3], Lukas Freund[1], Miriam Alb[1], Mariola Dragan[4], Ana Maria Waaga-Gasser[4], Rafael Jakubietz[5], Hermann Kneitz[6], Andreas Rosenwald[7,8], Silke Rebhan[1], Heike Walles[2,3] and Stephan Mielke[1,9]

[1]Department of Internal Medicine II, Center for Allogeneic Stem Cell Transplantation, Würzburg University Medical Center, Würzburg, Germany; [2]Department for Tissue Engineering and Regenerative Medicine, Würzburg University Medical Center, Würzburg, Germany; [3]Translational Center for Regenerative Therapies, Fraunhofer Institute for Silicate Research ISC, Würzburg, Germany; [4]Department of Surgery I, Würzburg University Medical Center, Würzburg, Germany; [5]Department of Surgery II, Würzburg University Medical Center, Würzburg, Germany; [6]Department of Dermatology, Section for Histopathology, Würzburg University Medical Center, Würzburg, Germany; [7]Institute of Pathology, University of Würzburg, Würzburg, Germany; [8]Comprehensive Cancer Center Mainfranken, Würzburg, Germany; [9]Karolinska Institute and University Hospital, Department of Laboratory Medicine and Medicine Huddinge, Department of Cell Therapy and Allogeneic Stem Cell Transplantation (CAST), Stockholm, Sweden

Abstract

Due to the rapidly increasing development and use of cellular products, there is a rising demand for non-animal-based test platforms to predict, study and treat undesired immunity. Here, we generated human organotypic skin models from human biopsies by isolating and expanding keratinocytes, fibroblasts and microvascular endothelial cells and seeding these components on a collagen matrix or a biological vascularized scaffold matrix in a bioreactor. We then were able to induce inflammation-mediated tissue damage by adding pre-stimulated, mismatched allogeneic lymphocytes and/or inflammatory cytokine-containing supernatants histomorphologically mimicking severe graft versus host disease (GvHD) of the skin. This could be prevented by the addition of immunosuppressants to the models. Consequently, these models harbor a promising potential to serve as a test platform for the prediction, prevention and treatment of GvHD. They also allow functional studies of immune effectors and suppressors including but not limited to allodepleted lymphocytes, gamma-delta T cells, regulatory T cells and mesenchymal stromal cells, which would otherwise be limited to animal models. Thus, the current test platform, developed with the limitation that no professional antigen presenting cells are in place, could greatly reduce animal testing for investigation of novel immune therapies.

1 Introduction

The rise of cellular therapies during the last decade has not only been challenging for healthcare providers but also for regulators and Good Manufacturing Practice (GMP) facilities around the globe owing to the associated risk of eliciting undesired immunity, i.e. graft versus host disease (GvHD). GvHD is a severe, potentially life-threatening complication of allogeneic hematopoietic stem cell transplantation (SCT) (Zeiser and Blazar, 2017) requiring immediate local or systemic immunosuppression. Both acute and chronic GvHD affect roughly every second transplant patient. Immunosuppression with calcineurin inhibitors or tacrolimus in combination with methotrexate has reduced severe and life-threatening acute GvHD rates and can be regarded as a broadly accepted clinical standard in GvHD prophylaxis, while steroids are recommended for the treatment of GvHD (Penack et al., 2011). Severe GvHD of the skin is characterized by apoptotic epithelial damage that can lead to complete loss of the protective epithelial layer (Rowlings et al., 1997; Horowitz et al., 1990; Appelbaum, 2001; Falkenburg et al., 2002).

[#] contributed equally

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Correspondence: Stephan Mielke, MD
Professor of Hematology and Cellular Therapy
Karolinska Institute and University Hospital
Department of Laboratory Medicine and CAST
SE-14186 Stockholm, Sweden
(stephan.mielke@ki.se)

GvHD has been extensively studied in several well-established mouse models such as the full MHC class I mismatch model C57BL/6 to BALB/c. Both transgenic and gene-deficient mouse strains have contributed significantly to the mechanistic understanding of GvHD and to the development of allogeneic stem cell transplantation as we know it today (Blazar et al., 1991; van Leeuwen et al., 2002).

However, cell populations and their expansion patterns as well as drug metabolism and pharmacology differ between humans and mice (Boieri et al., 2016), therefore translation of results to humans may be complicated and can potentially delay drug development and even affect patient safety (Perel et al., 2007; Cook et al., 2012; Johnson et al., 2006; Hunig, 2012). Thus, there is an urgent medical need for a predictive human *in vitro* test system for GvHD (Hofmeister et al., 2004).

Unfortunately, current *in vitro* test systems are weak predictors of GvHD due to a lack of physiological network interactions and organotypic structures. Similarly, humanized animal models have proven their limitation in predicting the GvHD potential of cellular products (Schroeder and DiPersio, 2011). So far, the only reliable predictor of skin GvHD has been developed by Vogelsang and colleagues, who used skin biopsies as a test platform for allogeneic lymphocytes and were indeed able to predict the appearance of skin GvHD (Dickinson et al., 2002; Sviland and Dickinson, 1999; Vogelsang et al., 1985). However, the number of tests this valuable model can be used for is limited.

We have developed authentic three-dimensional human organotypic models of the skin (Groeber et al., 2016a,b; Rossi et al., 2015) that find themselves already applied as human test platforms for risk assessment or efficacy testing in the cosmetic industry and in research and that are gaining increasing attention from regulators (Groeber et al., 2011, 2015). Here, we were able for the first time to induce inflammatory damage in these models using mismatched lymphocytes as a surrogate for an incompatible allograft. Our skin models can be built from expanded cellular components of various skin samples and thus allow multiple testing, thereby satisfying the needs of extensive GMP testing as well as allowing mechanistic studies involving various types of immune suppressors that may modulate GvHD or cytokine-mediated damage. Hence, the models could serve as alternatives to animal-based test procedures and may lead to the reduction or replacement of animal testing in GvHD research, not only in mice but also in non-human primates, dogs, pigs and other large animal models.

2 Material and methods

Cell isolation and culture

All volunteers or their legal representatives provided their written informed consent and all personal information was pseudonymized. The study protocol was approved by our local ethics committee (*AZ 182/10* and *2/12*).

Primary skin cells (keratinocytes, fibroblasts and microvascular endothelial cells) were isolated from human juvenile foreskin biopsies (donor age ≤ 7 years) or from adult skin biopsies from plastic surgeries (abdominoplasty, brachioplasty, dermolipectomy surgeries from upper arms and abdomen) according to previously published protocols (Rossi et al., 2015; Pudlas et al., 2011; Groeber et al., 2013).

Juvenile foreskin biopsies were used to establish the skin models initially as the proliferative capacity of keratinocytes was expected to be higher as shown previously by our group (Lange et al., 2016). Indications for the circumcisions were not available, however all samples underwent a visual inspection before being used in the models so that inflamed or infected tissues could be sorted out. The use of adult skin was expected to be more challenging, but these skin samples came together with peripheral blood samples from the same volunteer donors. With later translation to the clinic in mind, we also explored, for the first time, the use of 4 mm punch biopsies from the above described adult tissues. However, due to the limitation in size of these biopsies, only fibroblasts and keratinocytes but no endothelial cells could be isolated.

The punch biopsies were washed and treated with dispase (2 U/mL, Life Technologies GmbH, Darmstadt, Germany) for 60 min at 37°C. Afterwards, the epidermal and dermal parts were separated and washed. The epidermis was incubated with 0.05% (v/v) Trypsin/EDTA for 5 min at 37°C. After addition of 10% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich® Chemie GmbH, Schnelldorf, Germany), the cell suspension was mixed thoroughly for 5 min. Cells were washed and seeded in keratinocyte culture medium containing EpiLife® basal medium supplemented with Human Keratinocyte Growth Supplement, 100 U/mL penicillin and 100 µg/mL streptomycin (PenStrep, all from Life Technologies GmbH) on juvenile human fibroblasts as feeder cells treated with mitomycin C (4 µg/mL, Sigma-Aldrich® Chemie GmbH) (Aasen and Izpisua Belmonte, 2010). The feeder cells were derived from foreskin biopsies. Dermal parts of the punch biopsies were incubated for 45 min at 37°C in collagenase (500 U/mL, Roche Diagnostics GmbH, Mannheim, Germany) and washed twice. The suspension with dermal tissue residues was resuspended in fibroblast culture medium containing Dulbecco's Modified Eagle's Medium (Life Technologies GmbH) supplemented with 10% (v/v) FBS and PenStrep. After fibroblasts had grown out, remaining tissue residues were washed away.

Microvascular endothelial cells were isolated as described before (Groeber et al., 2013) and cultured in VascuLife® (VascuLife® VEGF Endothelial Medium Complete Kit, LifeLine Cell Technology, Frederick, MD, USA) supplemented with PenStrep. For experiments, keratinocytes were used from 1st to 3rd passage, fibroblasts from 1st to 5th passage, and endothelial cells from 1st to 4th passage.

Peripheral blood mononuclear cells (PBMCs) were isolated from leukoreduction system chambers (Trima Accel apheresis apparatus, Terumo BCT, Lakewood, CO, USA) (Bock et al., 2002) of healthy platelet donors (personal data fully anonymized) or from adult skin donors (personal data pseudonymized) by density-gradient centrifugation using Biocoll Separating Solution (1.077 g/mL, Biochrom GmbH, Berlin, Germany). For experiments, PBMCs were either used directly after isolation or cryopreserved and thawed the day before the experiment.

Generation of human skin models

Two types of human skin models were used in this study as illus-

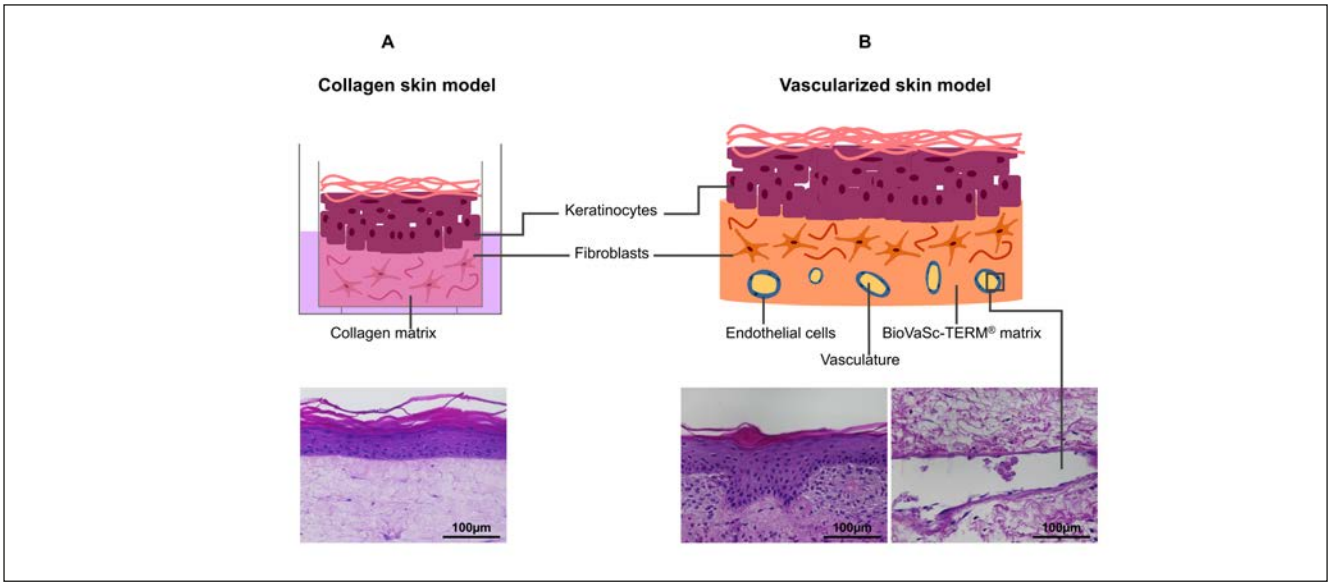

Fig. 1: Human artificial skin models

Human primary skin cells (keratinocytes, fibroblasts and microvascular endothelial cells) isolated from human juvenile foreskin or adult 4 mm skin biopsies were used for generation of artificial skin models. Every skin model was built from only one individual tissue donor. (A) For collagen-based full-thickness skin models, dermal equivalents were generated by mixing human fibroblasts with rat-tail collagen type I. To form the epidermal layer, keratinocytes were seeded on top of the dermal part. (B) For the vascularized skin models (SkinVaSc-TERM®), endothelial cells were injected into the vascular system of the porcine jejunum scaffolds. Fibroblasts and keratinocytes were seeded on top. Vascularized skin models were cultured under physiological perfusion. For both types of skin models, the surface of the epidermal layer was exposed to the air to allow epidermal differentiation.

trated in Figure 1. Every skin model was built from only one individual tissue donor.

Human full-thickness skin models were generated based on a collagen hydrogel in 24-well inserts (Polycarbonate Membrane Inserts in Multidish 24, Nunc™, Roskilde, Denmark) according to a previously published protocol using the EpiLife® medium system (Lange et al., 2016). Briefly, 500 µl dermal equivalents were prepared by mixing $5x10^4$ fibroblasts per insert with the collagen solution (collagen solution prepared in-house, 4 mg/mL final rat-tail collagen type I concentration). After 24 h, keratinocytes were seeded on the dermal equivalents at a density of $5x10^5$ cells/cm^2 in keratinocyte culture medium supplemented with 1.5 mmol/l $CaCl_2$ (Sigma-Aldrich® Chemie GmbH) and cultured overnight. Then, medium was replaced and supplemented with 50 µg/mL vitamin C (Caeser & Loretz GmbH, Hilden, Germany) followed by exposure of the surface to air for induction of epidermal differentiation. Medium change was performed every two or three days.

Human vascularized skin models (SkinVaSc-TERM®) were generated in-house on a biological vascularized scaffold (BioVaSc-TERM®) (Mertsching et al., 2009; Scheller et al., 2013) in a previously published perfusable bioreactor system (Groeber et al., 2013, 2016a) on the basis of the EpiLife® medium system. The matrix was prepared from jejunal segments of German Landrace pigs. Scaffolds used in the experiments described here were explanted from dead animals between 2014 and 2016, which did not require approval according to animal care guidelines. Briefly, vessels were saturated with VascuLife® medium overnight and reseeded with $1x10^7$ endothelial cells. The matrix was connected to the circulation of a bioreactor system (Groeber et al., 2013). Then, $8.9x10^5$ fibroblasts were seeded on the matrix surface. The perfusion was started and the pressure was increased stepwise to 100 mmHg, finally implementing a physiological pulsatile pressure profile with a 20-mmHg amplitude. After 5 days, medium was changed to keratinocyte culture medium supplemented with 1.5 mmol/l $CaCl_2$, and $4x10^6$ keratinocytes were seeded on the matrix surface. Dynamic culture was continued for 3 days; then medium was replaced and supplemented with 50 µg/mL vitamin C and the surface was exposed to air. Skin models were cultured for a further 11-14 days under dynamic conditions with medium change every 6 days.

Induction of tissue damage in human skin models

For simulation of GvHD and induction of tissue damage, autologous or allogeneic PBMCs pre-stimulated in a mixed lymphocyte reaction (MLR) were injected into the collagen skin models. Therefore, MLRs were prepared in an autologous (PBMCs from the adult tissue donor stimulated with irradiated PBMCs of the same individual) or an allogeneic setting (randomly mismatched allogeneic PBMCs stimulated with irradiated PBMCs from the autologous tissue donor). The MLR was set up with $1x10^6$ responder cells/mL in CellGro® GMP DC medium for seven days with or without the addition of 200 ng/mL cyclosporin A (CsA, Sandimmun® Injection, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA). Collagen skin models receiv-

ing PBMCs that had undergone the MLR in the presence of CsA were additionally pretreated with 200 ng/mL CsA one day before injection of the cells. Alternatively, allogeneic PBMCs were pre-stimulated with 25 ng/mL PMA and 1 µg/mL ionomycin overnight or left unstimulated. Then, cells were washed twice with CellGro® GMP DC medium.

Collagen skin models were used on day 13 of culture at the air-liquid interface. Pre-stimulated or non-stimulated PBMCs with or without pre-treatment of CsA were injected with insulin syringes at two locations into the dermal part of the skin models ($2x10^6$ cells per skin model). Injection of CellGro® GMP DC medium alone served as control. EpiLife® medium was replaced by fresh CellGro® GMP DC medium as culture medium. In another setting, conditioned supernatants of unstimulated or pre-stimulated PBMCs were collected and injected directly into the skin models and also used as culture medium under these conditions.

Using at least two different skin cell donors, triplicates were prepared for each condition. After incubation for 48 h, medium supernatants were collected from collagen skin models and stored at -80°C. The collagen skin models were fixed in paraformaldehyde (Roti®-Histofix, Carl Roth GmbH & Co.KG, Karlsruhe, Germany) for 2 h and further processed for histological analysis.

For the vascularized skin models, PBMCs were first labeled with 2.5 µM carboxyfluorescein succinimidyl ester (CFSE, CellTrace™ CFSE Cell Proliferation Kit, Life Technologies GmbH) and then pre-stimulated with 50 ng/mL PMA and 0.8 µg/mL ionomycin overnight. Afterwards, PBMCs were washed twice with CellGro® GMP DC medium. Vascularized skin models were used after 11 to 14 days of culture at the air-liquid interface. CFSE-labeled, pre-stimulated PBMCs were injected into the arterial access of the vascularized skin models and also added into the pressure flask ($1.5\ 2x10^8$ cells in each case). Injection of CellGro® GMP DC medium alone into the arterial access and into the pressure flask of a second bioreactor served as control. Experiments were run for 48, 72 or 96 h. Medium supernatants were collected and stored at -80°C at the indicated time points. PBMCs were recollected from circulation and prepared for flow cytometry. Vascularized skin models were fixed in paraformaldehyde overnight and embedded in paraffin for histological analysis. All vascularized skin models were prepared with cells from one skin cell donor, but with PBMCs from three different donors.

Histological analysis

Morphological features of the paraffin-embedded tissue sections were visualized by hematoxylin/eosin (HE) staining. For immunohistochemical staining, sections were stained with monoclonal mouse anti-human antibodies for CD3 (F7.2.38), CD4 (4B12) and CD8 (C8/144B, all from Dako, Glostrup, Denmark). For detection, the Dako REAL™ Detection System, Peroxidase/AEC, Rabbit/Mouse (biotinylated goat anti-mouse/rabbit antibodies, Dako) was used.

Flow cytometric analysis

Cells were stained for 15 min at 4°C with CD3 PerCP (SK7, BioLegend Inc., San Diego, CA, USA) and CD25 APC (BC96, BioLegend Inc.). Data were obtained using a FACSCalibur flow cytometer (BectonDickinson, Heidelberg, Germany) or CytoFlex (Beckman Coulter Inc., Brea, CA, USA) and analyzed using FlowJo 10 software (Tree Star, Ashland, OR, USA).

Cytokine measurement

Cytokine levels of IFNγ, interleukin (IL)2, IL17A, and tumor necrosis factor alpha (TNFα) in medium supernatants were analyzed using the Luminex® technology platform (Milliplex Human Cytokine MAGNETIC Kit, HCYTOMAG-60K-04, Merck Millipore, Darmstadt, Germany) according to the manufacturer's recommendations.

Statistical analysis

Statistical analyses were conducted applying Prism software v5.04 (GraphPad Software Inc., San Diego, CA, USA). Normal distribution was evaluated using the Kolmogorov Smirnov normality test. For comparison of two groups, the unpaired t-test for values with Gaussian distribution was performed. For comparison of more than two groups, the one-way analysis of variances (ANOVA) with Bonferroni's multiple comparisons test for values with Gaussian distribution was performed. A p value ≤ 0.05 was considered significant.

Data sharing

For original data, please contact the corresponding author.

3 Results

3.1 Immunogenic properties of human primary skin cells

As a first step, we investigated the ability of the individual cellular components, i.e., keratinocytes, fibroblasts and endothelial cells isolated from adult skin biopsies, to stimulate or suppress previously activated or non-activated randomly mismatched PBMCs. All cell types investigated showed marked expression of HLA class I molecules and HLA DR upon stimulation with IFNγ. However, the costimulatory molecule CD80 was expressed at low levels in keratinocytes, fibroblasts and endothelial cells, whilst CD86 expression lacked completely, even following previous stimulation with IFNγ. Notably, the expression of the inhibitor PD-L1 was upregulated in all three cell types after stimulation with IFNγ (Fig. S1[1]).

Irradiated endothelial cells, keratinocytes and fibroblasts proved their general ability to stimulate allogeneic PBMCs (Fig. S2[1]). However, attempting to increase their antigen-presenting capacity by unspecific pre-stimulation prior to irradiation did not increase overall proliferative activity as measured in one-way MLRs (Fig. S2[1]). Consequently, allogeneic PBMCs were also

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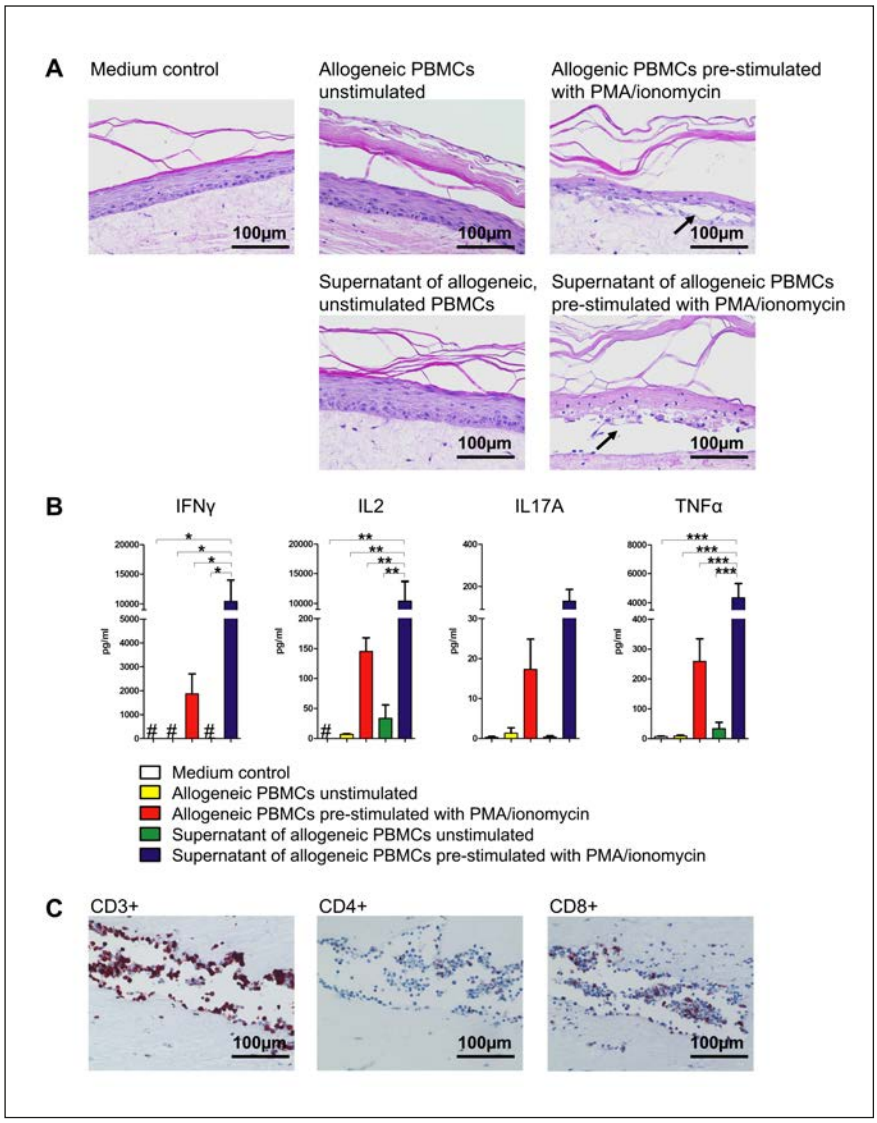

Fig. 2: Inflammation-induced damage in collagen skin models (juvenile foreskin skin cells)
Collagen skin models were generated using primary keratinocytes and fibroblasts isolated from human juvenile foreskins. For each experiment, every skin model was built from only one individual tissue donor. Allogeneic PBMCs were used from another donor. Culture medium, unstimulated PBMCs or allogeneic pre-stimulated PBMCs (unspecific stimulation with PMA/ionomycin) were injected into the matrix of collagen skin models and co-cultured for 48 h. Three experiments were performed in triplicates (n = 3). (A) HE-stained histological cross sections of collagen skin models are presented. Representative sections from skin models derived from a single skin donor are shown. Triplicate sections from all three donors are shown in Fig. S3[1]. The arrow indicates disruption of the epidermal cellular organization and detachment of the epidermal layer from the dermal part. Scale bars represent 100 µm. (B) Cytokine levels of IFNγ, IL2, IL17A and TNFα in the supernatants of the culture medium of the skin models were measured using Luminex® technology. Results are expressed as mean and SEM (n = 3). (C) Representative immunohistochemical staining of CD3, CD4 and CD8 molecules in PBMC infiltrates of one collagen skin model treated with pre-stimulated PBMCs is shown. Scale bars represent 100 µm. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$; # cytokine level not detectable.

pre-stimulated before adding them to the tissue models to overcome the missing lymph node function.

3.2 Pre-stimulated PBMCs induce tissue damage in collagen skin models

As a next step, we exposed the collagen skin model built from juvenile skin cells to allogeneic PBMCs with and without pre-stimulation. Here, previously unstimulated PBMCs did not cause significant alterations as compared to medium controls (Fig. 2A). In contrast, PBMCs pre-stimulated with PMA/ionomycin resulted in disruption of the epidermal cellular organization and complete detachment of the epidermal layer from the dermal part (mimicking histological grade IV acute GvHD). The same damage could be induced using the medium supernatant from PBMCs previously stimulated with PMA/ionomycin. We could show that these supernatants contained high levels of pro-inflammatory cytokines such as IFNγ, IL2, IL17A and TNFα (Fig. 2B). Immunohistochemical staining of the lymphocytic infiltrates revealed their identity as $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 2C).

Additionally, we exposed collagen skin models built from adult skin cells isolated from 4 mm punch biopsies to autologous and allogeneic PBMCs that had been pre-stimulated in respective MLRs. The injection of allogeneic stimulator cells resulted in disruption of the epidermal cellular organization and partial detachment of the epidermal layer from the dermal part (mimicking histological grade IV acute GvHD) (Fig. 3A). No such changes were observed in the autologous setting (negative control). PBMCs pre-stimulated with PMA/ionomycin served as a positive control, where lymphocytes infiltrated the model and induced dermal-epidermal detachment. Tissue damage was not observed after addition of medium supernatants from allogeneic or autologous PBMCs to the skin models, only from PBMCs pre-stimulated with PMA/ionomycin, probably due to the high concentration of proinflammatory cytokines (Fig. 3B). Addition of 200 ng/

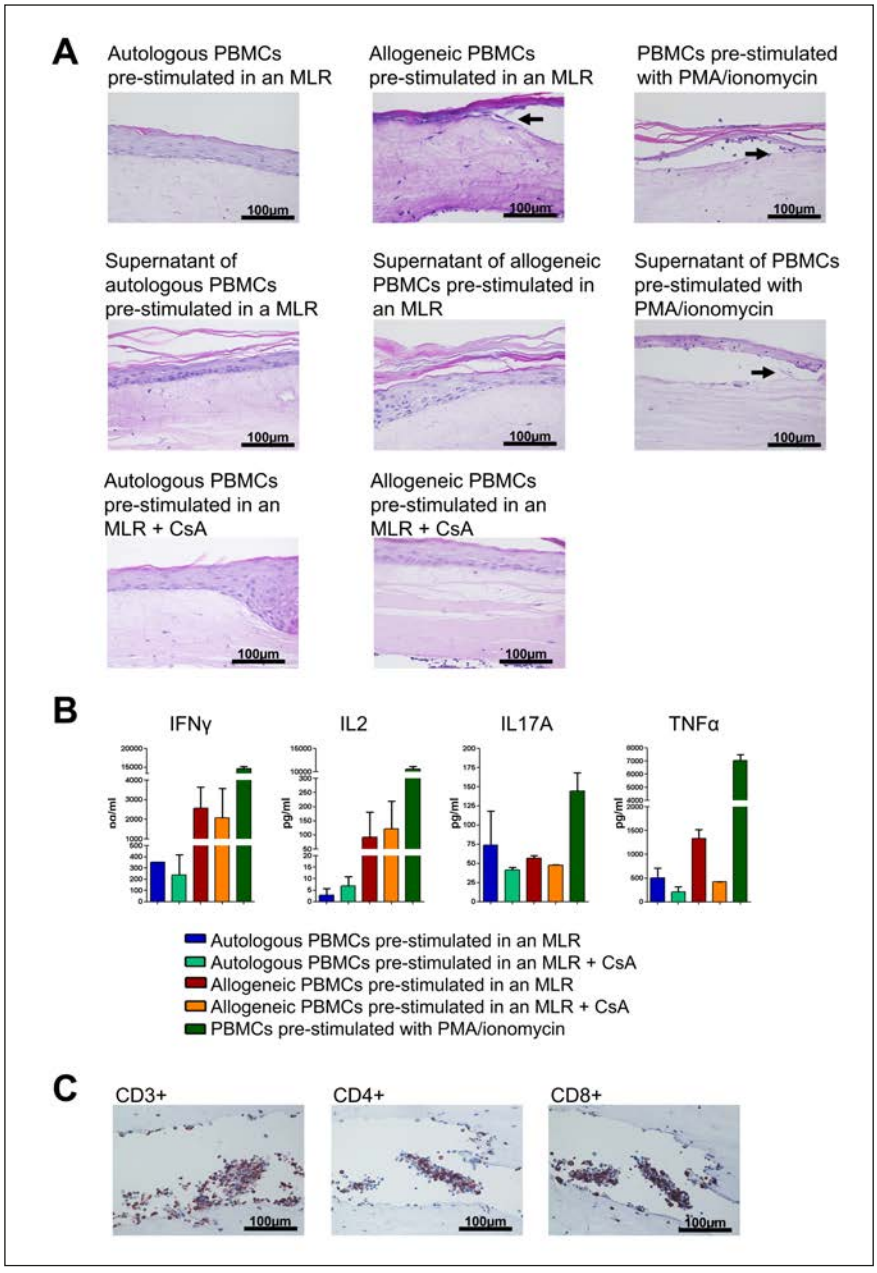

Fig. 3: Inflammation-induced damage in collagen skin models (adult skin cells from punch biopsies)
Collagen skin models were built from adult skin cells isolated from 4 mm punch biopsies. For each experiment, every skin model was built from only one individual tissue donor. Culture medium (data not shown), autologous, allogeneic or PMA/ionomycin pre-stimulated PBMCs were injected into the matrix of collagen skin models. Additionally, medium supernatants were collected from unstimulated or pre-stimulated PBMCs, injected into collagen skin models and used as culture medium. Furthermore, autologous and allogeneic PBMCs pre-stimulated in an MLR cultured with 200 ng/mL CsA were injected into the matrix. Collagen skin models were pre-treated with CsA one day before injection. Each condition was co-cultured for 48 h. Two experiments were performed in triplicates (n = 2). (A) Representative sections from skin models derived from a single donor are shown. Triplicate sections from both donors are shown in Fig. S4[1]. The arrow indicates disruption of the epidermal cellular organization and detachment of the epidermal layer from the dermal part. HE-stained histological cross sections of collagen skin models are presented.
(B) Cytokine levels of IFNγ, IL2, IL17A and TNFα in the supernatants of the culture medium of the skin models were measured using Luminex® technology. Results are expressed as mean and SEM (n = 2).
(C) Representative immunohistochemical staining of CD3, CD4 and CD8 molecules in PBMC infiltrates of one collagen skin model treated with allogeneic PBMCs is shown. Scale bars represent 100 µm.

mL CsA to the collagen skin models as well as to the pre-stimulated PBMCs led to prevention of tissue damage in the collagen skin models (Fig. 3A). Furthermore, we could show that supernatants contained pro-inflammatory cytokines such as IFNγ, IL2, IL17A and TNFα (Fig. 3B). Immunohistochemical staining of the lymphocytic infiltrates revealed their identity as $CD4^+$ and $CD8^+$ T lymphocytes (Fig. 3C).

3.3 Pre-stimulated PBMCs induce tissue damage in vascularized skin models

As a final step, we anticipated to induce the same inflammation in an even more sophisticated vascularized bioreactor-based skin model built from juvenile skin cells by injecting PMA/ionomycin pre-stimulated PBMCs into the circulation medium within the skin vessels. Skin models treated solely with medium served as a control. Bioreactors were run for 48, 72 or 96 h. Controls showed intact multilayered, cornified epidermis attached to the dermal part (Fig. 4A). In contrast, vascularized skin models treated with pre-stimulated PBMCs showed significant signs of cellular damage in the epidermal layer beginning after 48 h (mimicking histological grade II acute GvHD). Disruption of the epidermal organization as well as detachment of the epidermis from the dermis became more severe after 72 h (mimicking histological grade III acute GvHD). After 96 h, the epidermal layer was completely detached and infiltrates of PBMCs were found within the matrix (mimicking histological grade IV acute GvHD) (Fig. 4B).

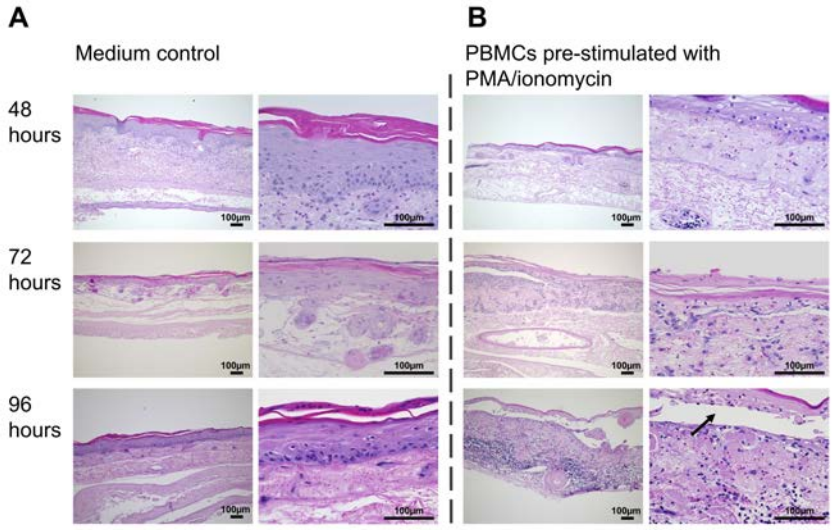

Fig. 4: Inflammation-induced damage in vascularized skin models (juvenile skin cells)
Vascularized skin models (SkinVaSc-TERM®) were generated in a perfusable bioreactor system. Every skin model was built from only one individual tissue donor. Pre-stimulated, CFSE-labeled PBMCs were introduced into the circulation of the bioreactor system. As a control, culture medium only was injected accordingly. Bioreactors were run for 48, 72 or 96 h. Representative HE-stained histological cross sections of vascularized skin models are presented for (A) medium control and (B) PBMCs pre-stimulated with PMA/ionomycin. Vascularized skin models were prepared from one skin cell donor, but with PBMCs from different donors for each experiment. The arrow indicates detachment of the epidermal layer from the dermal part. Scale bars represent 100 µm.

After 96 h, these infiltrates were shown by immunohistochemical staining to consist of high proportions of $CD4^+$ helper T lymphocytes and $CD8^+$ cytotoxic T lymphocytes (Fig. 5A). We found a pro-inflammatory cytokine profile including IFNγ, IL2, IL17A and TNFα (Fig. 5B). These cytokines were not detectable in the medium control at relevant levels. Most interestingly, PBMCs drawn from the bioreactor vessels after 96 h continued to proliferate without re-stimulation (Fig. 5C).

4 Discussion

Owing to the increasing development and use of cellular therapies worldwide, particularly in cancer immunotherapy, there is a strong upcoming demand for test platforms that allow prediction of immunologically induced tissue damage caused by novel cellular products such as genetically modified T lymphocytes (e.g., suicide genes, chimeric antigen receptors), expanded and/or modified autologous or allogeneic NK cells, expanded tumor infiltrating lymphocytes (TILs), allodepleted lymphocyte products as well as allogeneic or autologous antigen-specific T lymphocytes against viruses and tumor-associated autoantigens (TAAs) (Schuster et al., 2017; Mielke et al., 2008; Barkholt et al., 2009; Maude et al., 2014; Stevanovic et al., 2015; Lutz et al., 2015; Björklund et al., 2018).

With allogeneic SCT representing the most commonly applied cellular immunotherapy to date, GvHD remains one of the most important complications that can be neither fully predicted nor effectively avoided. However, advances in high-resolution HLA-typing of unrelated donors (Fleischhauer et al., 2018), novel immunosuppressive drugs (Zeiser et al., 2015; Mielke et al., 2014; Lutz and Mielke, 2016), and advanced graft manipulation techniques (Handgretinger et al., 2016; Montero et al., 2006; Barrett et al., 1998) have certainly contributed to reducing these risks. Furthermore, recent and highly promising developments in the field of biomarkers may allow early detection and treatment of clinically relevant GvHD (Major-Monfried et al., 2018; Ahmed et al., 2015). *Ex vivo* graft manipulation techniques such as selective allodepletion prevented or reduced the likelihood of high-grade GvHD in HLA-mismatched allotransplantation, allowing to reduce immunosuppression, thereby enhancing graft-versus-leukemia effects (Roy et al., 2019, 2020). Thus, tests predicting severe GvHD after *ex vivo* selection or manipulation of allogeneic immune cells could be of significant value for patient safety.

MLRs between donor and recipient have been proven to have predictive value in mismatched transplantation (Mielke et al., 2008; Amrolia et al., 2003), whilst in matched allogeneic SCT, the skin explant assay and the helper T-lymphocyte precursor frequency (HTLp) assay were the only assays beyond HLA typing that were able to predict GvHD based on minor antigen differences (Kaminski et al., 1989; Sharrock et al., 1990; Schwarer et al., 2004; Fleischhauer et al., 2006). HTLps are very much dependent on the type of antigen-presenting cells (APCs) used in the assay. However, using PBMCs or expanded T lymphocytes as stimulators has proven to be predictive for overall GvHD (Solomon et al., 2005). The skin explant system requires a skin biop-

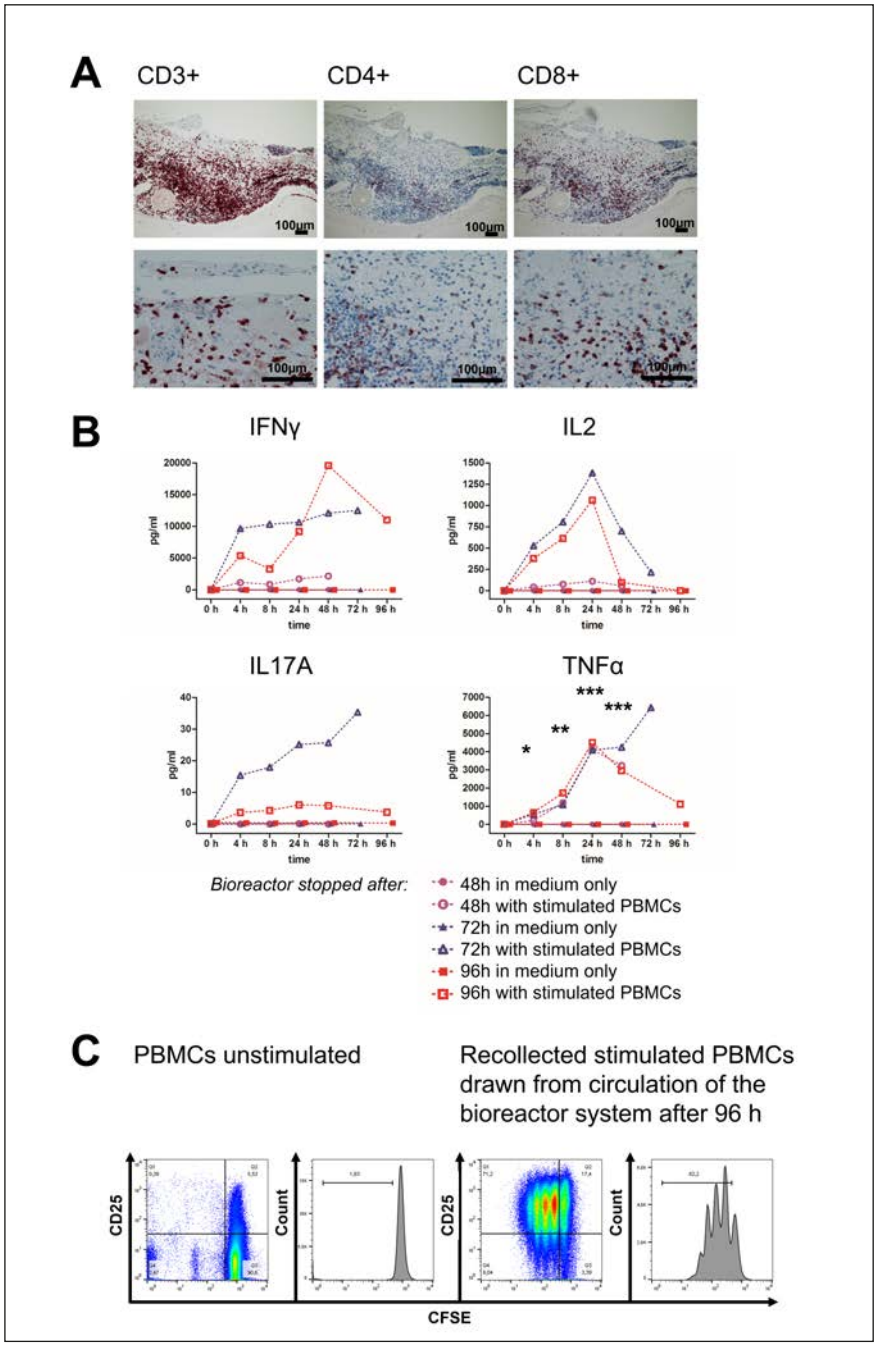

Fig. 5: Inflammation-induced damage in vascularized skin models (juvenile skin cells)
Vascularized skin models (SkinVaSc-TERM®) were generated in a perfusable bioreactor system. Every skin model was built from only one individual tissue donor. Pre-stimulated, CFSE-labeled PBMCs were introduced into the circulation of the bioreactor system. As a control, culture medium only was injected accordingly. Vascularized skin models were prepared from one skin cell donor, but with PBMCs from different donors for each experiment. (A) Immunohistochemical staining of cross sections for CD3, CD4 and CD8 is presented. Representative sections from vascularized skin models with pre-stimulated PBMCs after 96 h are shown. Scale bars represent 100 µm. (B) Cytokine levels of IFNγ, IL2, IL17A and TNFα in supernatants of the culture medium after 0, 4, 8, 24, 48, 72 and 96 h were measured using Luminex® technology. The results are depicted as timelines for vascularized skin models with medium only (closed symbols) and vascularized skin models with pre-stimulated PBMCs (open symbols). (C) After bioreactors were stopped, pre-stimulated, CFSE-labeled PBMCs were recollected from circulation, prepared for flow cytometric analysis and compared to unstimulated PBMCs cultured for the same period of time in a culture flask. Representative results are shown for the bioreactor at 96 h. Cells were gated for lymphocyte properties (forward and side light scatter). CD3 positive cells were analyzed for CD25 expression and proliferation (CFSE dilution, dot plots). Percentages in CFSE histograms correspond to the proportion of cells having undergone at least one cell division.

sy from the recipient and lymphocytes from the donor that are then co-cultured. The advantage of this system is, of course, that a representative fraction of recipient skin containing professional APCs is investigated. The sole but important drawback, however, is the very limited number of experiments that can be done on a single biopsy.

Here, we were able to mimic for the first time GvHD-like changes in artificial human skin models induced by randomly mismatched and pre-stimulated lymphocytes. Comparable to the skin explant assay, which served as a theoretical template for our GvHD-model, the great advantage in contrast to animal models is the use of human cells in a realistic, three-dimensional organotypic setting. With a view to the testing of immunotherapeutic drugs, the ability to use cells from the same species is an important prerequisite, as HLA molecules across species borders can induce severe immune reactions. In the past, artificial human skin models were built from preputium or major sections of skin derived from surgical interventions. Here, we succeeded in building several collagen skin models from single 4 mm punch biopsies following isolation, expansion and structural reshaping of the three-dimensional organ. Therefore, the advantage of the organ models presented here is that several models (at least 30

skin models per donor) can be generated, allowing multiple and complex investigations in a human test platform and thereby facilitating mechanistic research that otherwise would not be feasible in a human setting. In the future, FBS could be replaced by the use of serum-free media or normal AB serum to work in a truly human environment. Certainly, these models need to undergo further optimization. However, such research is deeply warranted, as the exact mechanistic pathways of several clinically-applied immune effector cells, e.g., mesenchymal stromal cells, remain unknown to date because such basic research so far has been limited to animal models and simple *in vitro* test systems. Due to the complexity of the system, such mechanistic pathways are difficult to reveal in clinical trials which involve far more variables.

We employed both a collagen skin model under static conditions and a vascularized skin model under dynamic conditions in a bioreactor system (Groeber et al., 2016a; Rossi et al., 2015; Lange et al., 2016). Pre-stimulated or unstimulated randomly HLA-mismatched lymphocytes injected either directly into the model or into the circulation medium induced a histomorphological pattern typical for GvHD. Cytokine measurements confirmed a GvHD typical profile of strong inflammation, whilst immunohistochemical analyses revealed the presence of CD3+CD8+ lymphocytes. A GvHD-like pattern of damage also could be induced by the sole use of supernatants of PBMC cultures that had been pre-stimulated with an artificial, unphysiological polyclonal stimulator (PMA/ionomycin) and consequently contained extremely high levels of pro-inflammatory cytokines. Consequently, such models may indeed harbor the potential to study the damaging effects of high-level cytokines on the skin as observed, for example, in different forms of cytokine release syndromes.

On a more critical note, it is clear that the human artificial skin models presented here cannot mimic the full pathway of GvHD development (Ferrara and Reddy, 2006). Comparable to the skin explant assay, these models lack lymph nodes and, therefore, the natural priming of T lymphocytes by activated professional APCs in the periphery. In our experiments, all types of skin cells lacked sufficient expression of co-stimulatory molecules, even after pre-stimulation, and therefore professional APC features. However, sufficient pre-stimulation of lymphocytes mimicking the function of the lymph nodes can overcome this limitation, even if such a scenario may not be fully reflective of the original pathways and complexity in the human body. Although keratinocytes, fibroblasts and endothelial cells do not belong to professional APCs, it has been reported that non-hematological APCs are sufficient for induction of GvHD in mice (Koyama et al., 2011). However, to date the individual ability of fibroblasts, keratinocytes and endothelial cells to exert APC-like functions is discussed controversially in the literature (Koyama et al., 2011; van Dijk et al., 2000; Teshima et al., 2002; Takagi et al., 2006; Haniffa et al., 2007; Dominguez-Castillo et al., 2008). In our study, unstimulated fibroblasts, keratinocytes and endothelial cells were able to induce proliferation without prior stimulation. Nonetheless, previous exposure to other GvHD-relevant cytokines such as TNFα, IL17A and IL6 or combinations thereof could have had a much stronger effect (Pechhold et al., 1997; Tawara et al., 2011; Ju et al., 2014). This finding is in line with accumulating evidence that endothelial cells play a significant role in GvHD pathogenesis (Penack et al., 2011; Zeiser et al., 2011) and may develop into sufficient APCs as a consequence of exposure to a pro-inflammatory environment, thereby enhancing the GvHD cascade (Dietrich et al., 2013; Schmid et al., 2014).

Therefore, the vascularized model may indeed have advantages in simulation of secondary GvHD effects over the collagen model. On the other hand, the construction of the vascularized skin model is technically more demanding and time-consuming. Taking into account that a minimum number of bioreactors needs to be available for one experiment, we believe that for daily clinical purposes of product testing and for multiple comparisons in complex experiments, the collagen model appears to be the more feasible option, although the vascularized model harbors unique opportunities such as allowing drug delivery directly into a vessel-like circulation system. Finally, ease of use and reliability will determine clinical applicability.

Our results clearly show that allogeneic PBMCs pre-activated in an MLR were an essential requirement to exert skin destruction in our skin models, thereby overcoming the primary absence of professional APCs such as Langerhans cells. Nonetheless introduction of professional APCs into these models would certainly enhance the authenticity of simulated immunological processes (Chau et al., 2013).

Taken together, we were able to induce inflammation-based histomorphological patterns of tissue damage clearly mimicking GvHD to both collagen and vascularized human skin models by pre-stimulated lymphocytes. Nonetheless, these models of GvHD-like tissue damage are limited in their ability to reflect all pathophysiological aspects of GvHD. To be able to assess these models' ability to predict GvHD, we are planning to investigate the residual skin GvHD potential of *ex vivo* manipulated, selectively allodepleted lymphocytes in haploidentical blood SCT using full-collagen models built from recipients' skin samples. With regard to the world-wide rise of haploidentical SCT (Passweg et al., 2017), methods to allow *ex vivo* testing of manipulated T lymphocytes are likely to be highly demanded. Currently, we are planning to expand these models to other GvHD organs such as gut and liver. Nonetheless, the predictive value of the artificial skin models for GvHD in organs other than skin may still be sufficient as known from the skin explant assay and HTLPs where both assays could predict overall GvHD development despite lacking original APCs from liver and gut (Sviland and Dickinson, 1999; Schwarer et al., 2004; Zhang et al., 2002). These models will allow sophisticated mechanistic studies of mesenchymal stromal cells, regulatory T cells or novel immunosuppressive drugs such as ruxolitinib for the prevention and/or treatment of GvHD in a fully human organ model. So far, only cyclosporine A has been tested, but other drugs may result in different findings. This also includes a unique opportunity to perform time-dependent studies of biomarkers heralding GvHD development and severity (Major-Monfried et al., 2018; Ahmed et al., 2015).

Developing these models into a sophisticated, reliable test system would not only reduce animal testing but also enhance accuracy of GvHD prediction. A further development would be to in-

corporate professional APCs and add lymph node-like structures to these models. This work can contribute to our overall understanding of the underlying mechanisms and support the development of novel immunotherapeutics for GvHD treatment.

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Conflict of interest

S.M. reports personal fees and non-financial support from Gilead, Mitenyi, Novartis, Celgene/BMS, Kiadis and Bellicum.

Author contributions

J.W. and L.B. collected samples, performed experiments, analyzed data and wrote the paper. F.G.-B. advised on scientific design and on generation of the skin models and wrote the paper. L.F. collected samples, performed experiments and analyzed data. M.A. advised on scientific design and wrote the paper. M.D. performed experiments. A.W. advised on scientific design. R.J. advised on scientific design and provided samples. H.K. and A.R. provided histological evaluation. S.R. collected samples and performed experiments. H.W. provided the laboratory platform for tissue modelling and advised on scientific design. S.M. designed the study, analyzed data and wrote the paper. All authors critically revised a final version of the manuscript for important intellectual content and approved the manuscript to be published.

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