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Faculty of Medicine with the Dentistry
and Division of Medical Education in English

Doctoral dissertation in medical sciences

IMMUNOMODULATORY PROPERTIES OF NOVEL ABDOMINOPLASTY SKIN-DERIVED ACELLULAR DERMAL MATRICES

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This dissertation prepared with great thanks to supervisors

Dr. hab Andrzej Eljaszewicz

prof. dr hab. Marcin Moniuszko

as well as to my parents

Mike & Lisa Holl

I. Scientific Output

1. Articles included in the dissertation

1.1. Review article included in this dissertation

- **Holl, Jordan**; Kowalewski, Cezary; Zimek, Zbigniew; Fiedor, Piotr; Kaminski, Artur; Oldak, Tomasz; Moniuszko, Marcin; Eljaszewicz, Andrzej. Chronic Diabetic Wounds and Their Treatment with Skin Substitutes. ***Cells*** 2021, *10*, 655.

IF: 6.600; MNiSW: 140

1.2. Original research article included in this dissertation

- **Holl, Jordan**; Pawlukianiec, Cezary; Corton Ruiz, Javier.; Groth, Dawid; Grubczak, Kamil; Hady, Hady Razak; Dadan, Jacek; Reszec, Joanna; Czaban, Slawomir; Kowalewski, Cezary; Moniuszko, Marcin; Eljaszewicz, Andrzej. Skin Substitute Preparation Method Induces Immunomodulatory Changes in Co-Incubated Cells through Collagen Modification. ***Pharmaceutics*** 2021, 13, 2164.

  IF: 6.321; MNiSW: 100

2. List of publications not included in the dissertation

2.1 Original articles

- Groth, Dawid; Poplawska, Izabela; Tynecka, Marlena; Grubczak, Kamil; **Holl, Jordan**; Starosz, Aleksandra; Janucik, Adrian; Borkowska, Klaudia; Juchniewicz, Dorota; Hady, Hady Razak; Czaban, Slawomir; Reszec, Joanna; Kaminski, Artur; Czech, Tomasz; Kowalewski, Cezary; Fiedor, Piotr; Zimek, Zbigniew; Lewandowska, Hanna; Oldak, Tomasz; Moniuszko, Marcin; Eljaszewicz, Andrzej. Abdominoplasty Skin-Based Dressing for Deep Wound Treatment—Evaluation of Different Methods of Preparation on Therapeutic Potential. ***Pharmaceutics*** 2021, *13*, 2118;

IF: 6.321; MNiSW: 100

- Lewandowska, Hanna; Eljaszewicz, Andrzej; Poplawska, Izabela; Tynecka, Marlena; Walewska, Alicja; Grubczak, Kamil; **Holl, Jordan**; Hady, Hady Razak; Czaban, Slawomir; Reszec, Joanna; Przybytniak, Grazyna; Głuszewski, Wojciech; Sadło, Jaroslaw; Dąbrowska-Gralak, Malgorzata; Kowalewski, Cezary; Fiedor, Piotr; Oldak, Tomasz; Kaminski, Artur; Zimek, Zbigniew; Moniuszko, Marcin. Optimization of Novel Human Acellular Dermal Dressing Sterilization for Routine Use in Clinical Practice. ***International Journal of Molecular Science.*** 2021, *22*, 8467.

IF: 5.924 ; MNiSW: 140

- Grubczak, Kamil; Kretowska-Grunwald, Anna; Groth, Dawid; Poplawska, Izabela; Eljaszewicz, Andrzej; Bolkun, Lukasz; Starosz, Aleksandra; **Holl, Jordan**; Mysliwiec, Marta; Kruszewska, Joanna; Wojtukiewicz, Marek Z.; Moniuszko, Marcin. Differential Response of MDA-MB-231 and MCF-7 Breast Cancer Cells to In Vitro Inhibition with CTLA-4 and PD-1 through Cancer-Immune Cells Modified Interactions. ***Cells*** 2021, *10*, 2044.

IF: 6.600; MNiSW: 140

2.2. Conference abstracts

- **Holl, Jordan**; Pawlukianiec, Cezary; Corton Ruiz, Javier.; Groth, Dawid; Grubczak, Kamil; Hady, Hady Razak; Dadan, Jacek; Reszec, Joanna; Czaban, Slawomir; Kowalewski, Cezary; Moniuszko, Marcin; Eljaszewicz, Andrzej. Differential preparation of acellular dermal matrices induces distinct immunomodulatory characteristics. *15th digital World Immune Regulation Meeting "Immune Activation, Effector Functions and Immune Tolerance with a special focus on Autoimmunity and Allergy", 30 June - 3 July 2021.* **(Registration grant award winner)**

- Pawlukianiec Cezary; **Holl Jordan**; Corton Ruiz, Javier; Moniuszko, Marcin; Eljaszewicz, Andrzej. Evaluation of the immunomodulatory effects of acellular

dermal matrices prepared by various methods. *LXXV International Scientific and Practical Conference for Students and Young Scientists Actual Problems of Modern Medicine and Pharmacy 2021. Republic of Belarus, Minsk, 14-16 April 2021.* **(Poster 1st place award)**

- Holl Jordan; Pawlukianiec Cezary; Groth Dawid; Grubczak Kamil; Hady, Hady Razak; Reszeć Joanna; Czaban Slawomir; Eljaszewicz Andrzej; Moniuszko Marcin. Evaluation of different decellularization protocols of human skin on acellular dermal matrix immunogenic and immune modulatory properties. *11th South Eastern European Immunology School (SEEIS2019). Program and Abstracts. Pristina, Kosovo. September 27-30, 2019.* **(Full registration and travel award)**

- Pawlukianiec Cezary; **Holl Jordan**; Groth Dawid; Grubczak Kamil; Popławska Izabela; Eljaszewicz Andrzej; Moniuszko Marcin. The effects of different decellularization protocols on the immune modulatory properties of human acellular dermal matrices. *6th Lublin International Medical Congress for Students and Young Doctors, Lublin, 28-30 November 2019.*

2.3. Impact Factor and Ministry of Science Points Summary

Article type	**Number**	**Impact Factor**	**MNiSW points**
Articles in this dissertation	2	12.921	240
Articles not in this dissertation	3	18.844	380
Conference abstracts	4	-	-
Total	9	31.765	620

II. Abbreviations

ADM – Acellular Dermal Matrix

ATP – Adenosine 50-triphosphate

CCR – Chemokine Receptor

CFSE – Carboxyfluorescein Succinimidyl Ester

CXCL – Chemokine Ligand

DAMP – Danger Associated Molecular Pattern

DAPI – 4′,6-diamidino-2-phenylindole; a blue-fluorescent DNA stain

DFU – Diabetic Foot Ulcer

DNA – Deoxyribonucleic Acid

EB – Epidermolysis Bullosa

ECM – Extracellular Matrix

EGF – Epidermal Growth Factor

FBS – Fetal Bovine Serum

FGF – Fibroblast Growth Factor

FMO – Fluorescence Minus One

hADM – Human-derived Acellular Dermal Matrix

HMGB1 – High Mobility Group Box protein 1

IFNγ – Interferon Gamma

IL – Interleukin

M1 Macrophage – A broadly pro-inflammatory Macrophage

M2 Macrophage – A broadly anti-inflammatory & pro-regenerative Macrophage

MCP-1 – Monocyte Chemotactic Protein 1

MFI – Mean Fluorescence Intensity

MMP – Matrix Metalloproteinase

MSC – Mesenchymal Stem Cell

NaCl – Sodium Chloride

NET – Neutrophil Extracellular Trap

PAMP – Pathogen Associated Molecular Pattern

PBMC – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PDGF – Platelet-Derived Growth Factor

PHA-P – Phytohaemagglutinin P; a T cell activator

RBS – Red Breast Syndrome

SDF-1 – Stromach Cell-derived Factor 1

SDS – Sodium Dodecyl Sulfate

T2DM – Type 2 Diabetes Mellitus

TIE-2 – Angiopoietin-1 receptor

TGF-β – Transforming Growth Factor Beta

Th17 –T Helper 17 Cell

TIMP – Tissue Inhibitor of Metalloproteinase

TNF – Tumor Necrosis Factor

Treg – Regulatory T Cell

TSLP – Thymic Stromal Lymphopoietin

VEGF – Vascular Endothelial Growth Factor

III. Introduction

The management of deep (full thickness), hardly-healing wounds, and non-healing ulcers remain a significant clinical challenge. To date, standard treatment procedures aim at surgical wound preparation and its subsequent closure. However, the wounds that extend below the deep layers of the skin require a more complex treatment. In such cases, a full- or split-thickness skin graft is used to cover the wound. Unfortunately, a limited quantity of available autologous grafts and subsequent complications associated with the invasiveness of graft collection constitutes a significant limitation of this therapeutic option. Therefore, there is a substantial need to develop novel methods for deep wound care.

Normal wound healing is composed of 4 distinct but overlapping phases, namely:

a. Hemostasis. <1 day. Capillaries become permeabilized. Patrolling inflammatory cells are chemoattracted into the wound. Ultimately, coagulation occurs as platelets flood the damaged area.
b. Inflammation. 1-3 days. Pro-inflammatory cells such as neutrophils, mast cells, and some monocytes are chemically attracted to and extravasate from circulating blood into the wound with the goal of cleansing foreign particulates, dead/injured tissue, and other debris. This phase tapers off as debris is cleared, with reduced cell infiltration of neutrophils. A provisional extracellular matrix (ECM) is laid down near the end of this phase.[1-3]
c. Proliferation. 3-7 days. The wound microenvironment changes to become characterized by a pro-regenerative environment consisting of growth factors, cytokines, and matrix metalloproteinases (MMPs). This phase is characterized by intense angiogenesis, the chemoattraction of fibroblasts, and macrophage polarization to an anti-inflammatory M2 phenotype. The provisional ECM is extracted and replaced by a more permanent collagen structure.[4]
d. Remodeling. 7+ days. Total epidermal wound closure. Fibroblasts and macrophages tightly regulate MMPs and their inhibitors, re-organizing dermal proteins and ECM components, ultimately returning the damaged skin to its normal functionality[5-7].

In contrast to normal wounds, chronic wounds are characterized by a variety of mechanistic complications which contribute to poor wound healing. These include neuropathy, angiogenic dysregulation, hypoxia, persistent inflammation, impaired

fibroblast function, and an abundance of cytokine/matrix metalloproteinase (MMP)/growth factor dysregulations. This may be further contributed to by systemic disorders such as metabolic disorders like type 2 diabetes mellitus (T2DM), which is known to both glycate proteins as well as to induce severe epigenetic consequences on the hematopoietic stem cells, which will later differentiate into the inflammatory and regenerative cells which infiltrate wounds, respectively[8-14] (Figure 1).

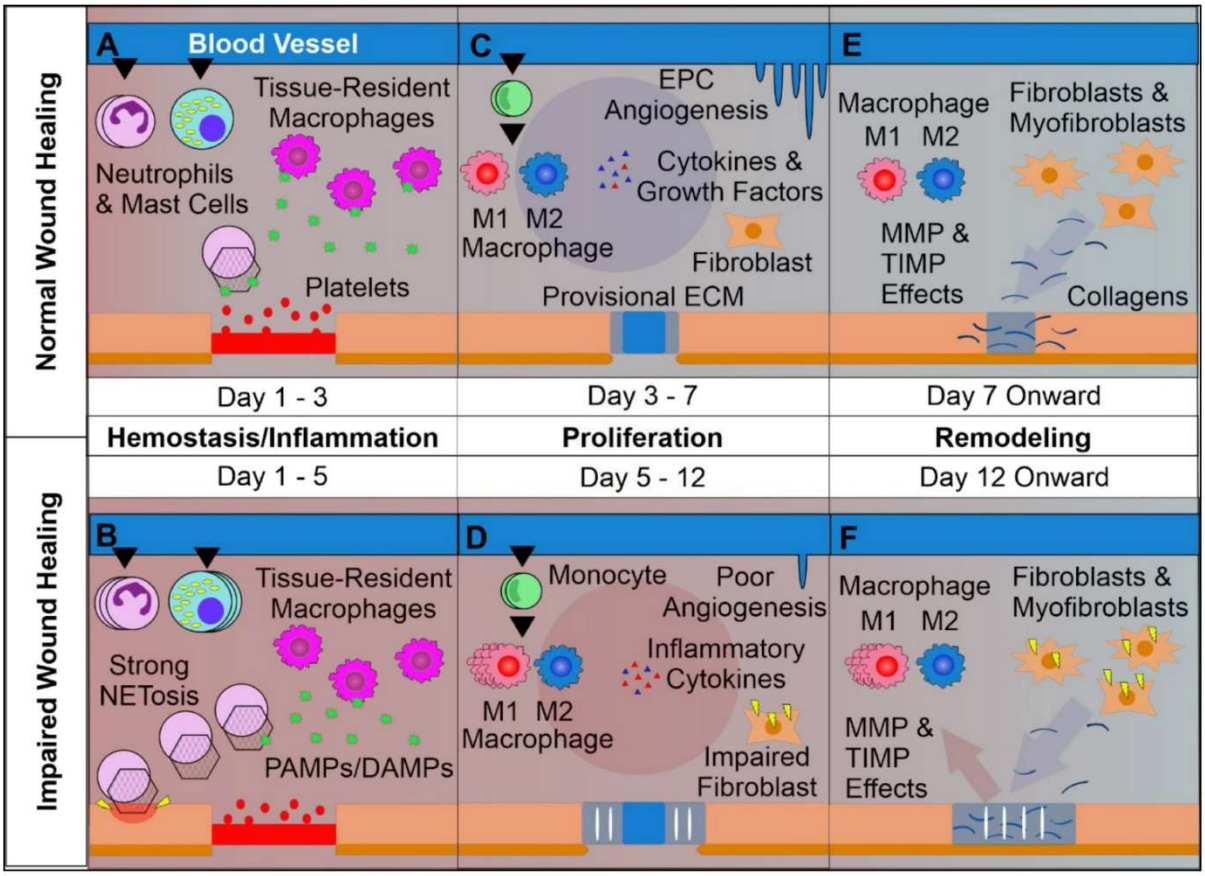

Figure 1: Overview of Normal vs. Impaired Wound Healing. A: The first phase of wound healing is hemostasis. Platelets form a clot at the injury site, and chemoattractants are released, recruiting key inflammatory cells. Next, inflammation takes charge, with infiltrating neutrophils and mast cells releasing pro-inflammatory cytokines and inducing strong sanitizing effects[15,16]. This is accompanied by neutrophil extracellular trap (NET) induction, which assists in capturing and destroying invading pathogens. Tissue-resident macrophages react to pathogen- and damage-associated molecular patterns (PAMPs & DAMPs), activating. Later a provisional matrix comprised of fibronectin and other provisional ECM components forms from the clot[2,4]. B: Impaired wounds see an upregulated influx of neutrophils and mast cells, leading to an overactive inflammatory response, causing collateral damage and extending the inflammatory phase to the detriment of subsequent phases[9,10,17]. C: Following the resolution of strong inflammation, the proliferative phase begins. Crucially, endothelial progenitor cells are stimulated by growth factors to induce angiogenesis. This angiogenesis allows for wound-resident cells to be supplied with oxygen and nutrients, facilitating their function. Infiltrating monocytes differentiate into M1 and M2 macrophage subsets[18]. M1 macrophages maintain a strong inflammatory profile, but are counterbalanced by pro-regenerative M2 macrophages which release anti-inflammatory cytokines, growth factors, and proteases which replace the provisional ECM with collagens, assisted by properly functioning fibroblasts. This process results in thick granular tissue and full keratinocyte coverage. D: Impaired wounds result in poor angiogenesis and, in the case of T2DM, glycated proteins. This hypoxic environment induces oxidative stress, driving inflammatory M1 macrophage polarization and impairment of fibroblasts, resulting in poor ECM reorganization and a persistent inflammatory environment[8,19,20]. E: Remodeling is carried out by macrophages, fibroblasts, and myofibroblasts re-organizing the provisional ECM into a coherent scar structure primarily by means of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), resulting in tissue with strong tensile strength and functionality[21,22]. F: Impaired wound-resident cells remain ineffective and pro-inflammatory. Collagen reorganization

resolves poorly, resulting in weak, non-functional skin that is apt to re-injure and potentially ulcerate, perpetually inflamed[5,23,24]. Figure adapted from Holl J et al. *Cells*. 2021.

Immune and progenitor cells play a crucial role in the effective healing of wounds (healing mechanisms and the role of different immune cells in the processes have been extensively presented in the review paper included in the dissertation, please see page 28). Briefly, T cells, neutrophils, monocytes, and fibroblasts act as mediators of inflammation and/or pro-regenerative signals which mediate the progression of wound healing through the subsequent phases of healing (Figure 1). The release of inflammatory mediators upon injury, including chemokines (such as CCL2, CCL3, and MCP-1), cytokines (for example IFNγ, TNF, and IL-6), danger-associated molecular patterns (DAMPs, such as adenosine triphosphate, high-mobility group box 1, heat shock protein, etc.), and lipid mediators, orchestrate local inflammation aimed at wound clearance from invading microorganisms, necrotic cells, and cell debris [15,24-29]. Moreover, it is well documented that the appropriately regulated inflammatory phase triggers the proliferation of tissue-resident and progenitor cells in the wound area[29-31]. However, in hardly healing wounds, prolonged or even uncontroled inflammation limits cell proliferation, extending the proliferative phase of wound healing [5,32,33]. Due to their pleiotropic biological activities, wound infiltrating monocytes and local macrophages have been proposed as central orchestrators of the healing phases transition from inflammatory to proliferative. In fact, monocytes/macrophages possess functional plasticity, allowing them to change their proinflammatory potential (characteristic for classically activated M1 phenotype) towards anti-inflammatory/reparatory function (typical form alternatively activated M2 phenotype). Therefore, in hardly healing wounds, M1 macrophages are presented with more significant quantities than their M2 counterparts [8,11,34]. Furthermore, monocytes/macrophages significantly contribute to the remodeling phase by the release of growth factors (such as insulin-like growth factor-I (IGF), vascular endothelial growth factor (VEGF), and TGF-β etc.), chemokines and cytokines (including CXCL4, CXCL8, CCL2, CCL5, IL-1 receptor agonist, and IL-10), MMPs and their inhibitors, and lipid mediators.

Skin substitutes represent a broad selection of synthetic and biologically-derived tissues which mimic the dermis and/or epidermis when transplanted into a wound environment, consequently covering the wound area, improving wound healing, and resolving the injury with minimal scarring. Although there exists a great variety of skin substitutes (Table 1), the acellular dermal matrix (ADM) are often an option in plastic surgery, as well as the treatment of burns, hardly healing wounds, and chronic wounds such as diabetic wounds

and epidermolysis bullosa. Notably, their effectiveness has been shown in some cases preferable to standard care procedures[35-39].

Table 1: Categories of Skin Substitutes

Composition	Material	Additives
Dermal	Autogenic	None—Fully Acellular
Epidermal	Allogenic	Acellular with Remnants
Full Skin	Xenogenic	Cellular (MSCs)
	Synthetic	Molecular (MMPs/TIMPs/Growth Factors/Cytokines)
	Mixed	

Table adapted from Holl J et al. *Cells*. 2021.

An ideal skin substitute should generally possess a variety of characteristics that have been linked with beneficial wound healing as a consequence of regenerative potential, low immunogenicity, and immune-modulatory activity, among others (Table 2). ADMs lack cellular repertoires and therefore possess only extracellular matrix components that are not only already present in the normal dermis but are biomimetic to recipient proteins. Consequently, when properly utilized, these unique features should reduce graft rejection and improve healing processes. Moreover, they can serve as a scaffold for stem cells, growth factors, and anti-oxidative compounds[39-41]. However, to date, the role of different preparation methods including decellularization protocols (the use of ionic detergents, nonionic detergents, or enzymes), asepticity of manufacturing (sterile preparation, irradiation, and/or chemical sterilization), and the source of skin grafts on ADMs immune-modulatory potential remains to be fully elucidated. In fact, the vast majority of available reports focus on the outcomes of specific skin substitutes in a variety of impaired or unimpaired wounds in experimental or clinical models, but do not elaborate on the mechanism of their beneficial effects.

Table 2: Properties of Ideal Skin Substitutes

Property	Elaboration
Non-immunogenic	• Components do not induce tissue rejection.
Bio-compatible	• Infiltrating cells can effectively adhere to scaffold material. • Integrates readily into existing ECM; cells can deposit/extract ECM
Regenerative	• Does not inhibit or promotes angiogenic function • Minimizes sub-optimal granulation of tissue & scarification • Beneficially modulates regenerative cells such as macrophages & fibroblasts • Facilitates rapid epithelial cell coverage
Protective	• Provides coverage for underlying structures • Minimizes disruptive "floating" in the wound bed • Retains & maintains a moist environment, reducing oxidative stress
Non-pathogenic	• The low number of applications to minimize infection risk • Sterile preparation method & donor source do not confer disease
Durable	• The substitute does not degrade before regenerative action. • Complicating conditions (infection, T2DM-induced glycosylation, etc.) do not compromise scaffold flexibility & function

Table adapted from Holl J et al. *Cells*. 2021.

These skin substitute characteristics are important due to the explicit interaction with and induction of function in regenerative cells such as monocytes/macrophages. As previously mentioned, monocytes/macrophages possess a variety of signaling, inflammatory, and pro-regenerative potential within the healing wound including the secretion of IL-1β, IL-6, IL-10, TNF, MMPs-1, -2, -9, the ability to orchestrate adaptive immune responses through T cell interaction, and the ability to differentiate into distinct pro-inflammatory M1 and anti-inflammatory/pro-regenerative M2 macrophage subsets[42-45]. The activation and regulation of monocytes can therefore affect the microenvironment cell composition and function, cytokine and growth factor milieu, and overall healing potential. Similarly, the inflammatory environment which is controlled by monocytes/macrophages can in turn influence fibroblasts, enhancing or degrading their angiogenic and ECM-

depository functional capacity through inflammatory regulation[5,22,46]. Therefore, examination of the ability of the preparation method to influence cellular behavior, especially monocytes, is paramount to revealing the true therapeutic potential of a given skin substitute. Notably, a more comprehensive literature review of both the normal wound healing process as well as the functional changes induced in ADM co-incubated cells thus far can be found in the review article in this dissertation.

Previously, members of the Department of Regenerative Medicine and Immune regulation proposed a new concept of human skin sourcing for hADM manufacturing. We established three distinct protocols for the creation of proprietary human bariatric surgery skin-derived acellular dermal matrices [ref]. Although the therapeutic potential of this novel hADM has been assessed in a preclinical murine model, its immune-modulatory potential remained elusive. Therefore, in my research, I wished to assess how our novel differentially prepared hADMs interact with immune cells *in vitro* (please see below).

Dissertation Aims:

1. To analyze the effect of different methods of human abdominoplasty skin processing on acellular dermal matrix immunogenicity
2. To evaluate immune-modulatory properties of differentially prepared human abdominoplasty skin-derived acellular dermal matrix
3. To evaluate the effects of immune cell on the structure of human abdominoplasty skin derived acellular dermal matrix.

IV. Materials & Methods

a. Skin Collection and Processing

Skin folds from bariatric patients were collected during abdominoplastic surgery at the 1st Clinical Department of Endocrine and General Surgery at the Medical University of Bialystok. Dermatome skin grafts were harvested from the resected skin fold, sealed in foil bags, and biobanked at -80° C for further processing. Skin was collected upon the approval of the Ethics Committee of the Medical University of Bialystok R-I-002/140/2016 from 2016-4-28. Study participants gave written informed consent, and the study was conducted following the provisions of the Helsinki Declaration

Dermal fragments were thawed in saline (Biomed-Lublin) at room temperature, followed by a decellularization step using chemical and/or enzymatic processing See table 3 for full details. Directly after decellularization and washing, acellular dermal matrices were lyophilized using automated -80° C lyophilization (Telstar LyoQuest), sealed in double foil bags, labeled, and biobanked in -80° C. The efficiency of the decellularization process was controlled using histochemical staining, when fragments of ADM were examined confocally for evidence of DAPI staining. No DAPI staining was observed.

Prior to use, 8mm fragments were created by biopsy punch. Next, 8mm acellular dermal matrix (ADM) fragments were re-hydrated in a 1mL complete culture medium – RPMI 1640 (Thermo Fisher) supplemented with 10% FBS (PAN Biotech) and 75 ug/mL gentamicin (Gibco) – for 24 hours in a 37° C, 5% CO^2 incubator. Each independent experiment utilized an hADM from a distinct decellularization series.

Table 3. hADM Decellularization Protocols.

hADM Preparation Method	Phase 1				Phase 2					Washing			Storage
	Reagents	time [h]	Temp. [°C]	[RPM]	Epidermal Removal	Reagents	time [h]	Temp. [°C]	[RPM]	Washing	Time [h]	[RPM]	Lyophilization Temp [°C]
hADM 1	1M NaCl + Antibiotic mix	24	37	40	Mechanical	SDS 0.1% + Antibiotic Mix	24	37	40	H_2O	5 × 24h	60	YES/ −70
hADM 2	1M NaCl + Antibiotic mix					3% Triton X-100 in PBS + Antibiotic Mix							
hADM 3	TrypLE Select + Antibiotic mix				Unnecessary	3% Triton X-100 in PBS + Antibiotic Mix							

NaCl—sodium chloride; SDS—Sodium dodecyl sulfate; PBS—Phosphate-Buffered Saline, H2O—distilled deionized water. Table adapted from Holl J et al. *Pharmaceutics*. 2021.

b. Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMCs were isolated from fresh buffy coats obtained from healthy donors at the Regional Blood Donation Center in Bialystok by means of density gradient centrifugation (Pancol, PAN Biotech), as previously described [28]. Pharm lyse buffer (BD) was used to remove residual red blood cells when needed. PBMC numbers were evaluated using a Bürker chamber. Freshly isolated PBMCs were resuspended in complete culture medium and used immediately for further research. Buffy coats were collected upon the approval of the Ethics Committee of the Medical University of Bialystok R-I-002/634/2018 from 2019-02-28. Study participants gave written informed consent, and the study was conducted following the provisions of the Helsinki Declaration.

c. T Cell Proliferation Assay

Freshly-isolated PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich) in PBS (Corning) for 5 min at room temperature in the dark. CFSE-labeled cells were washed three times in PBS (5 min, 400x g). Next, the cells were resuspended in complete culture medium and gently seeded on re-hydrated 8mm hADM fragments, 8mm unprocessed skin fragments, or left alone (vehicle/unstimulated control) in 24 well culture plates (Eppendorf) at the density of 1 x 10^6 cells/mL. Mitogen stimulation (5 ug/mL PHA-P, Gibco) was included as a positive control of PBMC proliferation. The cells were stimulated for 7 or 14 days with medium changes every four days. Finally, the cells were collected and stained with mouse anti-human CD3-FITC and CD8-PE conjugated antibodies (BD Biosciences) for 15 min in the dark. See table 4 for antibody information. Next, the samples were washed in PBS (Corning) and fixed using CellFix (BD) and analyzed by FACSCalibur (BD) flow cytometry. Obtained data were analyzed using FlowJo software (TreeStar inc.). Appropriate staining controls were used for setting the gates. Utilized gating strategy is figure 2. The results are presented as a ratio of proliferation versus the unstimulated control.

Table 4. Antibodies Utilized in Flow Cytometry.

	Cellular Marker	Fluorochrome	Origin/Isotype	Clone	Supplier
T Cells	CD3	PerCP	Mouse / IgG1, k	SK7	BD
	CD4	FITC	Mouse / IgG2b, k	OKT4	BioLegend
	CD8	PE	Mouse / IgG1, k	SK1	BD
	CD25	PE-Cy7	Mouse / IgG1, k	BC96	BioLegend
	CD127	PE	Mouse / IgG1	R34.34	Beckman
	CD161	APC	Mouse / IgG1, k	HP-3G10	BioLegend
	CD196	PerCP Cy5.5	Mouse / IgG2b, k	G034E3	BioLegend
	IL-17	PE	Mouse / IgG1, k	N49-653	BD
	IFNγ	PE-Cy7	Mouse / IgG1, k	B27	BD
Monocytes	CD14	PerCP	Mouse / IgG2b, k	MϕP9	BD
	CD16	FITC	Mouse / IgG1, k	3G8	BD
	CD163	PE	Mouse / IgG1, k	GHI/61	BioLegend
	TIE-2	Alexa Fluor 647	Mouse / IgG1, k	Ab33	BioLegend
	IL-10	PE	Rat / IgG2a, k	JES3-19F1	BioLegend
	TNF	PE	Mouse / IgG1	6401.1111	BD

Table adapted from Holl J et al. *Pharmaceutics*. 2021.

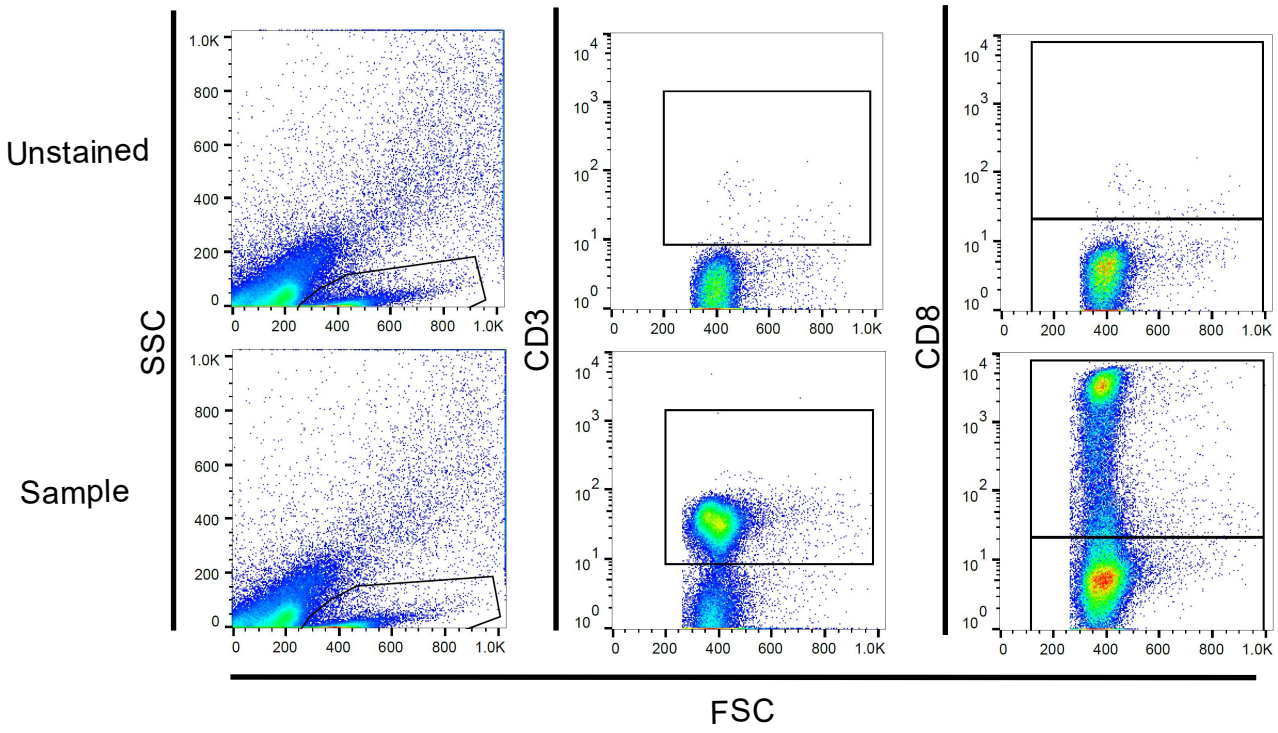

Figure 2. Representative Gating for T Cell Proliferation. This supplemental figure relates to Figure 1. CFSE-stained PBMCs cultured either alone or co-incubated with hADMs were collected, stained for CD3 & CD8, and underwent flow cytometry. Proliferation was quantified as shown on Figure 1 via histogram gating. Figure adapted from Holl J et al. *Pharmaceutics*. 2021.

d. Assessment of Monocyte and T Cell Phenotype

Freshly isolated PBMCs were gently seeded on re-hydrated 8mm hADM fragments or left alone (vehicle/unstimulated control) in 24 well culture plates (Eppendorf) at the density of 1 x 106 cells/mL in complete cell culture media. The cells were cultured for up to 72h and collected every 24h for flow cytometry analysis. For intracellular cytokine staining, brefeldin A (ThermoFisher) was added to culture wells 3h before cell acquisition. Additionally, cell culture supernatant was collected and biobanked at -80^{o} C for cytokine assay. Next, the cells were stained immediately with a panel of monoclonal antibodies (Table 4). Briefly, cells were incubated in the presence of monoclonal antibodies for 30 min at room temperature in the dark. Next, the specimens were washed twice in PBS and fixed with CellFix (BD) or subjected to intracellular staining. For the latter, samples were fixed and permeabilized using Perm2 Buffer (BD) according to manufacturer instructions, followed by washing in PBS and staining with fluorescent-conjugated antibodies (Table 4) for 30 min at 4^{o} C in the dark. Next, the cells were washed twice and fixed with CellFix (BD). Finally, the specimens were analyzed on a FACSCalibur flow cytometer (BD). Flow cytometry data analysis was performed using FlowJo software (Tree Star). Appropriate,

fluorescence-minus-one (FMO) controls were applied for setting correct gating. Utilized gating strategies for different T cell and monocyte subsets are presented in figures 3 & 4. Utilized gating strategies for T cell and monocyte function are presented in figures 5 & 6..

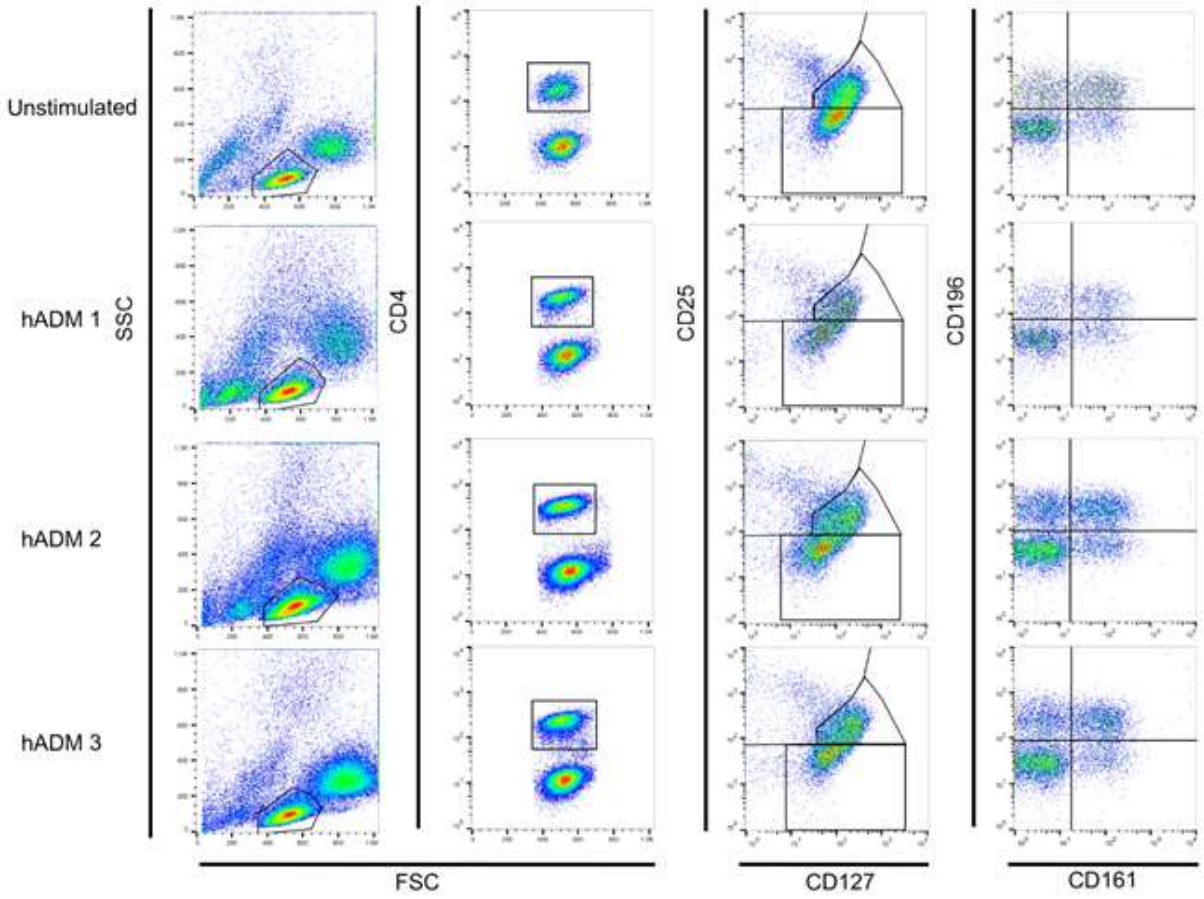

Figure 3. T Cell Phenotype is not Regulated by hADM Co-incubation. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2 or 3 days. Next, cells were stained extracellularly and examined by flow cytometry. (**A)** Representative schematic of flow cytometry gating of extracellularly-stained T cell subsets. (**B**) Frequencies of T cell subsets were noted for inactive T cells (CD25−/CD127++), active T cells (CD25+/CD127+), and Tregs (CD25++/CD127+/−). Frequency of Th17 cells were also examined by CD196+/CD161+. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed rank test used for B. $n = 5$ with 2 technical replicates; * $p < 0.05$. Figure adapted from Holl J et al. *Pharmaceutics*. 2021.

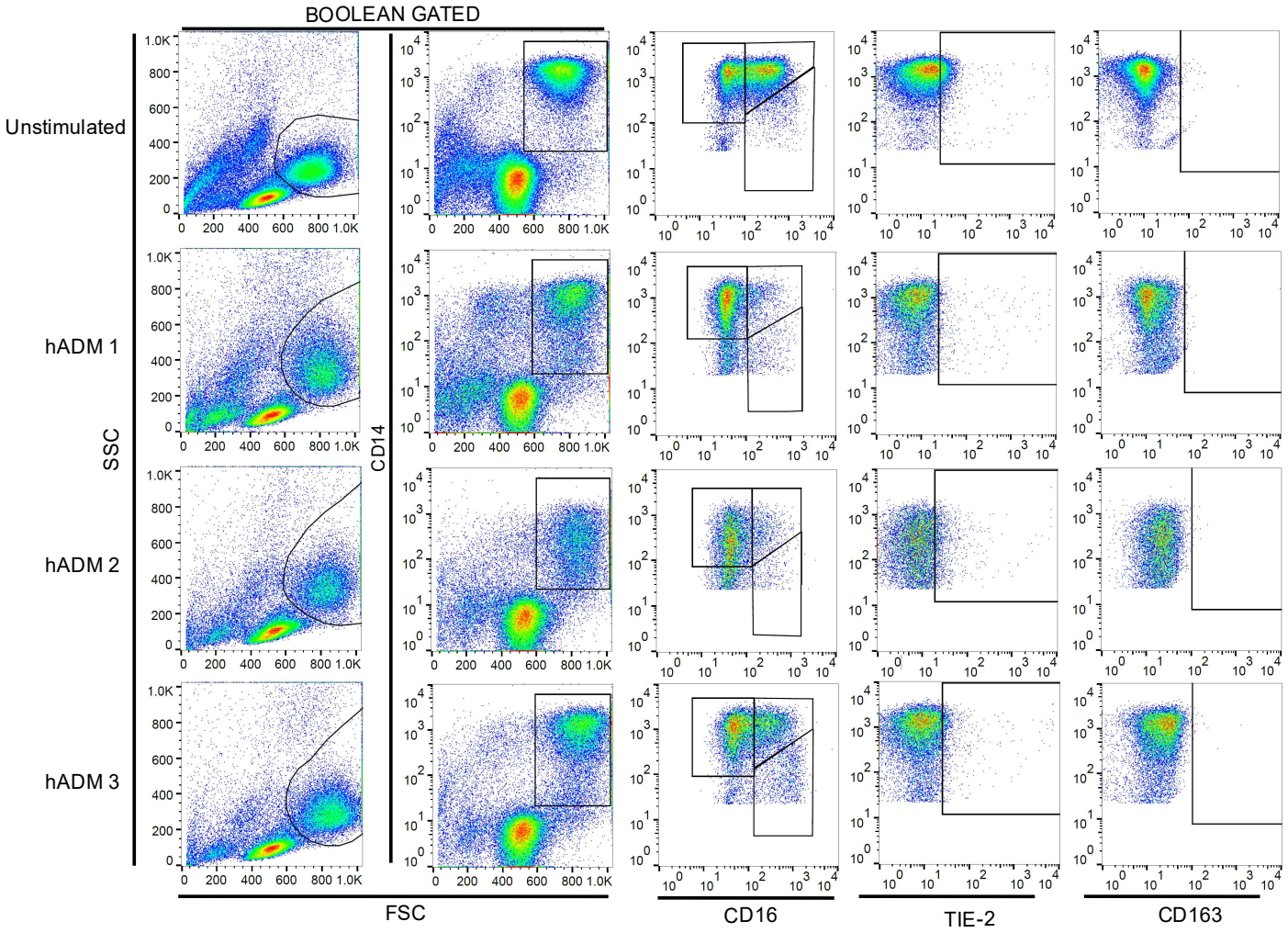

Figure 4. Representative Gating for Extracellularly-stained Monocytes. This supplemental figure relates to Figure 2. PBMCs cultured either alone or co-incubated with hADMs were collected, stained extracellularly for CD14, CD16, TIE-2 and CD163. Figure adapted from Holl J et al. *Pharmaceutics*. 2021.

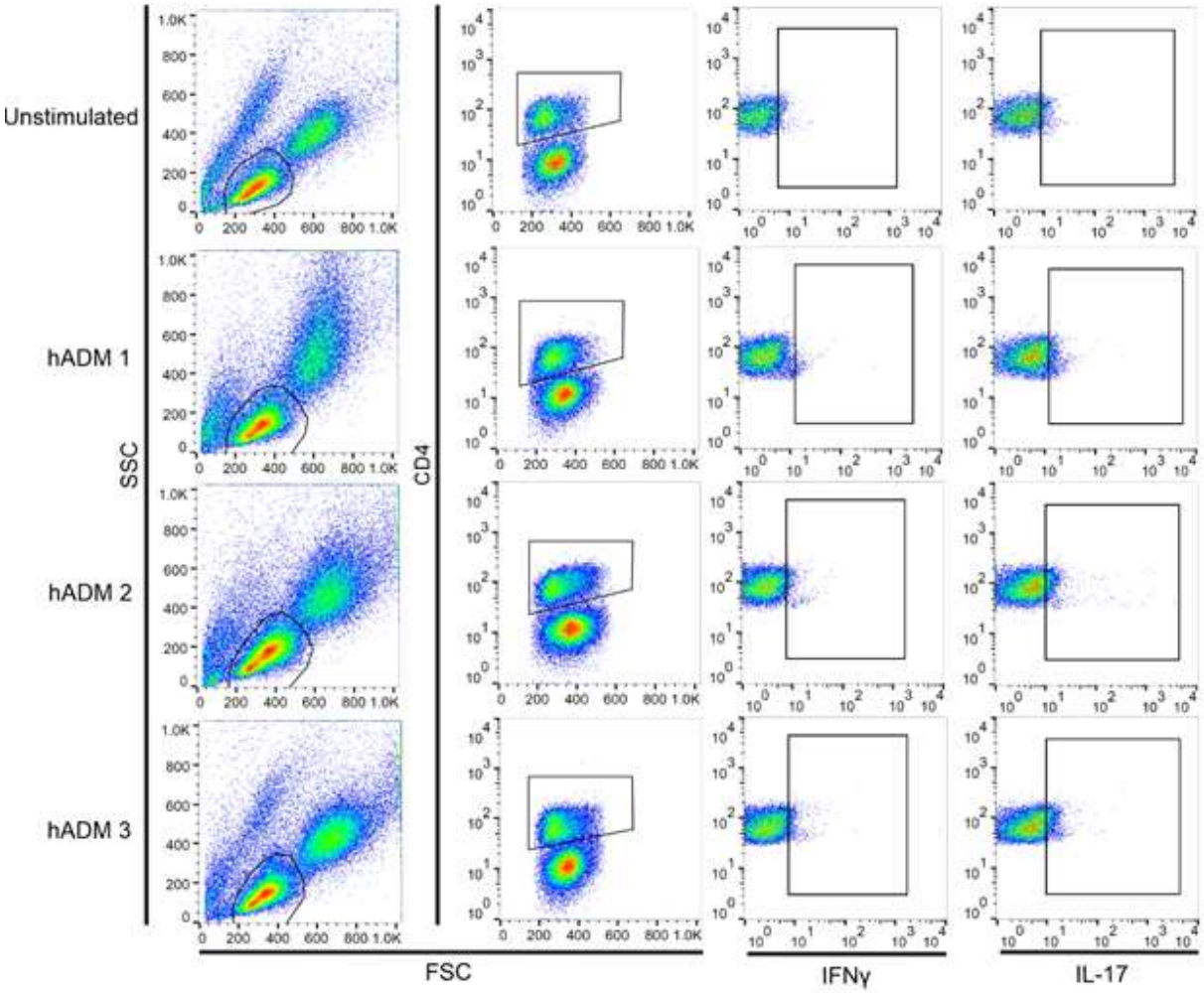

Figure 5. hADMs do not Induce Differential T Cell Function. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2 or 3 days. 2 hours before collection, protein transportation was inhibited. Finally, cells were permeabilized and stained extracellularly and intracellularly before being examined by flow cytometry. (**A**) Representative schematic for flow cytometry gating. (**B**) Quantification of CD4+/IFNγ+ or IL-17+ T cell frequency. (**C**) MFI quantification of CD4+/IFNγ+ or IL-17+ T cells. (**D**) Frequency and MFI ratios of inflammatory T cell cytokines. Results expressed as medians + interquartile range. Two-tailed Wilcoxon matched-pairs signed rank test used for B–E. $n = 5$ with 2 technical replicates; $*p < 0.05$. Figure adapted from Holl J et al. *Pharmaceutics*. 2021.

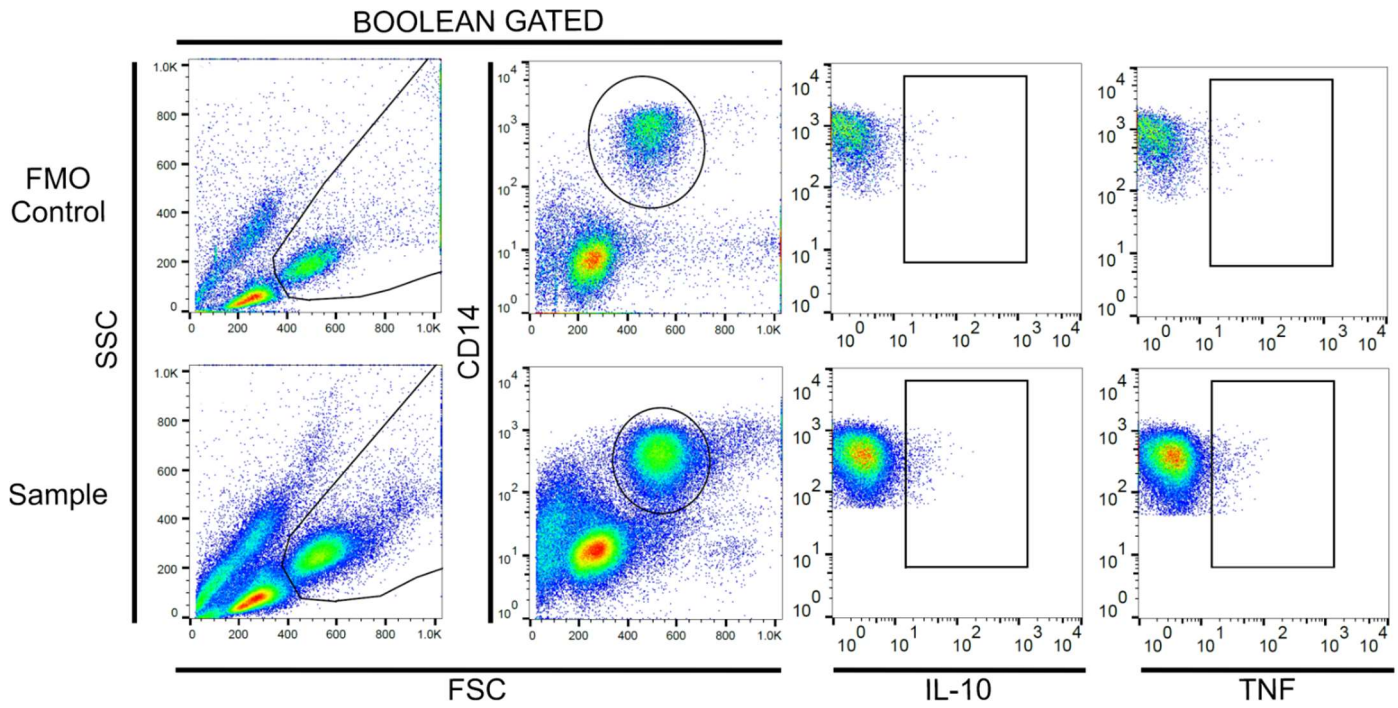

Figure 6. Representative Gating Strategy for Intracellularly-stained Monocytes. This supplemental figure relates to Figure 3. PBMCs cultured either alone or co-incubated with hADMs were collected, stained extracellularly for CD14, and intracellularly for IL-10 & TNF before undergoing flow cytometry. Figure adapted from Holl J et al. *Pharmaceutics*. 2021.

e. Cytokine Assay

Concentrations of factors TNF, IFNγ, IL-1β, IL-6, IL-10, IL-17, and TGF-β were measured by means of commercially available DuoSet ELISA (all from R&D Systems) according to suggested manufacturer protocol. The detection ranges for TNF (15.6 - 1,000 pg/mL) IFNγ (9.39 – 600 pg/mL), IL-1β (3.91 – 125 pg/mL) IL-6 (9.4 – 600 pg/mL), IL-10 (31.3 - 2,000 pg/mL), IL-17 (15.6 – 1,000 pg/mL), and TGF-β (31.3 – 2,000 pg/mL). Protein levels were analyzed with an automated microplate reader (LEDETEC96 system). The results were calculated according to the standard curve, generated by individual standard dilutions, by MicroWin 2000 Software.

f. Immunofluorescence Staining

hADMs were snap-frozen after coincubation with PBMCs mentioned previously. hADMs were cut using cryomicrotome into 20 uM longitudinal sections and seeded on glass slides. Next, cryosections were fixed with 4% paraformaldehyde (ChemCruz) and incubated in a detergent (0.1% Triton x-100 (Sigma) in 0.02% SDS-PBS (Sigma Aldrich and Corning, respectively), followed by incubation with blocking buffer (10% normal donkey serum (Abcam) in 1% BSA in PBS). Next, the slides were stained with a specific primary antibody for collagen types I, III, and IV or incubated in staining buffer (1%

protease-free bovine serum albumin (Sigma) in PBS) for 60 minutes in a high humidity chamber in the dark. Next, the slides were washed three times in washing buffer (Tween20-PBS) and stained with appropriate secondary antibodies. For primary and secondary antibodies, please see table 5. Finally, the specimens were mounted in Prolong Gold mounting medium with DAPI (ThermoFisher) and covered with cover slides (Menzel-Glasser), followed by overnight incubation at RT in the dark before analysis by confocal microscopy.

Table 5. Antibodies Utilized in Confocal Microscopy.

Primary Antibodies					
Marker		**Origin/Isotype**	**Clone**	**Supplier**	**RRID**
Collagen 1A1		Rabbit / IgG	Polyclonal	Invitrogen	AB_2547045
Collagen III		Rabbit / IgG	Polyclonal	Invitrogen	AB_2552139
Collagen IV		Mouse / IgG1	COL-94	Invitrogen	AB_558482
Secondary Antibodies					
Fluorochrome	**Reactivity**	**Origin/Isotype**	**Clone**	**Supplier**	**RRID**
Alexa Fluor 647	Mouse	Goat / IgG	Polyclonal	Invitrogen	AB_2536165
Alexa Fluor 700	Rabbit	Goat / IgG	Polyclonal	Invitrogen	AB_2535709

Table adapted from Holl J et al. *Pharmaceutics*. 2021.

g. Confocal Microscopy

Confocal pictures were acquired using an FV1200 Microscope (Olympus). Full-sized pictures were acquired in 3 channels using 405, 450, and 650nm lasers at 20us/pixel and 2048x2048 size using FluoView software. Full-sized photos were then examined using ImageJ software. Channel 1 (DAPI) was made blue, Channel 2 (autofluorescent collagen structure) was made green, Channel 3 (stained collagen fibers I, III, or IV) was made red. Z-stacks of each channel were created and merged. As many focused sections (500x500 pixels) as possible were extracted from each full-sized photograph - inside the hADM for collagens I & III or on the apical edge for collagen IV – and quantified. Each stained, quantified, focused section was divided by the mean of combined focused controls – slides stained without primary collagen-binding antibody but with secondary fluorescent antibodies specific to each collagen [Quantification = Stained Collagen / Staining Control]. Channels 1 & 2 (DAPI & autofluorescence, respectively) did not contribute to the semi-

quantitative measurement of collagens. In hADMs without co-incubated PBMCs, only one time point (24H) was used with quantification measured as mentioned above. However, when PBMC co-incubated hADMs were compared to those without PBMCs, the following equation was used [Quantification = (stained PBMC co-incubated focus section / control PBMC co-incubated focus section) / (stained non-PBMC focus section / control non-PBMC focus section)].

h. Quantification of DNA Within hADMs

hADMs were re-hydrated using 1mL of PBS in 24 well plates. Next, hADMs were digested using proteinase K in buffer solution provided in DNeasy blood & tissue DNA isolation kit (Qiagen). This resulted in the entire dissolution of hADMs into solution. This kit was used according to manufacturer protocol to isolate and extract DNA from the fibrous hADMs. Finally, DNA concentration was measured using a NanoDrop™ 2000/2000c Spectrophotometer (ThermoFisher) using NanoDrop 2000 software (ThermoFisher), specifically the nucleic acid measurement at 260/280nm.

i. Statistical Analysis

Graphs and statistics were calculated using GraphPad Prism 8 (GraphPad Software). Wilcoxon matched-pairs signed rank test was used to compare differences between analyzed conditions. To determinate the differences in residual DNA U-Mann Whitney test was used. The differences were considered statistically significant at $p < 0.05$. The results are presented as a median ± interquartile range.

V. Results

In theory, an ideal skin substitute should retain the aforementioned beneficial characteristics associated with successful clinical wound outcomes (Table 2). Therefore, my aim was to evaluate each of these novel preparation methods in the context of immunogenicity, beneficial cell polarization inductions, and ECM-retention capability. To this end, we began by examining the direct effect of each novel hADM decellularization process on immunogenicity and inflammatory potential – one of the underlying fundamental markers of clinical success.

When CFSE-stained PBMCs were cultured alone, with 1 of 3 hADMs, with full skin, or with a mitogenic control, differential levels of T cytotoxic cell (CD3+CD8+) and T helper cell (CD3+CD8-) proliferation was observed after 7 & 14 days. Notably, hADM 1 induced low T cell proliferation similar to the unstimulated control PBMCs. In contrast, hADMs 2 & 3 induced significantly higher proliferation of both analyzed T cell subsets. This result showed the low immunogenic effect of hADM 1 and the relatively higher immunogenicity of hADMs 2 & 3.

Next, PBMCs were co-incubated either alone or with 1 of 3 hADMs for 1, 2, or 3 days. First, T cells were examined for phenotype using flow cytometry analysis of non-activated T cells, activated T cells, putative regulatory T cells, and putative Th17 cells. In addition, interferon gamma (IFNy) & IL-17 producing T cells were evaluated after application of protein transport inhibitor. The obtained results suggest that analyzed hADMs have no effect on T cell phenotype. Next, monocytes were examined using flow cytometry for classical, intermediate, and non-classical subsets. I found significant differences in the composition of monocyte subsets after incubation in presence of differentially prepared abdominoplasty skin based acellular dermal matrices. Namely, I observed that these hADMs delay monocyte maturation towards the more mature intermediate and non-classical phenotypes when compared to the unstimulated control. Moreover, Tie-2 & CD163 expressing monocytes – cells with pro-angiogenic potential and anti-inflammatory potential (alternatively activated macrophage precursors), respectively – were examined. I observed slight differences between hADM co-incubated groups, with higher expression as time progressed and implied induction towards intermediate monocytes and therapeutically beneficial M2-like cells.

Next, the supernatants of PBMCs incubated alone or co-incubated with hADMs for 1, 2, or 3 days were examined to determine the release of pro-inflammatory IFNγ, IL-1β, IL-6, TNF, IL-17, as well as anti-inflammatory IL-10 and TGF-β, cytokines. Briefly, we found

that hADM 2 and hADM 3 induce pro-inflammatory profiles, with high levels of IFNγ, TNF, IL-1β, and IL-6 when compared to both unstimulated control and hADM 1. Additionally, anti-inflammatory IL-10 was found to be decreased in both unstimulated and hADM 1 in contrast to the significantly higher concentration seen in hADM 2 supernatants.

Having found significant differences in the immune responses to differentially manufactured novel abdominoplasty skin-derived hADMs, the influence of immune cells on the extracellular matrix structure, namely collagen architecture, was analyzed. Although no visible differences in the structural architecture of hADMs after PBMC co-incubation were observed, collagen IV fragmentation on the apical surface of hADMs was evidenced. Moreover, the localization of PBMCs (presumably monocytes) with collagen fibers was witnessed. To summarize, differences in collagen retention were revealed as well as the notable fragmentation of collagen IV.

Ultimately, we observed significant differences between hADM preparation methods in regard to co-incubated cell immunogenicity, cell polarization and functional capacity, and collagen retention. Notably, hADM 1 was found to induce T cell proliferation levels similar to that of the control, delay monocyte maturation towards a pro-inflammatory non-classical monocyte phenotype, induce similar inflammatory cytokine secretion versus control, and to retain crucial collagens I, III, & IV after processing, albeit with some collagen IV fragmentation and cellular adhesion to those fragments. Broadly speaking, each method of preparation induced unique and significantly different responses within the same co-incubated PBMC populations.

VI. Conclusions

a. Method of human abdominoplasty skin preparation induces differential immunogenic effects in co-incubated PBMCs. Notably, PBMCs co-incubated with hADM 1 (prepared using ionic detergent) behaved similarly to unstimulated controls while hADMs 2 & 3 (prepared using non-ionic and enzymatic detergents, respectively) induced strong immunogenic stimulatory effects after 7 & 14 days.
b. hADM co-incubation affected PBMC phenotype & function. Although T cells were not significantly altered in regard to phenotype & function after 3 days, monocytes were strongly affected, with an apparent delay of activation & maturation, as well as a shift into more pro-regenerative M2-like phenotype. Further, analysis of cytokine profiles revealed differential functional capacity of co-incubated PBMCs in hADM groups, with hADM 1 displaying a profile similar to the unstimulated control, while hADMs 2 & 3 were strongly pro-inflammatory.
c. Finally, hADM preparation retention of extracellular matrix collagens, especially at the apical surface where PBMCs were cultivated for up to 3 days. Importantly, collagen IV was fragmented, with PBMCs (presumably monocytes) further localizing with these fragments.
d. The method of hADM 1 preparation – through anionic detergent sodium dodecyl sulfate – may be suitable for the creation of non-immunogenic ADMs *in vivo*.

Vii. Dissertation Publications

cells

MDPI

Review

Chronic Diabetic Wounds and Their Treatment with Skin Substitutes

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Abstract: With the global prevalence of type 2 diabetes mellitus steeply rising, instances of chronic, hard-healing, or non-healing diabetic wounds and ulcers are predicted to increase. The growing understanding of healing and regenerative mechanisms has elucidated critical regulators of this process, including key cellular and humoral components. Despite this, the management and successful treatment of diabetic wounds represents a significant therapeutic challenge. To this end, the development of novel therapies and biological dressings has gained increased interest. Here we review key differences between normal and chronic non-healing diabetic wounds, and elaborate on recent advances in wound healing treatments with a particular focus on biological dressings and their effect on key wound healing pathways.

Keywords: wound healing; diabetes; chronic wounds; skin substitutes; skin dressings; matrices

1. Introduction

The global impact of diabetes, including type 2 diabetes mellitus (T2DM), is severe, costing over 760 billion dollars—constituting 10% of adults' annual health expenditure. More importantly, diabetes is projected to affect over 700 million individuals by 2045 (7.8% of the global population) [1,2]. In 2019 alone, more than 4 million adults died from direct and associated complications of diabetes. This prevalence and burden clearly outline diabetes and its associated complications as pressing global concerns.

Diabetic patients develop wounds characterized by impaired healing, prolonged inflammation, and reduced epithelization kinetics. Notably, 15% of patients suffering from T2DM develop ulcers localized on the lower limbs, referred to as diabetic foot ulcers (DFUs). DFUs represent the most severe form of diabetic wounds which may lead to lower limb amputation or death [3]. In fact, DFUs precede 84% of all diabetes-related lower limb amputations. Therefore, there exists a substantial need to elucidate the pathological processes causing ulceration, and which affect wound healing in diabetics.

Wound healing is defined as a natural physiological process occurring as the reaction to structural damage of tissues, including skin. These mechanisms involve sophisticated complimentary interactions between different cell types, acting through networks of soluble

mediators, including cytokines, chemokines, growth factors, and metabolites. Wound healing consists of four subsequent and overlapping phases: hemostasis, inflammation, proliferation (re-epithelization), and remodeling (scar maturation).

Interestingly, diabetic hyperglycemia contributes to a variety of systemic complications, causing an array of local pathologies manifesting within the wound microenvironment, including chronic inflammation, dysregulated angiogenesis, hypoxia-induced oxidative stress, neuropathy, advanced glycation end-products, and impaired neuropeptide signaling [4]. Here we discuss the influence of diabetes on wound healing and the formation of diabetic foot ulcers. Moreover, we discuss strategies for diabetic wound treatment concentrated on the use of skin substitutes and biological dressings.

2. Wound Healing Starts with Homeostasis

Immediately after wounding, degranulation of mast cells induces capillary permeability, in addition to vasodilation, increasing bleeding and allowing the influx of immune cells. Furthermore, the coagulation system is activated, and a scab is formed of provisional components [5]. Simultaneously, activated keratinocytes, fibroblasts, and platelets release soluble mediators: (a) growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF); (b) chemokines, including IL-8 (CXCL-8) and CXCL-2; (c) danger-associated molecular patterns (DAMPs) such as histones, genomic DNA, adenosine 5′-triphosphate (ATP), high mobility group box protein 1 (HMGB1); and (d) cytokines; namely, thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 [6–9]. Notably, all of the above-mentioned inflammatory mediators act as danger signals. Consequently, they trigger the infiltration of patrolling inflammatory cells and the induction of local immune responses (inflammation phase) and subsequent proliferative induction of tissue-resident cells.

3. Wound Inflammation Orchestrates Healing and Regeneration

A strong inflammatory cascade, initiated during hemostasis, now commences to clean the wound of debris, damaged cells, and microbes. The inflammatory phase is characterized by (a) an influx of inflammatory cells including neutrophils, monocytes/macrophages, mast cells, and T cells; (b) the accumulation of inflammatory mediators such as cytokines, chemokines, and lipid mediators; and c) the release of extracellular matrix degradation enzymes such as matrix metalloproteases (MMPs) and collagenases; causing swelling, heat, and pain (Figure 1) [4].

It is widely recognized that localized, properly controlled inflammation acts as a trigger for the proliferative and remodeling phases [10,11]. On the other hand, the uncontrolled or prolonged inflammatory responses frequently observed in diabetic wounds lead to the impairment of subsequent phases of the wound healing process, or are implicated as a contributor to ulceration [12,13]. Local inflammation is strictly associated with neutrophil infiltration and activation. Interestingly, neutrophils are absent in unwounded skin and their trafficking to the wound area is induced and controlled by tissue-resident T cells, mast cells, and macrophages [14]. In fact, neutrophils represent an important source of proteases (including elastase, cathepsin G, and urokinase-type plasminogen activator—that support re-epithelialization) [15–17]; reactive oxygen and nitrogen species, cytokines (including IL-1β, tumor necrosis factor (TNF), IL-6, IL-12p40, and transforming growth factor β (TGF-β)), and chemokines (including CCL2, CCL3, CCL5, CXCL1, and CXCL2) [18]. Moreover, generally high neutrophil counts within the wound, and the consequently increased neutrophil-to-lymphocyte ratio is recognized as a hallmark of impaired wound healing observed in T2DM-affected individuals [19]. Interestingly, T2DM is known to induce neutrophil extracellular trap induction (NETosis), a phenomenon which may be responsible for delayed wound healing, given that disruption of neutrophil ability to undergo NETosis led to accelerated wound closure in previous studies (Figure 1) [12,20]. This continual activation of neutrophils and induction of NETosis results in the induction of yet more inflammation by way of mitochondrial DNA and histone H4 [21] in contrast

with the normal process of inflammatory resolution by way of neutrophilic apoptotic body phagocytosis [11]. This uptake of neutrophil-derived apoptotic bodies' by infiltrating monocytes/macrophages helps resolve the inflammatory phase in a self-perpetuating manner by limiting inflammatory cell infiltration and shifting the production of eicosanoids from pro-inflammatory to anti-inflammatory mediators [22–24]. Unfortunately, however, in diabetic wounds the inflammatory phase is significantly prolonged by the disruption of mechanisms which both control the influx of neutrophils as well as regulate their inflammatory processes [12,21,25]. Interestingly, it seems that the cause of many observed dysregulations of the inflammatory phase is not directly associated with localized high glucose levels but rather the epigenetic polarization of innate immune cell pro-inflammatory function prior to wound infiltration, as in progenitor cell modification due to T2DM-related systemic complications such as hyperglycemia [26]. This polarization of innate immune cells towards pro-inflammatory phenotypes is additionally supported by systemic inflammatory effects observed in diabetic patients and animal models [27]. However, to date, mechanisms controlling the epigenetic regulation of neutrophils and monocytes/macrophages in diabetic individuals remain elusive.

Monocyte migration to injured skin is controlled by chemokines derived by mast cells, keratinocytes, and fibroblasts acting through CCR2, CCR5, and mainly monocyte chemotactic protein 1 (MCP-1) [28]. Notably, due to pleiotropic biological activities, monocytes and macrophages are recognized as central players in the resolution and regulation of the inflammatory, proliferative, and remodeling phases of wound healing [29]. Unlike normal wound-infiltrating macrophages differentiating into classically-activated (inflammatory) M1 and alternatively-activated (reparative/regulatory) M2 macrophages, T2DM-affected macrophages strongly polarize into the inflammatory M1 phenotype [26]. Classically activated macrophages possess high phagocytic properties and are efficient in their production of pro-inflammatory cytokines—namely IL-1α, IL-1β, IL-6, TNF, and IL-12—contributing to and extending the inflammation phase, increasing neutrophilic infiltration, and prolonging low-grade inflammation which is characteristic of chronic DFUs [26,30]. Consequently, a lower absolute number of M2 macrophages and a higher M1:M2 macrophage ratio within the wound reduces secretory levels of growth factors PDGF, FGF, and VEGF, as well as anti-inflammatory cytokines including IL-10, TGF-α and TGF-β—all of which are responsible for the induction of the proliferative phase and effective regulation of inflammation, respectively [13,31]. Moreover, monocytes/macrophages act as antigen-presenting cells, linking the innate and adaptive immune responses [29]. Despite this, to date, the role of mutual interactions between macrophages and T cells in wound healing has yet to be fully elucidated.

It is well established that skin resident T cells play an essential role in the maintenance and regulation of local skin inflammation in the course of wound healing. In fact, Th17 cells were shown to promote neutrophilic infiltration, and high levels of IL-17A were shown to reduce wound repair [32]. On the other hand, regulatory T cells (Tregs) are considered essential regulators of inflammation and constitute a significant source of IL-10 [33]. Importantly, depletion of Tregs significantly reduces wound closure [34]. It is tempting to speculate that the systemic inflammation observed in diabetic patients limits the migration of Tregs and increases the infiltration of Th17 cells in the diabetic wound and thus represents one of the mechanisms of increased neutrophilic inflammation and a prolonged inflammatory phase. Notably, the healing process of diabetic wounds may be accelerated by topical retinoic acid, thereby inducing T cell plasticity and differentiation of Th17 cells towards Tregs [35]. This confirms the crucial role of T cells in the regulation of the inflammatory phase of diabetic wound healing.

Taken together, the prolonged inflammation phase observed in diabetic wounds that impairs wound closure and remodeling originates not only from high levels of localized pro-inflammatory mediators, but also from deficiencies in anti-inflammatory cytokines derived by regulatory cells, including M2 macrophages and Tregs. Despite this observation, the regulation of this process needs attention in future research.

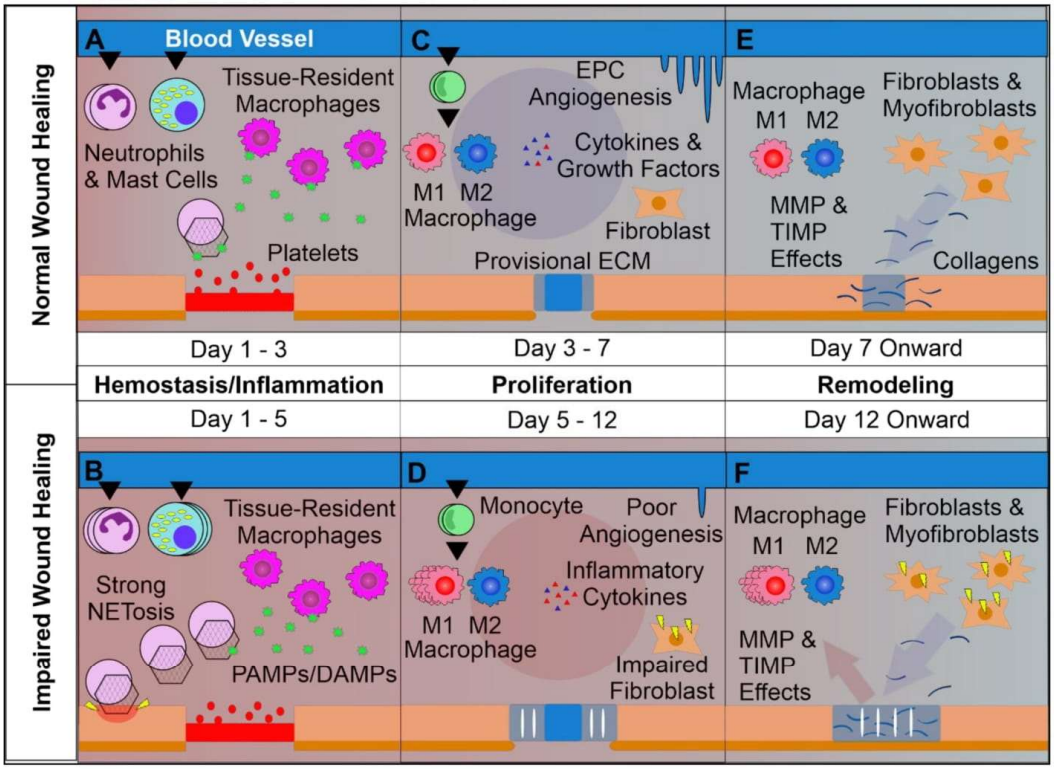

Figure 1. Overview of Normal vs. Impaired Wound Healing. (**A**): The first phase of wound healing is hemostasis. Platelets form a clot at the site of injury, and chemoattractants are released, recruiting key inflammatory cells. Next, inflammation takes charge, with infiltrating neutrophils and mast cells releasing pro-inflammatory cytokines and inducing strong sanitizing effects. This is accompanied by neutrophil extracellular trap (NETosis) induction, which assists in capturing and destroying invading pathogens. Tissue-resident macrophages react to pathogen- and damage-associated molecular patterns (PAMPs & DAMPs), activating. Later a provisional matrix comprised of fibronectin and other provisional extracellular matrix (ECM) components forms from the clot. (**B**): Impaired wounds see an upregulated influx of neutrophils and mast cells, leading to an overactive inflammatory response, causing collateral damage and extending the inflammatory phase to the detriment of subsequent phases. (**C**): Following resolution of strong inflammation, the proliferative phase begins. Crucially, endothelial progenitor cells are stimulated by growth factors to induce angiogenesis. This angiogenesis allows for wound-resident cells to be supplied with oxygen and nutrients, facilitating their function. Infiltrating monocytes differentiate into M1 and M2 macrophage subsets. M1 macrophages maintain a strong inflammatory profile, but are counterbalanced by pro-regenerative M2 macrophages which release anti-inflammatory cytokines, growth factors, and proteases which replace the provisional ECM with collagens, assisted by properly functioning fibroblasts. This process results in thick granular tissue and full keratinocyte coverage. (**D**): Impaired wounds result in poor angiogenesis and, in the case of T2DM, glycated proteins. This hypoxic environment induces oxidative stress, driving inflammatory M1 macrophage polarization and impairment of fibroblasts, resulting in poor ECM reorganization and a persistent inflammatory environment. (**E**): Remodeling is carried out by macrophages, fibroblasts, and myofibroblasts re-organizing the provisional ECM into a coherent scar structure primarily by means of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs), resulting in tissue with strong tensile strength and functionality. (**F**): Impaired wound-resident cells remain ineffective and pro-inflammatory. Collagen reorganization resolves poorly, resulting in weak, non-functional skin that is apt to re-injure and potentially ulcerate, perpetually inflamed.

4. Proliferation of Tissue Resident Cells Is Crucial for Wound Closure

Following the inflammatory phase of wound healing, the proliferative phase, characterized by formation of granulation tissue, begins (Figure 1). Granulation tissue consists of fibroblasts, immune cells, and newly formed blood capillaries which allow epithelial cell migration towards the apical wound surface in the process of re-epithelization [36]. As mentioned, fibroblasts, keratinocytes, mast cells, and M2 macrophages display potent regenerative activities, mainly through the secretion of cytokines (e.g., IL-10 and IL-35), growth factors (e.g., TGF-α, TGF-β, FGF and EGF), chemotactic factors for stem and progenitor cells (e.g., CXCL8 and SDF-1), and extracellular matrix reorganization through the activities of MMPs and their inhibitors (TIMPs) [13,23,29–32,37–39]. As discussed above, wounds with impaired healing kinetics and chronic wounds, including DFU, are known to significantly reduce skin-resident cell proliferation as well as stem and progenitor cell activation. Although this is partially the effect of the extended inflammatory phase, other compounding factors such as T2DM-mediated glycation of proteins, reduced angiogenic capability, and resultant oxidative stress contribute to the unnecessary extension of the proliferative phase, with the wounds failing to achieve closure in the most severe cases [26]. Detrimentally, the observation of dysfunctional and reduced numbers of circulating stem and progenitor cells, including endothelial progenitors, has been previously reported in T2DM patients [40,41]. This is in contrast to normally-healing wounds where neovascularization is the hallmark of the proliferation phase [4]. Contrastingly, the significantly reduced ability of endothelial progenitors to form new vessels accompanied by limited numbers of stem cells represents a significant contributor to disrupted re-epithelization in diabetic wounds [42,43]. Recently, stem cell-based therapies, including those based on mesenchymal stem cell (MSC) application, have become an attractive treatment strategy for impaired wounds, including DFUs. Novel strategies and treatment options for diabetic wounds will be discussed in the following paragraphs.

Wound closure requires reconstruction of the dermis before epithelial coverage by migratory basal keratinocytes can take place [38]. This stage requires the reconstruction of the three-dimensional collagen structure of the dermis upon which subsequent cell populations are located. Therefore, fibroblasts and myofibroblasts are considered central players in this process [44]. Their function is also supported by wound-resident macrophages, mast cells, and lymphocytes, in VEGF and TGF-β dependent mechanisms [29–31]. Importantly, this process is also closely associated with neovascularization of the wound bed, providing crucial nutrient and oxygen supplies to the healing site [45]. M2 macrophages additionally support wound angiogenesis by direct (macrophage-to-endothelial cell adhesion) and indirect (paracrine effect) mechanisms [46]. Interestingly, these activities are similar to those observed in tumor-associated macrophages, as recently discussed elsewhere [47–49]. Unfortunately, as mentioned before, in diabetic wounds, monocyte polarization towards M2 macrophages is inhibited, and pro-inflammatory polarization is promoted. Similarly, T2DM-impaired fibroblasts display a low activation level, decreased collagen deposition, and reduced paracrine signaling ability, including downregulation of TGF-β pathway activation [50].

A well-regulated proliferative phase is arguably the most crucial indicator of a successfully-healing wound, given the importance of both angiogenesis in tandem with epidermal coverage of the wound. Furthermore, when dysregulated, this process slows or even halts entirely, resulting in chronic ulcerative wounds. Although this mechanism remains to be fully elucidated due to the high number of participating entities, key cellular and molecular factors have been implicated in T2DM-induced or otherwise impaired wounds—namely fibroblasts, macrophages, key aforementioned growth factors, and unresolved/self-renewing inflammatory bodies from a persistent inflammatory phase.

5. Wound Remodeling

In the fourth phase of the healing process—wound remodeling—granulation tissue is strengthened by the accumulation of ECM proteins, which form scar tissue [44] (Figure 1).

Moreover, the decrease of cellular and vascular components as well as an increase in the concentration collagens is observed with the principal aim of recovering normal skin function.

As with their strong participation in the proliferative phase, wound-resident fibroblasts, myofibroblasts, and M2 macrophages play an integral role in remodeling [38,44,51]. Collagens comprise 85% of the dermis and are consistently re-organized during wound healing to determine terminal scar fate after the remodeling phase [52]. At this stage of normal wound healing, collagen III undergoes degradation, with the subsequent deposition of type I collagen controlled by TGF-β and FGF signaling [53]. Similarly, secreted matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) are able to guide the deposition and extraction of ECM components [54,55]. In this way, fibroblasts and macrophages can shape the final structure of a healing wound, with collagen fibers becoming thicker, denser, and intertwining—resulting in enhanced scar tissue strength in the normally-healing wound [56].

Notably, the ECM is dynamically subjected to constant changes throughout the remodeling phase, which results in the maturation of its structure. Its composition plays an essential role in skin repair via interactions of its protein structures, such as provisionally-deposited fibronectin and vitronectin, with different cell types [38]. Importantly, the delivery of ECM elements of decellularized skin structures during wound healing has been demonstrated to improve the wound healing process, and consequently become an attractive therapeutic approach by members of our and other groups (please see following chapters of this manuscript) [57–61].

In contrast to normally healing wounds, T2DM-affected wounds possess many structural and functional differences by comparison. Namely, abrogated angiogenesis resulting in a hypoxic wound environment and subsequent oxidative stress [26]. Recently, T2DM was shown to drive M1 macrophage polarization in the healing wounds of mice as a consequence of oxidative stress [26]. As previously mentioned, M1 macrophages promote inflammation and, their persistently increased numbers result in the differential expression of MMPs and TIMPs which are responsible for the reorganization of provisional ECM components in the late proliferative and remodeling phases of wound healing [38,62]. Therefore, guidance of tissue-infiltrating and resident macrophages towards a non-classical M2 phenotype, either by increased angiogenesis or elimination of oxidative stress, can result in a return to normative wound healing [26].

Similarly, fibroblasts in impaired wounds have their ECM deposition abilities significantly diminished. Using a 3D in-vitro culture, DFU-derived fibroblasts were observed to produce ECMs twofold thinner than normal [63]. Additionally, these thinner matrices were also shown to possess a greater composition of collagen type I and fibronectin content [63]. Additionally, topically-applied fibronectin has been previously shown to increase wound healing ability in DFUs, increasing angiogenesis while reducing inflammatory cytokine expression, apoptosis, and oxidative stress [64]. Taken together, these observations suggest that enhanced and/or corrective fibroblast activity can be potentiated by treatment with ECM components to compensate for the deficiency present in DFU fibroblasts.

6. Treatment Strategies for Diabetic Wounds and Ulcers

Our growing understanding of wound healing mechanisms has led to the development of a variety of potentially effective treatment strategies for hard healing wounds. Currently, well-established treatments for DFUs (standard care) include pressure off-loading from the wound site, debridement of necrotic tissue, pathogenic suppression, and topical wound dressings of varying types to minimize patient non-compliance and subsequent poor clinical outcome [65]. Frequently, these measures are used as control treatments in the evaluation of novel experimental therapies, although the material, treatment period, and other factors vary according to the type and severity of evaluated wounds [66,67].

Notably, experimental strategies (Table 1) include the (a) application of cell-based therapies—aimed at the systemic or local application of cells with regenerative potential (mainly stem and progenitor cells); (b) use of biologically-derived therapeutics; (c) appli-

cation of physical methods such as hyperbaric pressure, electrical stimulation); (d) use of dermal and epidermal skins substitutes; and (e) combination of these strategies in addition to standard care [4]. In fact, skin substitutes and biological dressings are readily available and considered safe, promising options to treat large skin defects and hard-healing wounds. Therefore, in the following section, we will discuss the use of dermal scaffolds and dressings in the context of diabetic wound healing.

Table 1. Clinical trials involving biological materials including or with potential secondary application with skin substitutes and acellular dermal matrices in diabetic foot ulcers and impaired wounds.

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
Acellular Dermal Matrices	Effect of Meso Wound Matrix in the Treatment of DFUs	22 October 2020	NCT04182451	Active, not recruiting	No results available	
	Comparative Effectiveness of Two Acellular Matrices (Dermacell vs. Integra) for Management of Deep Diabetic Foot Ulcers	2 September 2020	NCT03476876	Recruiting	No results available	
	DermACELL AWM® in Chronic Wagner Grade 3/4 Diabetic Foot Ulcers	6 September 2019	NCT03044132	Completed	DermACELL healed complex DFUs with exposed bone. Results suggest wound closure if given time.	[68] 31361269
	DermACELL in Subjects With Chronic Wounds of the Lower Extremities	14 March 2018	NCT01970163	Completed	DermACELL increases in healing rates in DFUs compared with conventional care options	[69] 26933467
					DermACELL-treated subjects had higher wound closure than those treated with ADM Graftjacket.	[70] 28544150
	OASIS Wound Matrix (Oasis) Mechanism of Action	9 June 2011	NCT00570141	Completed	7 of 13 wounds closed fully after 12 weeks.	
	Acellular Porcine Dermal Matrix Wound Dressing in the Management of Diabetic Foot Ulcers	7 June 2011	NCT01353495	Completed	Submitted; Pending	

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
	AMNIOEXCEL® Plus vs. A Marketed Comparator vs. SOC in the Management of Diabetic Foot Ulcers	20 July 2020	NCT03547635	Completed	13,000 treatments: All matrices roughly equivalent in closure over 12 weeks. SIS & UBM healed more quickly and cost less. Half of patients treated achieved wound closure vs. none with SOC	[71] 27681811 [72] 26978860
Skin Substitutes	Multi Center Site, Controlled Trial Comparing a Bioengineered Skin Substitute to a Human Skin Allograft	26 June 2019	NCT01676272	Completed	No results available	
	Clinical Outcomes After Treatment With RestrataTM Wound Matrix in Diabetic Foot Ulcers (DFU)	13 August 2018	NCT03312595	Completed	No results available	
	Pivotal Trial of Dermagraft(R) to Treat Diabetic Foot Ulcers	21 May 2018	NCT01181453	Completed	Benefit for chronic DFUs >6 weeks duration	[73] 12766097
	Dermagraft(R) for the Treatment of Patients With Diabetic Foot Ulcers	21 May 2018	NCT01181440	Completed	Dermagraft-treated patients have better healing than SOC.	[73] 12766097

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
Growth Factors	BB-101 (Recombinant Human for Treatment of Diabetic Lower Leg and Foot Ulcers	14 September 2020	NCT03888053	Recruiting	No results available	
	A Randomized Trial on Platelet Rich Plasma Versus Saline Dressing of Diabetic Foot Ulcers	16 September 2019	NCT04090008	Completed	No results available	
	Efficacy and Safety of Heberprot-P® in Patients With Advanced Diabetic Foot Ulcer in Dasman Diabetes Institute.	4 August 2017	NCT03239457	Completed	No results available	
	A Phase 3 Clinical Trial to Assess the Effectiveness of BioChaperone PDGF-BB In the Treatment of Chronic Diabetic Foot Ulcer	29 June 2017	NCT02236793	Completed	No results available	

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
	A Study Evaluating Topical Recombinant Human Vascular Endothelial Growth Factor (Telbermin) for Induction of Healing of Chronic, Diabetic Foot Ulcers	11 May 2017	NCT00069446	Completed	No results available	
	Comparative Study of 3 Dose Regimens of BioChaperone to Becaplermin Gel for the Treatment of Diabetic Foot Ulcer	15 December 2014	NCT01098357	Completed	No results available	
	Efficacy and Safety of rhEGF in Diabetic Foot Ulcer Patients With Uncontrolled Diabetic Mellitus	4 August 2014	NCT01629199	Completed	No results available	
	Evaluation of the Safety Follow-up of Becaplermin or Placebo Gel Following Treatment of Chronic, Full Thickness Diabetic Ulcers	8 June 2011	NCT00740922	Completed	No results available	
	Gene Therapy to Improve Wound Healing in Patients With Diabetes	20 November 2007	NCT00065663	Completed	No results available	

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
Misc.	Utilization of Platelet Gel for Treatment of Diabetic Foot Ulcers	4 December 2015	NCT02134132	Completed	No results available	
	Evaluation of the Effect of Vivostat Platelet Rich Fibrin (PRF) in the Treatment of Diabetic Foot Ulcers	12 October 2011	NCT00770939	Completed	No results available	
MMPs	Matrix Metalloproteinase-1/Tissue Inhibitor of Metalloproteinase-1 (MMP-1/TIMP-1) Ratio and Diabetic Foot Ulcers (DIAB-MMP2)	18 December 2013	NCT00935051	Completed	No results available	
Mixed	Phase 2b Study of GAM501 in the Treatment of Diabetic Ulcers of the Lower Extremities (MATRIX)	10 February 2010	NCT00493051	Completed		[74] 17199833

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
Stem Cells	Phase 1, Open-Label Safety Study of Umbilical Cord Lining Mesenchymal Stem Cells (Corlicyte®) to Heal Chronic Diabetic Foot Ulcers	6 August 2020	NCT04104451	Recruiting	No results available	
	Clinical Study of Adipose-derived Stem Cells in the Treatment of Diabetic Foot	16 April 2019	NCT03916211	Not yet recruiting	No results available	
	Comparison of Autologous MSCs and Mononuclear Cells on Diabetic Critical Limb Ischemia and Foot Ulcer	1 December 2010	NCT00955669	Completed	BMMSCs led to increased blood flow and wound healing compared to BMMNCs	[75] 30917698
Endothelial Progenitor Cells	Cryopreserved Human Umbilical Cord (TTAX01) for Late Stage, Complex Non-healing Diabetic Foot Ulcers (AMBULATE DFU II)	24 November 2020	NCT04450693	Recruiting	No results available	
	Cryopreserved Human Umbilical Cord (TTAX01) for Late Stage, Complex Non-healing Diabetic Foot Ulcers (AMBULATE DFU)	5 November 2020	NCT04176120	Recruiting	No results available	

Table 1. *Cont.*

	Name of Clinical TRIAL	Last Update	Clinical Trial ID	Status	Conclusions	Publications (PMID)
Antibodies	The Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of UTTR1147A in Participants With Neuropathic Non-Healing Diabetic Foot Ulcers	21 November 2018	NCT02833389	Completed	No results available	

* Table data taken from clinicaltrials.gov, updated on 17 January 2021. Key search words included: "Diabetic Foot Ulcer" and "Impaired Wound," as well as associated subcategories.

7. The Use of Skin Substitutes in Diabetic Wound Healing

Skin substitutes can be divided into subcategories based upon their composition, derivative source, and unique additives, if any exist (Table 2). Among implantable scaffolds, these include those with a dermal and/or epidermal component. Further distinguishment can be observed based on whether the biomaterials used are derived from a biologic source, fully synthetic, or a mixture of both. Further disambiguation can occur as to whether the materials are derived from the host of the transplant (autogenic), another human donor (allogenic), or derivative of another animal species (xenogenic). Lastly, scaffolds may be classified by whether they are completely acellular or not, with non-autologous cellular matrices theoretically possessing the risk of an adverse reaction as a consequence of host rejection. Notably, however, many successful skin substitutes are composed of a variety of individually-sourced materials, obfuscating the full contributory mechanism of individual components [76,77]. This is especially true given the complexity of chronic wound environments and the clinical variability within an individual at the local and systemic level. Hereafter we will focus on scaffolds which possess a dermal element—in particular fully acellular dermal matrices—due to dermal element (1) prevalence in DFU treatment, (2) ECM-related therapeutic mechanistic effects induced, (3) safety in regard to tissue rejection, 4) history of beneficial clinical outcomes, and (5) their abundance in recent clinical trials (Table 1).

Table 2. Categories of Skin Substitutes.

Composition	Material	Additives
Dermal	Autogenic	None—Fully Acellular
Epidermal	Allogenic	Acellular with Remnants
Full Skin	Xenogenic	Cellular (MSCs)
	Synthetic	Molecular (MMPs/TIMPs/Growth Factors/Cytokines)
	Mixed	

8. Dermal Scaffolds

Dermal scaffolds are dermal tissue-derived or dermis-like matrices that retain the ability to integrate into host ECM or are cleaved, thereby supporting re-epithelization and maturation of the healing wound [78]. Given the extensive role that the ECM plays in wound healing, the examination of wound substitutes that mimic native dermis has been

implicated as an effective ameliorative therapy, often in conjunction with supplementary cellular or molecular components [76,79,80]. Consequently, recent decades have seen innovation in 3D cell cultures and other substitutes which model human skin in vitro, allowing for the evaluation of skin substitutes more readily than with animal models alone [81]. Despite this, some skin substitutes (including dermal scaffolds) do not possess the ability to fully integrate with host fibroblast-derived ECM components, often leading to future complications in their extraction or inability to undergo desirable ECM/collagen deposition during the remodeling phase of wound healing. However, recent evidence has demonstrated the association of dermal scaffolds with beneficial therapeutic outcomes—particularly in impaired wound resolution [82,83].

Tissue engineering has become a valuable tool in the creation of scaffolds that can integrate with a recipient's tissue, due in large part to recent innovations in engineering technology and the success of traditional biologically-sourced scaffolds. Therefore, the creation of biomimetic engineered artificial, synthetic, or natural substitutes for bone, skin, and/or blood vessels has shown a marked interest. In fact, ideal scaffolds and tissue substitutes including skin matrices, be they bio-engineered or natural, should be characterized as: low- or non-immunogenic, bio-compatible, regenerative, protective, non-pathogenic, and durable (Table 3). In fact, acellular dermal matrices possess many of these characteristics and are considered useful dressings in skin wounds, including hard healing or non-healing wounds such as DFUs. Their therapeutic properties originate from and depend on their source, method of preparation, and further modification. Therefore, in the following part of this review, we will summarize current knowledge on the use of acellular dermal matrices and cell-covered dermal matrices in wound healing.

Table 3. Ideal properties of skin substitutes.

Property	Elaboration
Non-immunogenic	• Components do not induce tissue rejection.
Bio-compatible	• Infiltrating cells can effectively adhere to scaffold material. • Integrates readily into existing ECM; cells can deposit/extract ECM
Regenerative	• Does not inhibit or promotes angiogenic function • Minimizes sub-optimal granulation of tissue & scarification • Beneficially modulates regenerative cells such as macrophages & fibroblasts • Facilitates rapid epithelial cell coverage
Protective	• Provides coverage for underlying structures • Minimizes disruptive "floating" in the wound bed • Retains & maintains a moist environment, reducing oxidative stress
Non-pathogenic	• The low number of applications to minimize infection risk • Sterile preparation method & donor source do not confer disease
Durable	• The substitute does not degrade before regenerative action. • Complicating conditions (infection, T2DM-induced glycosylation, etc.) do not compromise scaffold flexibility & function

9. Acellular Dermal Matrices

Acellular dermal matrices are perhaps the most biomimetic scaffolds, as they can retain the primary functional structure of normal dermal tissue. This is particularly important

because, as mentioned above, intact ECM components strongly affect the healing potential of a wound and its subsequent reorganization during remodeling [52]. However, the method of decellularization and tissue source must be addressed, given that this process differentially affects the wound microenvironment [84] by way of retaining functional matrix proteins and components, as well as its physical characteristics—as in the case of collagen fiber cross-linkage [85,86].

Given that the most abundant dermal collagen is type I by a wide margin, followed by type III, acellular dermal matrices (ADMs) are generally similar in composition. As a consequence of their abundance and early research demonstrating the chemotactic attraction of human fibroblasts to collagens I, II, and III [87], they receive a great deal of attention. Notably, monocyte adhesion to collagen types I and III have a demonstrable effect on the secretion of the wound and ECM-affecting products including growth factors [88–90], cytokines [91], and enzymes [89] which play a crucial role in normative wound healing [31,92–94]. Similarly, 3D environments dense with collagen I fibers were recently shown to induce immunosuppressive effects in M2 macrophages [95], suggesting a beneficial therapeutic effect in DFU-resident macrophages. This is further supported by the observation that the overabundance of type III collagen, seen in hypertrophic wound scars is by itself insufficient to induce immunomodulatory effects sufficient to resume normative wound healing, as has been previously observed with collagen type I deposition [96].

Although the full contributory role of ADMs in DFUs has not yet been elucidated, the mechanistic effects of specific proprietary ADMs has been witnessed. In one study, application of a xenogenic ADM was able to return the M1:M2 macrophage polarization ratio normally seen in DFUs to that of normally-healing wounds [97]. Furthermore, ADMs have also been witnessed to induce increased levels of microvascular blood flow within DFUs [98]. These promising results firmly establish ADMs as favorable candidates for further research in the context of chronic wounds, especially given the crucial importance of macrophage ratio and angiogenesis therein.

Due to their ubiquity and history of effective clinical outcomes, ADMs are frequently utilized in a variety of pathologies besides DFU, including rare skin conditions, such as epidermolysis bullosa, plastic surgery, and burn treatment, among others [59,60,99,100]. Recent studies and meta analyses have shown the effectiveness of ADMs in regard to their ability to influence the immune response by differentially modulating key growth factors and cytokines, resulting in enhanced wound closure and faster resolution [57,61,101].

By highly mimicking normal dermal tissue and subsequently eliminating potential immunogenic antigens on the surface of donor cells, it is hoped that these scaffolds will induce a return to normative wound healing in the recipient. This is further buttressed by observable differences in cellular vs. non-cellular human dermal matrices—underpinning the importance of cell-associated immunogenic component removal as a means to minimize ADM rejection and associated complications [102,103].

Likely obfuscated by the inflammatory nature of DFUs, ADM-mediated immunogenic responses go largely unnoticed. However, this phenomenon can occasionally be witnessed in sterile, normally-healing wound environments such as breast reconstruction in the form of red breast syndrome (RBS). Although the etiology of RBS is speculative, it is limited in nature—resolving without treatment and theorized to clear as a consequence of ADM neovascularization [104]. RBS and other ADM-related adverse effects, although very rare, are likely related to the presence of endotoxin or wound contamination with microbes [105]. Despite the likelihood that ADM-treated DFUs face a similar sterile inflammatory response, ADMs have been witnessed to be exceedingly safe, with high healing rates vs. standard care and a lack of immunogenic, toxic, or carcinogenic complications [106].

Although the key elements regulating skin substitute-mediated wound healing mechanisms remain to be fully elucidated, recent evidence has shed light on essential modulations in the wound microenvironment, which subsequently lead to beneficial therapeutic outcomes. It seems that the induction of differing ADM physical characteristics and mechanistic effects within the wound micro-environment are based not only upon the source,

but crucially on the method of preparation that proprietary ADMs undergo—namely in relation to the decellularization and sterilization processes.

Notably, irradiation for sterilization purposes may fundamentally damage or change the structural components of skin substitutes depending on dose [85,86]. This process induces structural changes and can result in the damage and extraction of ECM components which may directly affect the healing process supporting the epithelization process and remodeling or inducing inflammation. In fact, it appears that modulation of scaffold degradability, among other physical characteristics, can be cultivated via physical and chemical modification in order to facilitate guided responses, including the infiltration, adhesion, and proliferation of regenerative cells [107]. On the other hand, when adversely affecting the therapeutic potential of biological dressing, the sterilization process may be bypassed by appropriate aseptic production, assuming that proper precaution against and screening for endotoxins is undertaken [106].

10. Cell-Supplemented Dermal Matrices

Having established the high customizability and effectiveness of keystone elements of ADMs, on their own they hold a great deal of potential. Even so, skin substitutes' therapeutic potential may be improved by the supplemental utilization of stem and progenitor cells with well-characterized activities supporting the healing process, as in the case of mesenchymal stem cells (MSCs) and fibroblasts [103,108,109]. Interestingly MSCs co-cultured with skin substitutes in vitro were shown to release trophic factors important for tissue regeneration [110] and to improve the healing of diabetic wounds when used in tandem skin substitutes, including ADMs [76,79,110–112]. This strong regenerative effect is associated primarily with MSC ability to improve neovascularization of the wound via paracrine activity in addition to their well-characterized anti-inflammatory effects [113]. Furthermore, the lack of notable differences in clinical outcomes when comparing autologous and allogeneic MSCs, suggests their low immunogenicity [114], leading to speculation as to entirely allogenic therapeutic possibilities without the risk of tissue rejection. In fact, a recent systematic review of adipose-derived stem cells used in conjunction with ADMs found them to be both safe and effective [115]. These promising results indicate the efficacy and safety of MSC-mediated therapy for DFUs, with the number of active clinical trials including MSCs in DFUs (Table 1).

Presently, acellular dermal matrices hold a great deal of promise for the treatment of hard-healing wounds, including diabetic wounds [57,99–101]. Their continued usage and examination in regard to source tissue, structural composition, and preparation are of supreme importance, with these factors playing key roles in the degradation of specific collagen fibers and their ability to integrate and provide the most beneficial immunomodulatory effects. Further, given the beneficial effects associated with additive cellular and molecular components such as growth factors, proteases, and easily-attainable MSCs, combinations of these additives with ADMs have been evidenced as safe and effective treatments leading to favorable therapeutic outcomes in DFUs, especially when compared against current standards of care (Table 1).

11. Conclusions

Diabetic wounds remain a significant clinical problem. The understanding of complex mechanisms of stem and progenitor cell dysfunctions and the dysregulation of systemic and local immune responses will significantly contribute to the efficacy of currently used therapies. However, the use of biological dressings, such as skin substitutes, additionally supported by stem cells or stem cell derived-fragments may represent a readily accessible and advantageous option for treating diabetic wounds. Notably, more studies focusing on specific biomaterials and their contributory influence to specific elements of the wound microenvironment are preferred. Effective guidance of skin substitute characteristics and the mechanistic contribution therein will help to develop innovative and effective protocols to treat chronic wounds in diabetic individuals well into the future.

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Skin Substitute Preparation Method Induces Immunomodulatory Changes in Co-Incubated Cells through Collagen Modification

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Abstract: Chronic ulcerative and hard-healing wounds are a growing global concern. Skin substitutes, including acellular dermal matrices (ADMs), have shown beneficial effects in healing processes. Presently, the vast majority of currently available ADMs are processed from xenobiotic or cadaveric skin. Here we propose a novel strategy for ADM preparation from human abdominoplasty-derived skin. Skin was processed using three different methods of decellularization involving the use of ionic detergent (sodium dodecyl sulfate; SDS, in hADM 1), non-ionic detergent (Triton X-100 in hADM 2), and a combination of recombinant trypsin and Triton X-100 (in hADM 3). We next evaluated the immunogenicity and immunomodulatory properties of this novel hADM by using an in vitro model of peripheral blood mononuclear cell culture, flow cytometry, and cytokine assays. We found that similarly sourced but differentially processed hADMs possess distinct immunogenicity. hADM 1 showed no immunogenic effects as evidenced by low T cell proliferation and no significant change in cytokine profile. In contrast, hADMs 2 and 3 showed relatively higher immunogenicity. Moreover, our novel hADMs exerted no effect on T cell composition after three-day of coincubation. However, we observed significant changes in the composition of monocytes, indicating their maturation toward a phenotype possessing anti-inflammatory and pro-angiogenic properties. Taken together, we showed here that abdominoplasty skin is suitable for hADM manufacturing. More importantly, the use of SDS-based protocols for the purposes of dermal matrix decellularization allows for the preparation of non-immunogenic scaffolds with high therapeutic potential. Despite these encouraging results, further studies are needed to evaluate the beneficial effects of our hADM 1 on deep and hard-healing wounds.

Keywords: skin substitute; acellular dermal matrix; preparation method; collagen structure; collagen adhesion; dermal architecture

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1. Introduction

Chronic and hard-healing wounds are a pervasive global health concern. The elevated mortality [1] and reduced quality of life associated with these types of wounds, combined with presently limited therapeutic options, highlight the need for novel ameliorative therapies to improve these healing processes.

Wound healing is composed of four overlapping phases: hemostasis, inflammation, proliferation and remodeling [2]. In each of these phases, distinct mechanisms take place to induce effective wound closure and scar formation. During coagulation in the hemostasis phase, the inflammatory cascade is initiated by the degranulation of mast cells, with the central role of wound-infiltrating inflammatory cells such as neutrophils and monocytes clearing the wound of debris and pathogens. Next, the proliferative ability of epidermal keratinocytes and extracellular matrix (ECM)-depositors, including myofibroblasts, facilitates the rapid coverage of the wound area by dermal and epidermal tissue, inducing scar formation. Following wound closure, the remodeling of crucial ECM components takes place, resulting in scar maturation. However, in contrast to normal wound healing, chronic wounds possess a variety of mechanistic dysfunctions, which result in prolonged or ultimately non-existent wound healing [3,4]. To date, several hallmarks of chronic wounds have been identified, such as persistent inflammation [3–6], low levels of growth factors and high oxidative stress [7–10], poor angiogenesis [11–13], high levels of matrix metalloproteinases [14–17] and the dysfunction of dermal fibroblasts [10,18]. In fact, proliferative coverage of the wound area is significantly inhibited in chronic wounds, and the healing process is blocked in a self-perpetuating cycle of inflammatory reaction. Therefore, it is well recognized that the ideal dressing for hard-healing wounds should not only cover the wound but also modulate the wound microenvironment through its immunoregulatory effects.

Notably, skin substitutes or skin-derived dressings have been used as an effective complementary treatment for deep, extensive, and hard-healing wounds, among others [19–27]. Skin substitutes are biologically or synthetically derived tissues applied to the wound to induce favorable wound healing effects. In fact, skin substitutes have a long history of use in treating wounds and ulcers of different etiology. However, their therapeutic effects depend on the derivative source, structural composition, preparation method, and means of sterilization/aseptic creation, among others [2,26,28].

Recent studies demonstrated the regenerative potential of ADM application in diabetic foot ulcer (DFU) healing and mechanisms associated with beneficial effects [22,29–31]. Moreover, the members of our group have developed a novel approach to hADM manufacturing from the resected skin fold of living abdominoplastic surgery patients. Previously, we were able to generate a novel abdominoplasty skin-derived hADM using a distinct method of decellularization and sterilization. We previously evaluated the effects of different methods of preparation on the purity and structure of our novel hADM. Moreover, we showed that human abdominoplasty skin-derived ADMs may serve as dressing for deep wound treatment [32]. More importantly, this dermal matrix was able to serve as a skin substitute for hard-healing wounds or as a biological scaffold for BIOOPA dressing to treat epidermolysis bullosa patients [23,28,33,34]. Here, we examined three differentially prepared hADMs and aimed to evaluate how these methods of aseptic preparation may influence their immunomodulatory properties and therapeutic potential.

2. Materials and Methods

2.1. Skin Collection and Processing

Skin folds from bariatric patients were collected during abdominoplastic surgery. Dermatome skin grafts were harvested from the resected skin fold, sealed in foil bags, and biobanked at −80 °C for further processing.

Dermal fragments were thawed in saline at room temperature, followed by a decellularization step using chemical and/or enzymatic processing (Supplementary Table S1). Directly after decellularization and washing, acellular dermal matrices were lyophilized us-

ing automated −80 °C lyophilization, sealed in double foil bags, labeled, and biobanked in −80 °C. The efficiency of the decellularization process was controlled using histochemical staining.

Prior to use, 8 mm fragments were created by biopsy punch. Next, 8 mm acellular dermal matrix (ADM) fragments were re-hydrated in a 1 mL complete culture medium RPMI 1640 (Thermo Fisher, Waltham, MA, USA) supplemented with 10% FBS (PAN Biotech, Aidenbach, Germany) and 75 µg/mL gentamicin (Gibco, Waltham, MA, USA) for 24 h in a 37 °C, 5% CO_2 incubator. Each independent experiment used an hADM from a distinct decellularization series.

2.2. Peripheral Blood Mononuclear Cell (PBMC) Isolation

PBMCs were isolated from fresh buffy coats obtained from healthy donors by means of density gradient centrifugation (Pancol, PAN Biotech), as previously described [28]. Pharm lyse buffer (BD, Franklin Lakes, NJ, USA) was used to remove residual red blood cells when needed. PBMC numbers were evaluated using a Bürker chamber. Freshly isolated PBMCs were resuspended in complete culture medium and used immediately for further research. Buffy coats were collected upon the approval of the Ethics Committee of the Medical University of Bialystok.

2.3. T Cell Proliferation Assay

Freshly isolated PBMCs were stained with carboxyfluorescein succinimidyl ester (CFSE, Sigma-Aldrich, St. Louis, MO, USA) in PBS (Corning Inc., Corning, NY, USA) for 5 min at room temperature in the dark. CFSE-labeled cells were washed three times in PBS (5 min, 400× *g*). Next, the cells were resuspended in complete culture medium and gently seeded on re-hydrated 8 mm hADM fragments, 8 mm unprocessed skin fragments, or left alone (vehicle/unstimulated control) in 24 well culture plates (Eppendorf, Hamburg, Germany) at the density of 1 × 106 cells/m. Mitogen stimulation (5 µg/mL PHA-P, Gibco) was included as a positive control of PBMC proliferation. The cells were stimulated for 7 or 14 days with medium changes every four days. Finally, the cells were collected and stained with mouse anti-human CD3-FITC and CD8-PE conjugated antibodies (BD Biosciences) for 15 min in the dark. See Supplementary Table S2 for antibody information. Next, the samples were washed in PBS and fixed using CellFix (BD) and analyzed by FACSCalibur (BD) flow cytometry. Obtained data were analyzed using FlowJo v10 software (TreeStar Inc., Ashland, OR, USA). Appropriate staining controls were used for setting the gates. The used gating strategy is presented in Supplementary Figure S1. The results are presented as a ratio of proliferation versus the unstimulated control.

2.4. Assessment of Monocyte and T Cell Phenotype

Freshly isolated PBMCs were gently seeded on re-hydrated 8 mm hADM fragments or left alone (vehicle/unstimulated control) in 24 well culture plates (Eppendorf) at the density of 1 × 106 cells/mL in complete cell culture media. The cells were cultured for up to 72 h and collected every 24 h for flow cytometry analysis. For intracellular cytokine staining, brefeldin A (Thermo Fisher) was added to culture wells 3 h before cell acquisition. Additionally, cell culture supernatant was collected and biobanked at −80 °C for cytokine assay. Next, the cells were stained immediately with a panel of monoclonal antibodies (Supplementary Table S2). Briefly, cells were incubated in the presence of monoclonal antibodies for 30 min at room temperature in the dark. Next, the specimens were washed twice in PBS and fixed with CellFix (BD) or subjected to intracellular staining. For the latter, samples were fixed and permeabilized using Perm2 Buffer (BD) according to manufacturer instructions, followed by washing in PBS and staining with fluorescent-conjugated antibodies (Supplementary Table S2) for 30 min at 4 °C in the dark. Next, the cells were washed twice and fixed with CellFix (BD). Finally, the specimens were analyzed on a FACSCalibur flow cytometer (BD). Flow cytometry data analysis was performed using FlowJo software (Tree Star). Appropriate, fluorescence-minus-one (FMO) controls were

applied for setting correct gating. Used gating strategies for different T cell and monocyte subsets are presented in Supplementary Figures S2 and S3. Used gating strategies for different T cell and monocyte functions are presented in Supplementary Figures S4 and S5.

2.5. Cytokine Assay

Concentrations of factors TNF, IFNγ, IL-1β, IL-6, IL-10, IL-17 and TGF-β were measured by means of commercially available DuoSet ELISA (all from R&D Systems, Minneapolis, MN, USA), as previously described [28,34,35]. The detection ranges for TNF (15.6–1000 pg/mL) IFNγ (9.39–600 pg/mL), IL-1β (3.91–125 pg/mL), IL-6 (9.4–600 pg/mL), IL-10 (31.3–2000 pg/mL), IL-17 (15.6–1000 pg/mL) and TGF-β (31.3–2000 pg/mL). Protein levels were analyzed with an automated microplate reader (LEDETEC96 system). The results were calculated according to the standard curve, generated by individual standard dilutions, by MicroWin 2000 Software.

2.6. Immunofluorescence Staining

hADMs were snap-frozen after coincubation with PBMCs mentioned previously. hADMs were cut using cryomicrotome into 20 µM longitudinal sections and seeded on glass slides. Next, cryosections were fixed with 4% paraformaldehyde (Santa Cruz Biotechnology, Dallas, TX, USA) and incubated in a detergent (0.1% Triton X-100 (Sigma)) in 0.02% SDS-PBS (Sigma-Aldrich and Corning, respectively), followed by incubation with blocking buffer (10% normal donkey serum–Abcam, Cambridge, UK) in 1% BSA in PBS). Next, the slides were stained with a specific primary antibody for collagen types I, III and IV or incubated in staining buffer (1% protease-free bovine serum albumin (Sigma) in PBS) for 60 min in a high humidity chamber in the dark. Next, the slides were washed three times in washing buffer (Tween20-PBS) and stained with appropriate secondary antibodies. For detailed characteristics of primary and related secondary antibodies, please see Supplementary Table S3. Finally, the specimens were mounted in Prolong Gold mounting medium with DAPI (Thermo Fisher) and covered with cover slides (Avantor, Gliwice, Poland), followed by overnight incubation at RT in the dark before analysis by confocal microscopy.

2.7. Confocal Microscopy

Confocal pictures were acquired using an FV1200 Microscope (Olympus, Tokyo, Japan). Full-sized pictures were acquired in 3 channels using 405, 450 and 650 nm lasers at 20 µs/pixel and 2048 × 2048 size using FluoView software (https://www.olympus-lifescience.com; Olympus) accessed on 1 March 2021. Full-sized photos were then examined using ImageJ software (https://imagej.nih.gov/ij/; Public domain) accessed on 1 May 2021. Channel 1 (DAPI) was made blue, Channel 2 (autofluorescent collagen structure) was made green, Channel 3 (stained collagen fibers I, III or IV) was made red. Z-stacks of each channel were created and merged. As many focused sections (500 × 500 pixels) as possible were extracted from each full-sized photograph inside the hADM for collagens I and III or on the apical edge for collagen IV, and quantified. Each stained, quantified, focused section was divided by the mean of combined focused controls; slides stained without primary collagen-binding antibody but with secondary fluorescent antibodies specific to each collagen [Quantification = Stained Collagen/Staining Control]. Channels 1 and 2 (DAPI and autofluorescence, respectively) did not contribute to the semi-quantitative measurement of collagens. In hADMs without co-incubated PBMCs, only one time point (24 h) was used with quantification measured as mentioned above. However, when PBMC co-incubated hADMs were compared to those without PBMCs, the following equation was used [Quantification = (stained PBMC co-incubated focus section/control PBMC co-incubated focus section)/(stained non-PBMC focus section/control non-PBMC focus section)].

2.8. Quantification of DNA Present within hADMs

hADMs were re-hydrated using 1 mL of PBS in 24 well plates. Next, hADMs were digested using proteinase K in buffer solution provided in DNeasy blood and tissue DNA isolation kit (Qiagen, Hilden, Germany). This resulted in the entire dissolution of hADMs into solution. This kit was used according to manufacturer protocol to isolate and extract DNA from the fibrous hADMs. Finally, DNA concentration was measured using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher) using NanoDrop 2000 software (http://isogen.nl/nanodrop-software; Thermo Fisher) accessed on 3 October 2021. Specifically, the nucleic acid measurement at 260/280 nm was used.

2.9. Statistics

Graphs and statistics were calculated using GraphPad Prism 8 (http://graphpad.com; GraphPad Software, San Diego, CA, USA) accessed on 1 August 2020. Wilcoxon matched-pairs signed-rank test was used to compare differences between analyzed conditions. To determinate the differences in residual DNA, Mann-Whitney U-test was used. The differences were considered statistically significant at $p < 0.05$. The results are presented as a median ± interquartile range.

3. Results

3.1. Different Methods of Human Abdominoplasty Skin Preparation Influence Acellular Dermal Matrix Immunogenicity

It is implied that an ideal skin substitute or dermal dressing should be low- or non-immunogenic and support healing processes [2]. However, due to the nature of tissue sourcing, method of processing, and the sterilization that ADMs undergo, various allogenic tissue-derived immunogenic mediators may be retained within and later released from their structure after wound implantation. Therefore, we first aimed to analyze whether aseptic preparation procedures of our novel abdominoplasty skin-derived ADMs may affect their immunogenicity.

First, we used a CFSE-based assay to analyze the proliferation of CD3+ T cells and their two main subsets, namely cytotoxic (CD3+CD8+ T cells) and helper (CD3+CD8−) T cells (Figure 1A and Supplementary Figure S2). hADM 1 had low immunogenicity and did not induce T cell proliferation, as no significant differences were observed when compared to the unstimulated control in both analyzed time points (Figure 1B). In contrast, hADM 2 and hADM 3 induced greater T cell proliferation comparable to unprocessed skin. Moreover, hADM 3 showed the highest immunogenicity among all tested groups (Figure 1B). Moreover, the same differences were observed in both cytotoxic and helper T cells (Figure 1C).

Taken together, we showed here that different method of novel human abdominoplasty skin-derived ADM preparation can influence their immunogenicity and induce T cell proliferation. Further, hADM 1 induced the least immunogenic effect in co-incubated T cells, indicating putatively beneficial effects.

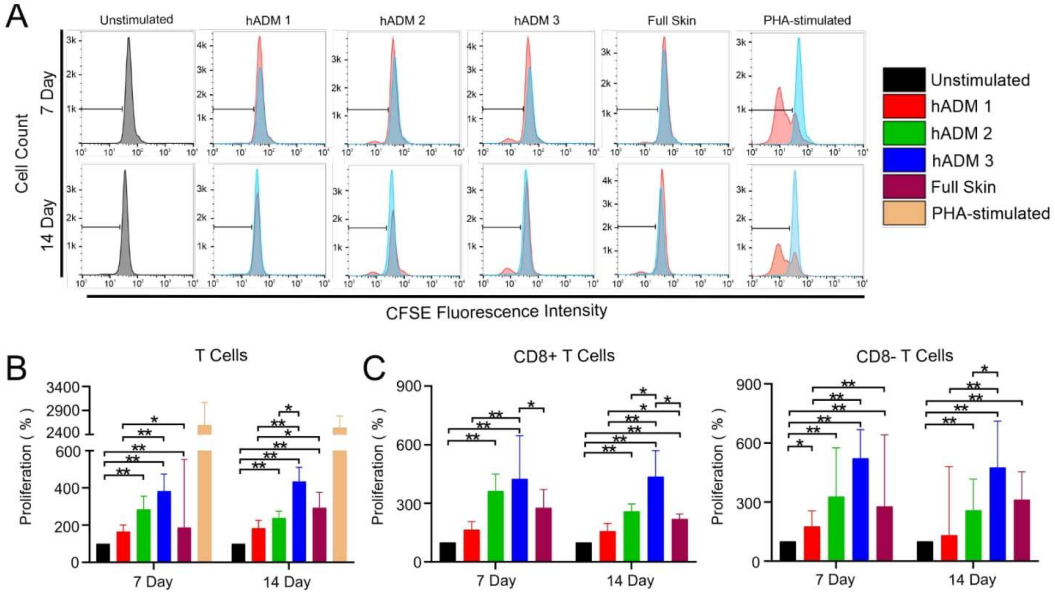

Figure 1. hADM preparation method induces differential T cell proliferation. Flow cytometric analysis of T cell proliferation. CFSE-stained PBMCs were co-incubated alone, with hADMs, with full skin, or with mitogenic control (lectin PHA) for 7 or 14 days. (**A**) Histograms displaying proliferation of whole T cells for 7 and 14 days. (**B**) Quantification of whole CD3+ T cell proliferation. (**C**) Quantification of CD3+/CD8+ and CD3+/CD8− T cell proliferation. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed-rank test used for (**B**,**C**). $n = 5$ with 2 technical replicates; * $p < 0.05$. ** $p < 0.01$.

3.2. *Abdominoplasty Skin-Derived ADM Preparation Methods Do Not Influence T Cell Phenotype*

Recent evidence indicates the crucial role of T cells in wound healing and scar formation, showing that T cell depletion significantly impairs the healing process and increases scar size [36–38]. Therefore, having found that the differential preparation method of hADMs affects immunogenic T cell proliferation, we next wished to analyze whether observed differential T cell responses are associated with changes in their phenotype in the early stage of coincubation mimicking the inflammatory phase of wound healing.

First, we aimed to investigate changes in the composition of different T cell subsets, namely non-activated T cells (CD4+/CD25−/CD127+), activated T cells (CD4+/CD25+CD127+), putative regulatory T cells (CD4+/CD25+/CD127low/−), and putative Th17 cells (CD4+/CD161+/CD196+) (Supplementary Figure S2A). We found no significant differences in the frequency of non-activated, activated, and putative Th17 cells (Supplementary Figure S2B). However, the frequency of putative Tregs was slightly increased at 24 and 48 h, but not 72 h (Supplementary Figure S2B). Next, we aimed to analyze whether the manufacturing procedure of abdominoplasty skin-derived hADM may influence the frequency of pro-inflammatory IFNγ and IL-17 producing T cells incubated in the presence of our novel hADMs. However, we observed no significant differences in the frequency of analyzed subsets (Supplementary Figure S4B). Similarly, no differences were observed in the mean fluorescence intensity of IFNγ and IL-17 in T cells (Supplementary Figure S4C).

Taken together, we showed that the differential abdominoplasty skin-derived hADM preparation method does not significantly influence T cell phenotype composition.

3.3. Novel Abdominoplasty-Derived Skin Substitutes Cause Differential Monocyte Activation

Given monocytes/macrophages central role in all phases of wound healing and their ability to orchestrate adaptive immune responses in the process of antigen presentation (including T cell proliferation induction) [39–43], we wished to evaluate the activation of monocytes after coincubation with differentially manufactured abdominoplasty skin-derived hADMs, hypothesizing that they may influence the previously witnessed differential T cell proliferation.

First, we aimed to analyze the composition of three major monocyte subsets, namely the frequency of classical (CD14++CD16−, non-activated), intermediate (CD14++CD16+), and non-classical (CD14+CD16++) monocytes (Figure 2A). In fact, we found significant differences in the composition of monocyte subsets after incubation with all analyzed hADMs compared to the unstimulated control (Figure 2B). The frequency of classical monocytes was higher in all analyzed conditions at 24 h. Consequently, we found a lower frequency of activated monocytes (cells with CD16 expression), namely intermediate but not non-classical monocytes (Figure 2B). Contrastingly, no significant differences in the frequency of classical monocytes were observed at 48 h; however, we found a significantly lower frequency of intermediate monocytes after incubation with hADM 2 and hADM 3. Moreover, a higher frequency of non-classical cells was observed after incubation with hADM 1 and hADM 2, but not hADM 3 (Figure 2B). At 72 h, we observed a significantly lower frequency of classical monocytes after incubation with hADM 1 and hADM 3. No differences were observed in intermediate monocytes, while non-classical monocyte frequency was higher in all analyzed conditions when compared to the unstimulated control (Figure 2B).

Having found significant changes in the composition of different monocyte subsets, we next sought to analyze whether these observed differences were associated with the frequency of Tie-2- and/or CD163-expressing cells, these markers being associated with pro-angiogenic and anti-inflammatory properties, respectively [44,45]. In addition, we wished to analyze the frequency of anti-inflammatory IL-10 and pro-inflammatory TNF-producing monocytes.

We found no differences in the frequency of Tie-2-expressing monocytes after 24 h incubation (Figure 2C). However, at 48 h, the frequency of analyzed cells was lower after incubation with hADM 2, while no differences were observed at 72 h compared to the unstimulated cells (Figure 2C). The frequency of CD163-expressing monocytes did not change after 24 h stimulation when compared to the unstimulated control. However, at 48 h, we found a lower frequency of analyzed cells incubated with hADM 3 compared to the unstimulated control, and similarly at 72 h in cells incubated with hADM 2 and hADM 3 (Figure 2C). Similarly, no differences in CD163 expression were observed at 24 h. In the latter time points, we found a significantly lower expression level of CD163 on monocytes when compared to unstimulated control. Interestingly, cells incubated with hADM 2 and hADM 3 showed lower levels of analyzed molecules when compared to hADM 1 (Figure 2D). Unexpectedly we found no differences in the frequency of IL-10 and TNF-producing monocytes (Figure 3B). However, at the 48 and 72 h time points, IL-10 and TNF expression (defined as MFI) was lower in hADM 2 and hADM 3, but not hADM 1 co-incubated cells versus unstimulated control (Figure 3C).

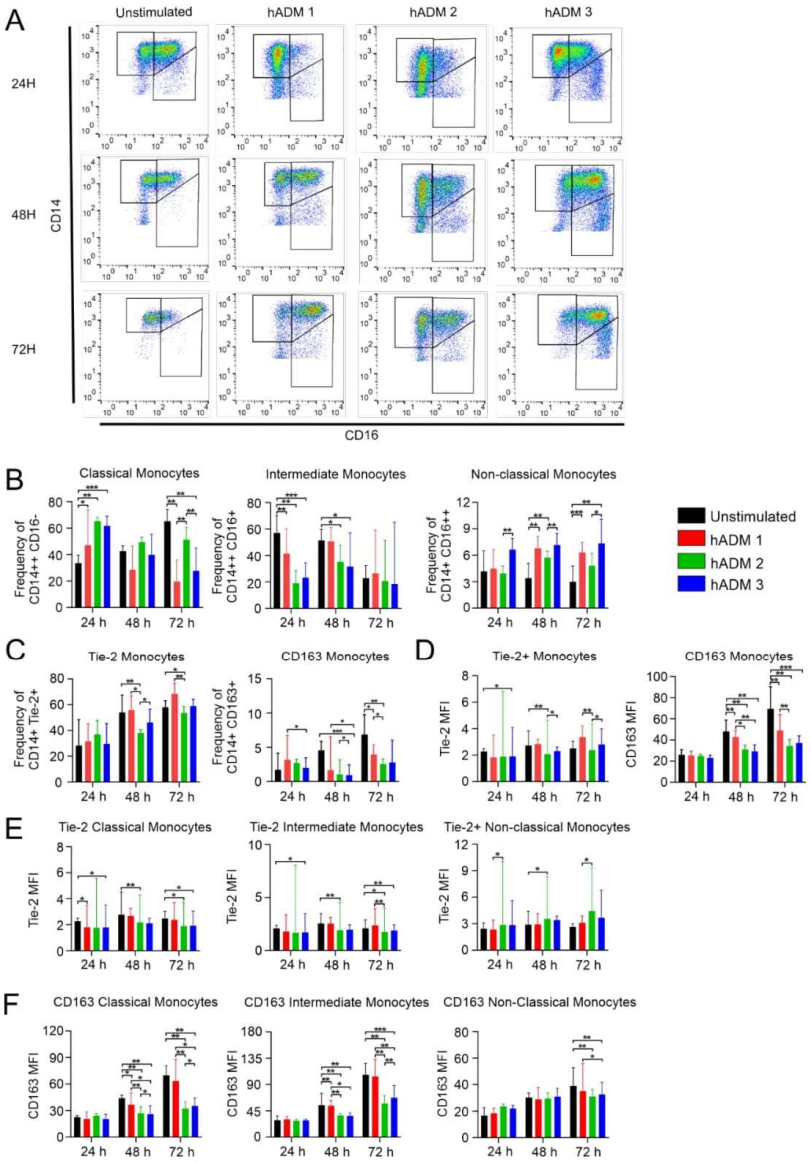

Figure 2. Monocyte phenotype is altered by hADM preparation method. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2 or 3 days. Next, cells were stained extracellularly and examined by flow cytometry. (**A**) Representative CD14 × CD16 gating plots for each unstimulated and hADM condition at 24 h, 48 h and 72 h. (**B**) Subset frequencies for classical (CD14++/CD16−), intermediate (CD14++/CD16+), and non-classical (CD14+/CD16++) monocytes. (**C**) Frequency of CD14+/Tie-2 or CD163+ monocytes. (**D**) MFI quantification of CD14+ monocytes for Tie-2 or CD163. (**E**) Tie-2 MFI for specific monocyte subsets. (**F**) CD163 MFI for specific monocyte subsets. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed-rank test used for (**B**–**F**) $n = 5$ with 2 technical replicates; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

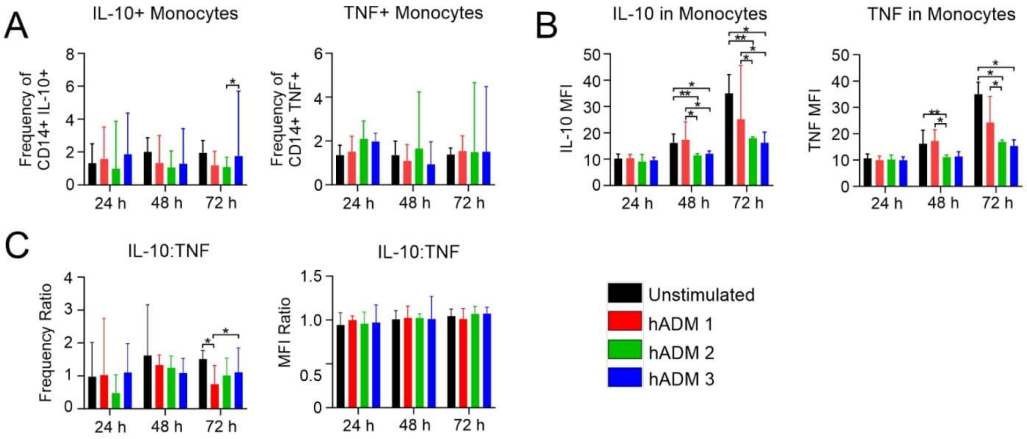

Figure 3. Monocyte effector function is modulated by hADM coincubation. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2, or 3 days. A total of 2 h before collection, protein transportation was inhibited. Finally, cells were permeabilized and stained extracellularly and intracellularly before being examined by flow cytometry. (**A**) Frequency of CD14+/IL-10+ or TNF+ monocytes. (**B**) MFI quantification of CD14+/IL-10+ or TNF+ monocytes. (**C**) Frequency and MFI ratios of IL-10:TNF. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed-rank test used for (**A**–**C**) $n = 5$ with 2 technical replicates; * $p = 0.05$, ** $p = 0.01$.

Here we showed how differentially produced hADMs may modulate monocyte maturation, initially delaying the surface expression of CD16 and maturation into intermediate and non-classical subsets when compared to the control. Further, we observed a declining expression of CD163 in hADM co-incubated groups over time and no difference in IL-10 and TNF-producing monocytes. Crucially, the method of hADM preparation can be seen to induce differential monocyte subset polarization.

3.4. Differentially Manufactured Abdominoplasty Skin-Derived Acellular Dermal Matrices Induce a Distinct Pattern of Cytokine Responses

Having found significant changes in T cell proliferation and monocyte activation, we next wished to analyze levels of pro-inflammatory IFNγ, IL-1β, IL-6, TNF, IL-17 and anti-inflammatory IL-10 and TGF-β in cell culture supernatants (Figure 4). We found that hADM 2 and hADM 3 induced pro-inflammatory profiles, with high levels of IFNγ, TNF, IL-1β, and IL-6 when compared to both unstimulated control and hADM 1 (Figure 4A–D) in all analyzed time points. In contrast, levels of IL-17 were relatively low at 24 h, with increasing concentrations observed in the later time points. In fact, hADM 3 showed the highest level of IL-17 in analyzed samples. In hADM 2 and hADM 3, we observed a trend to increase IL-17 levels at 48 h, and which reached statistical significance at 72 h time point (Figure 4E). The highest levels of IL-10 were observed in PBMCs after incubation with hADM 2 (Figure 4F). Although hADM 3 induced moderate IL-10 secretion, this was still significantly greater than hADM 1 and the unstimulated control. Finally, TGF-β was measured, but the concentrations observed in the supernatant were very low (Figure 4G). Significantly higher levels of TGF-β were observed only in cell culture supernatants from PBMC incubation with hADM 2 at 48 h and hADM 3 at 72 h time points (Figure 4G).

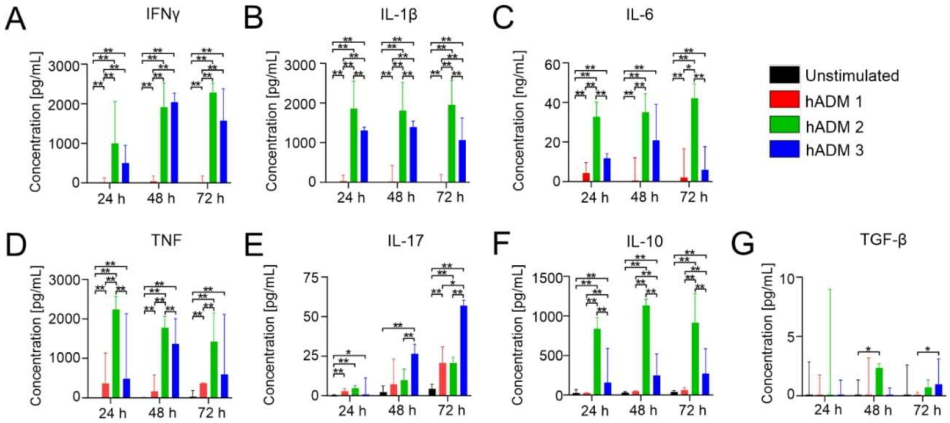

Figure 4. Method of hADM preparation induces differential cytokine secretion in vitro. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2, or 3 days. Supernatants were collected, and ELISA was performed on 100 uL fractions. (**A**–**E**) Pro-inflammatory and (**F**,**G**) Anti-inflammatory cytokine and growth factor concentrations. Results expressed as medians + interquartile range. Two-tailed Wilcoxon matched-pairs signed-rank test used for (**A**–**G**) $n = 6$ with 2 technical replicates. * $p = 0.05$, ** $p = 0.01$.

Ultimately, our results demonstrate the broad pro-inflammatory profiles associated with PBMCs co-incubated with hADMs 2 and 3 in regard to IFNγ, IL-1β, IL-6, TNF and IL-17. Contrasting, hADM 1 possessed an attenuated inflammatory cytokine profile. Finally, anti-inflammatory IL-10 levels were high for hADM 2 and moderate for hADM 3, while low in hADM 1 and the control.

3.5. hADM Apical Architecture Is Differentially Extracted by Prepatory Method

The dermal layers of the skin are mainly composed of different types of collagen fibers [46,47]. Furthermore, it is well established that immune cells may change collagen architecture by the release of proteolytic enzymes, such as metalloproteinases [17,48,49]. Therefore, having found significant differences in the immune responses to differentially manufactured novel abdominoplasty skin-derived hADMs, we next wished to analyze the influence of immune cells on the extracellular matrix structure, namely collagen architecture (Figure 5).

First, we found no visible differences in the structures of all three analyzed hADMs after coincubation with PBMCs (Figure 5A). PBMCs were located on the apical portion of hADMs and did not penetrate to the deeper layers of the matrix. Interestingly, in the apical site, fragmentation of collagen IV was observed. Moreover, we found the co-localization of mononuclear cells with collagen IV fragments, suggesting phagocytosis of released fibers (Figure 5A). Next, we quantified type I, III and IV collagens after hADM coincubation with PBMCs relative to hADMs incubated alone (Supplementary Figure S6). Interestingly, we observed slight but significant differences in the density of various collagen fibers in all three analyzed time points, namely at 24, 48 and 72 h. The presence of collagen I was lowest in hADM 2 compared to analyzed counterparts in all analyzed time points, while hADM 3 presented the highest content of collagen I after 24 and 72 h incubation. In contrast, collagen III was higher in hADM 1 at 24 h and further decreased at 48 and 72 h when compared to hADM 3. Again, hADM 2 showed the lowest density of collagen III fibers after incubation in the presence of PBMC among analyzed matrices. In contrast, no differences were observed in the density of collagen IV among analyzed hADMs at 24 h, while at 48 h, hADM 2 showed a slightly higher level of this collagen. However, at 72 h, the collagen IV level in hADM 1 was lower when compared to hADM 2.

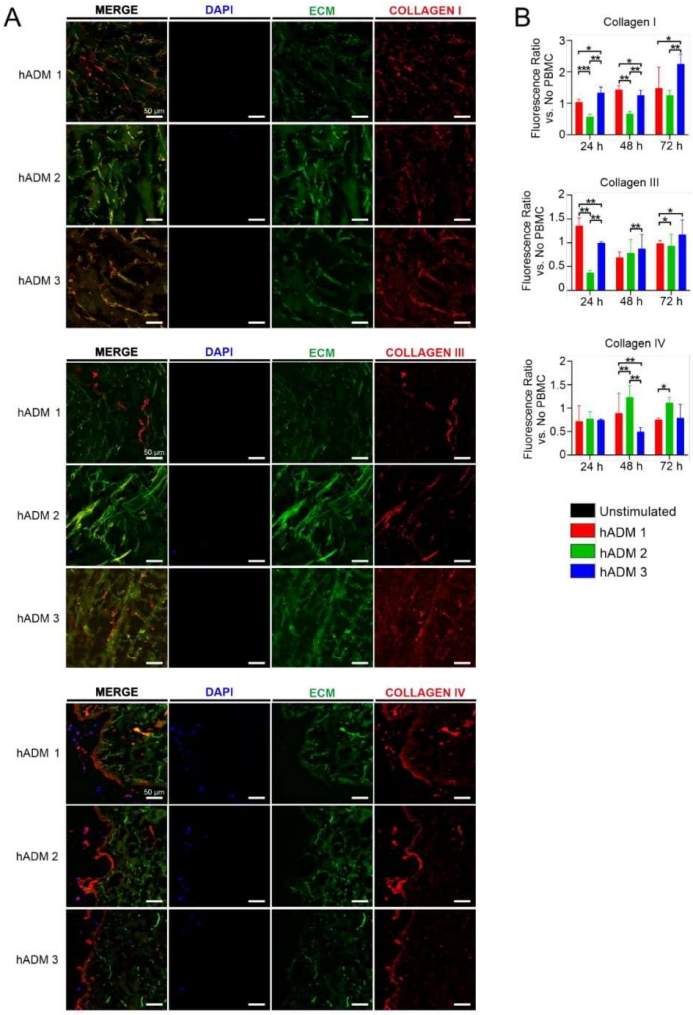

Figure 5. Collagen fibers are retained after coincubation with PBMCs. hADMs were examined confocally after fluorescence-conjugated antibody staining for collagens I, III and IV. Quantifications are derived from focused z-stack photographs using whole channel fluorescence. (**A**) Images for collagens I, III and IV can be seen for each hADM and in 3 separate channels as well as merged together. MERGE: combined representation of channels 1–3, detailed below. Channel 1 (DAPI): cell nuclei staining via DAPI using a 405 nm laser. Channel 2 (ECM): autofluorescent collagen fibers and ECM using a 488 nm laser. Channel 3 (specific collagen): specific collagen fibers were stained using a primary and subsequently a secondary fluorescence-conjugated antibody using a 647 nm laser. Process of acquisition and analysis described fully in Materials and Methods section. (**B**) Comparison of collagens in PBMC co-incubated hADMs vs. hADMs without cell culture. Z-stack sections had their absolute fluorescences divided by control sections. Quantification formula can be seen in the Materials and Methods section. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed-rank test used for B. $n = 3$ with 2 technical replicates; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All scale bars represent 50 µm.

Here we showed differences in collagen retention in PBMC co-incubated hADMs, finding that collagen I was extracted in hADM 2 at all time points. Further, we found differences in the extraction of collagen III, noting slight extraction of hADM 1 over time. hADM appeared to possess slight collagen III depositions, but this still ultimately left it with the lowest collagen III concentration at 72 h. Finally, we examined collagen IV, finding only small differences in its overall quantification, although significant fragmentation was witnessed.

4. Discussion

Here we showed that similarly sourced but differentially manufactured novel human abdominoplasty skin-derived ADMs possess different immunogenic and immunomodulatory properties. Furthermore, our results indicate the potentially favorable immunomodulatory properties of hADM 1, while hADM 2 and hADM 3 seem to be more immunogenic, notably characterized by high inflammatory cytokine profiles.

The preparation of biological scaffolds, including ADMs, aims to remove all cellular and nuclear components from tissue while preserving the three-dimensional ultrastructure of the ECM. In fact, decellularization procedures should be balanced to preserve the delicate structure of the scaffold and remove all unwanted components that may trigger strong inflammatory reactions [50,51]. Notably, these residual components, including nucleic acids, may act as danger-associated molecular patterns that stimulate pattern recognition receptors of the innate immune system, such as toll-like receptors (TLR9), RIG-I-like receptors and AIM2-like receptors [52,53]. A greater quantity of matrix-bound DNA (data presented in Supplementary Figure S7) partially explain the observed increased inflammatory responses of hADMs 2 and 3 co-incubated PBMCs, including the proliferative response of T cells monocyte activation and higher cytokine release. Moreover, it may also explain the unexpectedly observed increase in the proliferation of hADM 2 and 3 co-incubated T cells when compared to unprocessed skin [54].

Nearly 80% of the ECM is composed of insoluble or hardly soluble proteins, mainly collagen, which, upon application to the wound, serves as a scaffold for different cell subsets [48,55–57] and improves healing potential [58]. Recent evidence shows that particular compositional elements of the ECM can elicit immune cell activation, which in turn triggers specific cytokine responses in vitro and in vivo [59–61]. These immune-modulatory properties may be induced by direct antigenic effects of ECM structure or released components, including collagen fragments, laminin, hyaluronan, and integrins [60,62]. Notably, the use of enzymes with proteolytic activity, such as trypsin and its modifications (as in hADM 3), allows for the cleavage of proteins adherent to cells, thereby separating cellular contents from the ECM. It has been shown that enzymatic decellularization is more destructive to elastin and collagen fibers in comparison to ionic and non-ionic detergents, such as those used in our study (sodium dodecyl sulfate (SDS) and Triton X-100, respectively) [63–67]. This ECM-extracting effect explains the observed higher immunogenicity of hADM 3 in our study. On the other hand, however, the detergents modulate fibrillar collagen structure, which may result in decreased mechanical strength of the end product [68–70]. SDS is an anionic detergent that effectively extracts and denatures proteins, allowing for the efficient removal of cellular and antigenic contents of processed tissue [71–73]. In addition, SDS has been shown to reduce soluble collagen content by alteration of its molecular structure, bringing it to the point of insolubility [74]. In contrast, Triton X-100 disrupts DNA-protein, lipid-protein, and lipid-lipid interactions while maintaining the native structure of ECM [75,76]. It is believed that the use of both ionic and non-ionic detergents decreases the immunogenicity of decellularized grafts [63,77]. Similarly, in our study, the use of detergents allows for the manufacture of hADMs with relatively low immunogenicity. However, the observed use of SDS seems to be more effective in the reduction in T cell proliferation and cytokine release.

T cell responses are regulated by changes in the composition and function of T cell subsets. They may be regulated by the induction of cellular plasticity (involving epigenetic

machinery in response to microenvironmental changes) or induced in the process of antigen presentation by antigen-presenting cells (such as monocytes/macrophages and dendritic cells) [40,41,78,79]. In our study, we found no direct effect of novel human abdominoplasty skin-derived ADMs on differential T cell subset composition. Therefore, we assumed that our hADMs do not directly induce T cell plasticity.

Decellularization procedures can preserve ECM fragments that can regulate cell migration and modulate local inflammatory responses, improving graft integration with recipient tissues and supporting healing mechanisms. In the wound bed, infiltrating monocytes and dermal macrophages play a central role, orchestrating all steps of healing by (a) clearing the wound from pathogens and debris; (b) releasing growth factors-guiding local progenitor cells proliferation and differentiation; (c) producing inflammatory mediators-regulating immune responses and inducing migration of immune cells and progenitor cells to the wound; (d) releasing pro-angiogenic factors-ultimately regulating, both directly and indirectly, tissue neovascularization; and (e) releasing ECM modulating enzymes-contributing to scar maturation [45,79–81]. In addition, monocytes/macrophages have been shown to therapeutically modulate pathological processes in hard-healing wounds and ulcers (such as diabetic foot ulcers) treated with ADMs [31,82,83]. Therefore, the ideal dermal dressing should target the pro-inflammatory function of monocytes and macrophages and induce their reparatory properties, among others [84,85].

Peripheral blood monocytes constitute of three functionally distinct cell subsets, namely (a) classical CD14++CD16− monocytes (non-activated cells with high phagocytic activities that, upon activation by cytokines, chemokines, growth factors, the danger-associated or pathogen-associated molecular patterns (DAMPS and PAMPS, respectively) acquire CD16 expression); (b) intermediate (CD14++CD16+) monocytes (putative precursors of alternatively activated macrophages referred to as M2 cells with high reparatory and anti-inflammatory potential); and (c) non-classical (CD14+CD16++) monocytes (putative precursors of classically activated M1 macrophages with high pro-inflammatory potential) [86,87]. Notably, in vitro monocyte maturation toward macrophages can be induced by their adhesion to plastic or glass surfaces [88]. Similarly, in our study, monocytes incubated without the presence of hADMs start their maturation process, which was observed as a significant decrease in classical monocyte frequency and the subsequent increase in CD16+ cells. It seems, however, that initially (at 24 h), our novel hADMs reduce the activation and maturation of monocytes toward macrophage-like cells. However, in the later time points, maturation was induced predominantly toward cells with reparatory and pro-angiogenic potential, namely intermediate monocytes and Tie-2 expressing cells [45,89]. It is well established that the implementation of M2 macrophages to biomaterials improves vascularization and healing more effectively when compared to M1-like cells [90–92]. However, to date, the mechanistic role of ECM components in monocyte differentiation toward macrophages and their polarization remains elusive. Recent evidence shows that decellularized ECM supports the generation of M2 monocyte-derived macrophages with different CD23, CD163 and EGR2 expressions [31]. It seems that monocytes differentiate on the surface of ECM and do not penetrate to its deeper layers [93]. Although this is also broadly supported by our observations, in some contrast to previous reports, we found no increase in the frequency of CD163-expressing monocytes. This may be associated with a relatively short culture period and/or a different source of decellularized tissue. Moreover, we found that the hADM surface is partially degraded by collagen IV release, which is further digested by monocytes. In some contrast, collagen I and III seem to remain intact.

Finally, the distinct cytokine profiles induced by differential hADM production must be acknowledged. Previously, an in vitro model of sterile tissue inflammation that induced pro-inflammatory M1-like effects in macrophages was able to be abrogated by coincubation with an artificial dermal matrix composed of collagen and modified hyaluronan [94], in addition to the aforementioned immunomodulatory effects witnessed in macrophage interactions with integrins [61]. Therefore, the observed elevated inflammatory cytokine expression in hADM 2 and 3 co-incubated PBMCs appears consistent with M1-like cell

activation, providing a compelling future target in the deeper examination of modified and/or extracted ECM components that may induce distinct immunomodulatory effects. Taken together, we showed here that decellularization of human abdominoplasty skin using anionic detergent (SDS) could be used for novel non-immunogenic hADM preparation (hADM 1). Given its high potential to induce monocyte polarization toward anti-inflammatory/reparatory M2-like cells, hADM 1 represents a candidate who can serve as a dressing for deep, hard-healing, and/or chronic wounds. However, further in vivo studies are needed to elucidate its therapeutic potential. Moreover, there is still a substantial need to better understand the effects of different decellularization procedures and the role of constituent structural components of decellularized ECM regarding their ability to improve wound healing processes.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pharmaceutics13122164/s1, Figure S1: Representative gating for T cell proliferation, Figure S2: T cell phenotype is not regulated by hADM coincubation, Figure S3: Representative gating for extracellularly stained monocytes, Figure S4: hADMs do not induce differential T cell function, Figure S5: Representative gating strategy for intracellularly stained monocytes, Figure S6: Representative photos of ADMs without PBMC coincubation, Figure S7: Quantification of residual DNA, Table S1: hADM decellularization protocols, Table S2: Antibodies used in flow cytometry, Table S3: Antibodies used in confocal microscopy.

Author Contributions: M.M. and A.E. designed the study; J.H., C.P. and K.G. performed flow cytometry experiments. J.H. and J.C.R. performed confocal microscopy experiments. J.H. analyzed the data; D.G., H.R.H., J.D., J.R. and S.C. were responsible for the qualification of patients, acquisition, and biobanking of skin samples; D.G., J.H., J.R. and A.E. skin decellularization; J.H. and A.E. wrote the manuscript draft prepared figures and tables. C.K., K.G., J.R. and A.E. validated the results; M.M. and A.E. revised the manuscript and figures, J.H., C.K., M.M., and A.E. project administration; M.M. and A.E. project supervision; J.H., C.K. and M.M. funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. All samples were collected upon approval of the Ethics Committee of the Medical University of Bialystok, Poland (Decision # R-I-002/634/2018; approved 28 February 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Abbreviations

CFSE	Carboxyfluorescein Succinimidyl Ester
ECM	Extracellular Matrix
hADM	Human-derived Acellular Dermal Matrix
IFNγ	Interferon Gamma
IL	Interleukin
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
PBMC	Peripheral Blood Mononuclear Cells
TGF-β	Transforming Growth Factor Beta
Th1/17	T helper Types 1 or 17, respectively
TNF	Tumor Necrosis Factor

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pharmaceutics

MDPI

Supplementary Materials

Skin Substitute Preparation Method Induces Immunomodulatory Changes in Co-Incubated Cells through Collagen Modification

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Abbreviations

CFSE–Carboxyfluorescein succinimidyl ester
ECM–Extracellular Matrix
hADM–Human-derived Acellular Dermal Matrix
IFNγ–Interferon Gamma
IL–Interleukin
LPS–Lipopolysaccharide
MFI–Mean Fluorescence Intensity
PBMC–Peripheral Blood Mononuclear Cells
TGF-β–Transforming Growth Factor Beta
Th1/17–T helper Types 1 or 17, respectively
TNF–Tumor Necrosis Factor

Table S1. hADM Decellularization Protocols.

hADM Preparation Method	Phase 1					Phase 2						Washing			Storage
	Reagents	time [h]	Temp. [°C]	[RPM]	Epidermal Removal	Reagents	time [h]	Temp. [°C]	[RPM]	Washing	Time [h]	[RPM]	Lyophilization Temp
hADM 1	1M NaCl + Antibiotic mix	24	37	40	Mechanical	SDS 0.1% + Antibiotic Mix	24	37	40	H_2O	5 × 24h	60	YES/ −70
hADM 2	1M NaCl + Antibiotic mix					3% Triton X-100 in PBS + Antibiotic Mix							
hADM 3	TrypLE Select + Antibiotic mix				Unnecessary	3% Triton X-100 in PBS + Antibiotic Mix							

NaCl—sodium chloride; SDS—Sodium dodecyl sulfate; PBS—Phosphate-Buffered Saline, H_2O—distilled deionized water.

Table S2. Antibodies Utilized in Flow Cytometry.

	Cellular Marker	Fluorochrome	Origin/Isotype	Clone	Supplier
T Cells	CD3	PerCP	Mouse / IgG1, k	SK7	BD
	CD4	FITC	Mouse / IgG2b, k	OKT4	BioLegend
	CD8	PE	Mouse / IgG1, k	SK1	BD
	CD25	PE-Cy7	Mouse / IgG1, k	BC96	BioLegend
	CD127	PE	Mouse / IgG1	R34.34	Beckman
	CD161	APC	Mouse / IgG1, k	HP-3G10	BioLegend
	CD196	PerCP Cy5.5	Mouse / IgG2b, k	G034E3	BioLegend
	IL-17	PE	Mouse / IgG1, k	N49-653	BD
	IFNγ	PE-Cy7	Mouse / IgG1, k	B27	BD
Monocytes	CD14	PerCP	Mouse / IgG2b, k	MϕP9	BD
	CD16	FITC	Mouse / IgG1, k	3G8	BD
	CD163	PE	Mouse / IgG1, k	GHI/61	BioLegend
	TIE-2	Alexa Fluor 647	Mouse / IgG1, k	Ab33	BioLegend
	IL-10	PE	Rat / IgG2a, k	JES3-19F1	BioLegend
	TNF	PE	Mouse / IgG1	6401.1111	BD

Table S3. Antibodies Utilized in Confocal Microscopy.

Primary Antibodies				
Marker	**Origin/Isotype**	**Clone**	**Supplier**	**RRID**
Collagen 1A1	Rabbit / IgG	Polyclonal	Invitrogen	AB_2547045
Collagen III	Rabbit / IgG	Polyclonal	Invitrogen	AB_2552139
Collagen IV	Mouse / IgG1	COL-94	Invitrogen	AB_558482
Secondary Antibodies				
Fluorochrome	**Reactivity**	**Origin/Isotype**	**Clone**	**Supplier**	**RRID**
Alexa Fluor 647	Mouse	Goat / IgG	Polyclonal	Invitrogen	AB_2536165
Alexa Fluor 700	Rabbit	Goat / IgG	Polyclonal	Invitrogen	AB_2535709

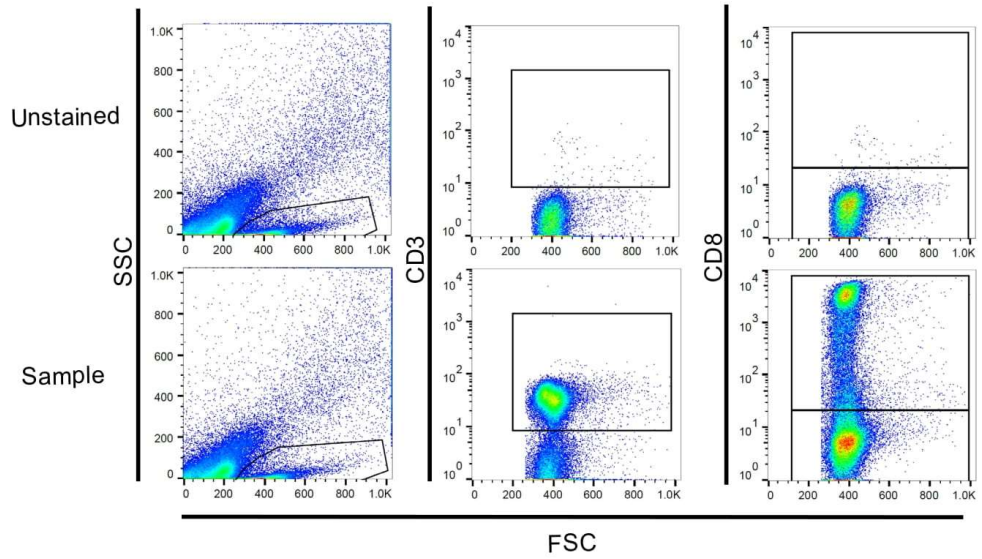

Figure S1. Representative Gating for T Cell Proliferation. This supplemental figure relates to Figure 1. CFSE-stained PBMCs cultured either alone or co-incubated with hADMs were collected, stained for CD3 & CD8, and underwent flow cytometry. Proliferation was quantified as shown on Figure 1 via histogram gating.

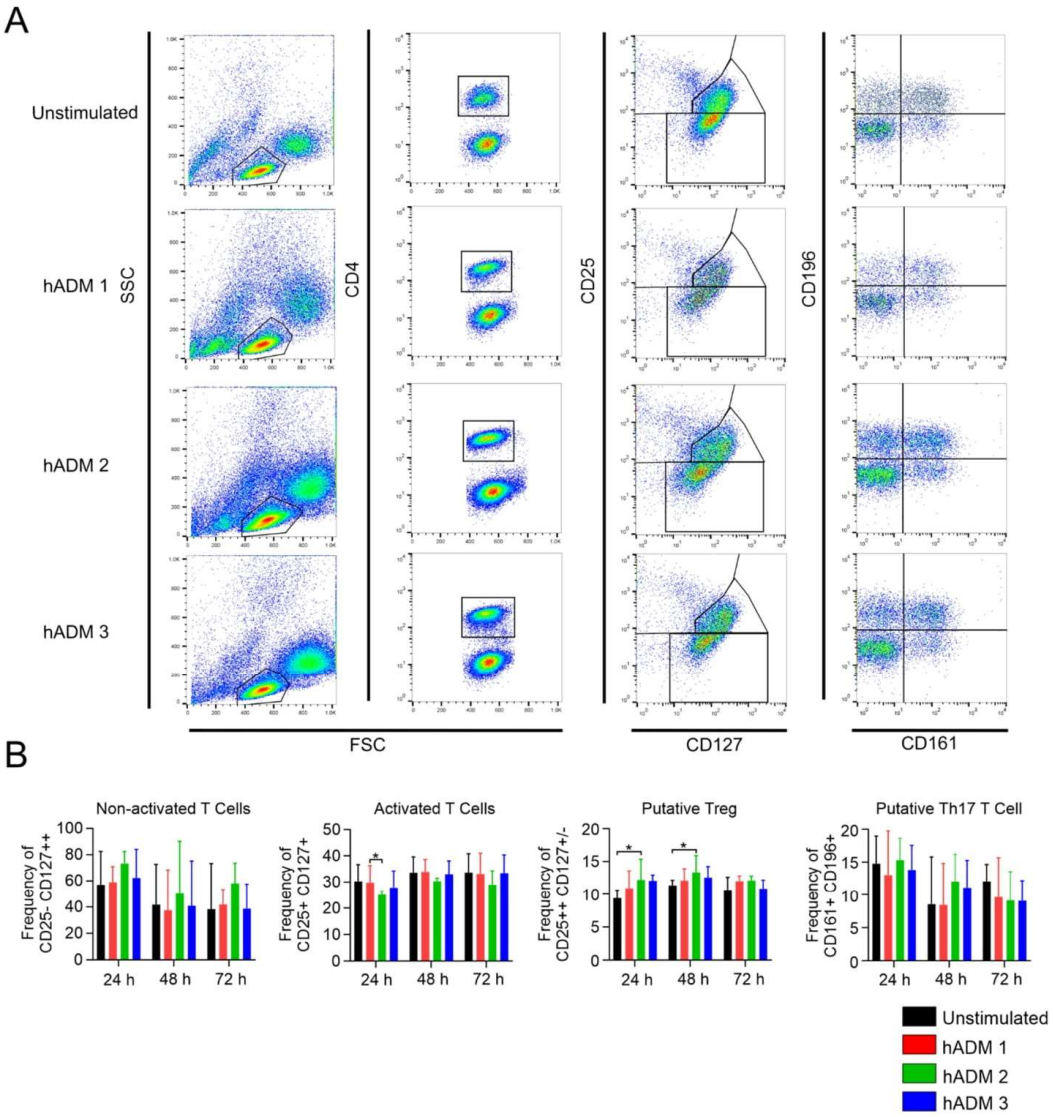

Figure S2. T Cell Phenotype is not Regulated by hADM Co-incubation. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2 or 3 days. Next, cells were stained extracellularly and examined by flow cytometry. (**A**) Representative schematic of flow cytometry gating of extracellularly-stained T cell subsets. (**B**) Frequencies of T cell subsets were noted for inactive T cells (CD25−/CD127++), active T cells (CD25+/CD127+), and Tregs (CD25++/CD127+/−). Frequency of Th17 cells were also examined by CD196+/CD161+. Results expressed as medians + interquartile ranges. Two-tailed Wilcoxon matched-pairs signed rank test used for B. $n = 5$ with 2 technical replicates; * $p < 0.05$.

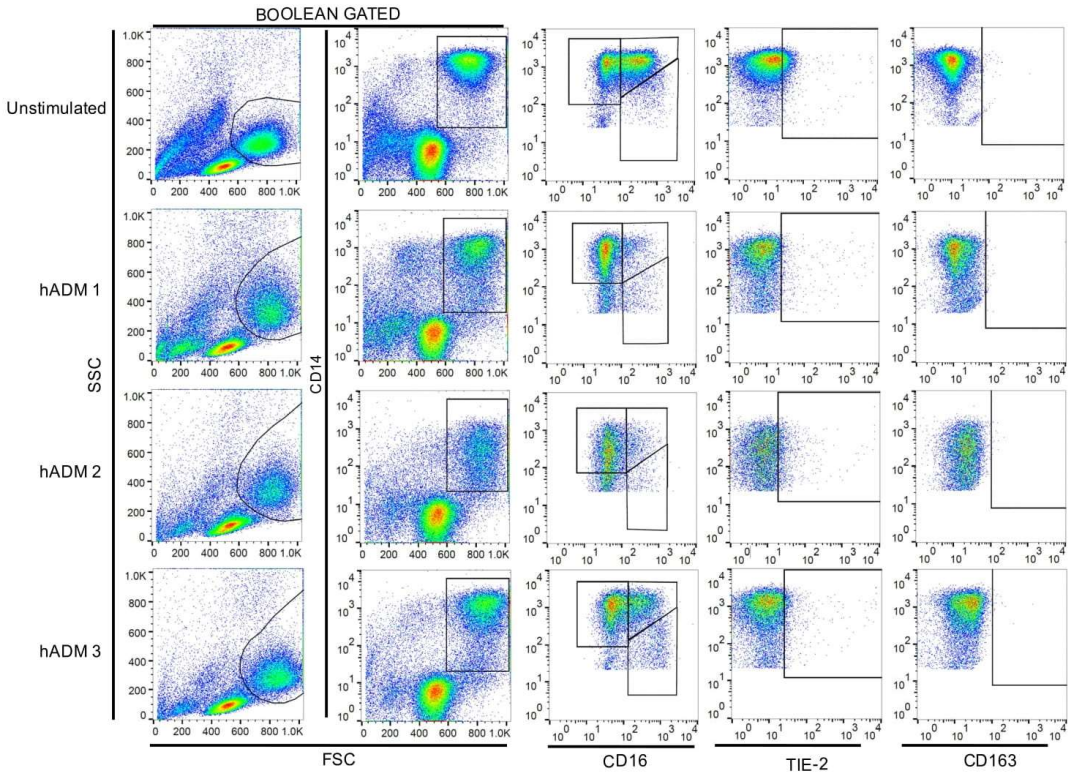

Figure S3. Representative Gating for Extracellularly-stained Monocytes. This supplemental figure relates to Figure 2. PBMCs cultured either alone or co-incubated with hADMs were collected, stained extracellularly for CD14, CD16, TIE-2 and CD163. Frequency of monocyte subsets (by CD14/CD16) as well as frequency and MFI of surface receptors TIE-2 & CD163 can be seen in Figure 2.

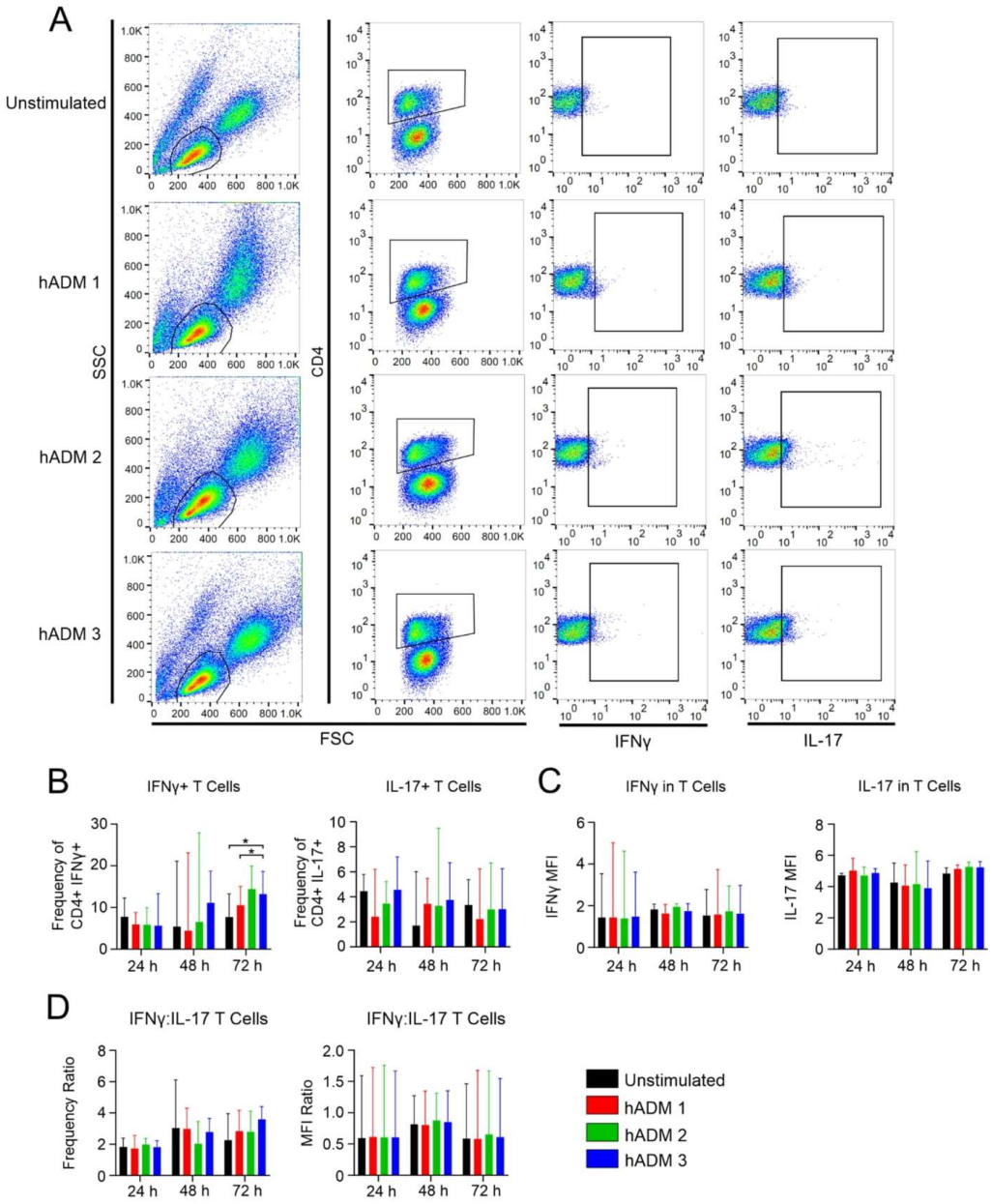

Figure S4. hADMs do not Induce Differential T Cell Function. Healthy donor PBMCs were co-incubated alone or with hADMs for 1, 2 or 3 days. 2 hours before collection, protein transportation was inhibited. Finally, cells were permeabilized and stained extracellularly and intracellularly before being examined by flow cytometry. (**A**) Representative schematic for flow cytometry gating. (**B**) Quantification of CD4+/IFNγ+ or IL-17+ T cell frequency. (**C**) MFI quantification of CD4+/IFNγ+ or IL-17+ T cells. (**D**) Frequency and MFI ratios of inflammatory T cell cytokines. Results expressed as medians + inter-quartile range. Two-tailed Wilcoxon matched-pairs signed rank test used for B–E. $n = 5$ with 2 technical replicates; $^{*}p < 0.05$.

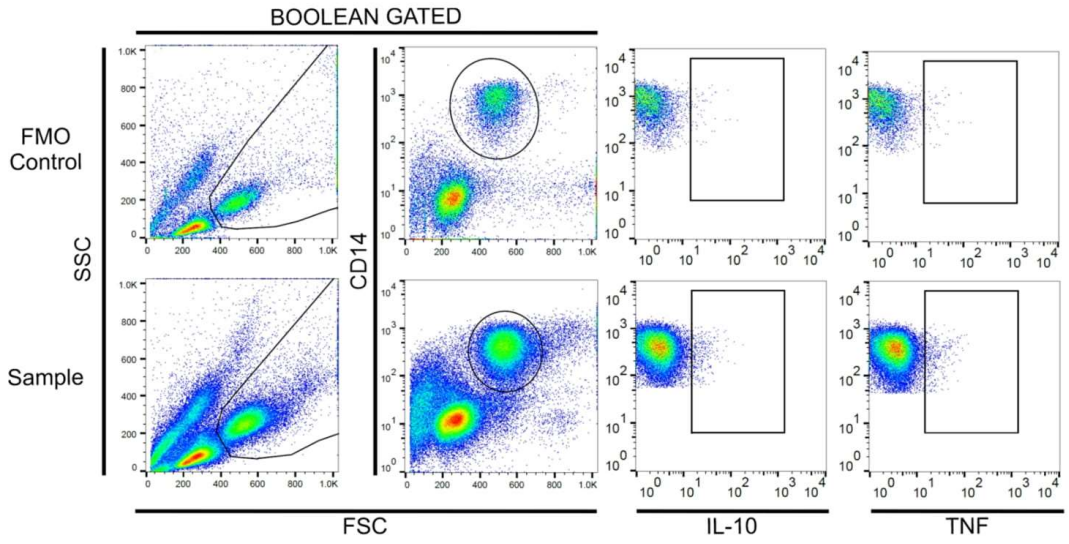

Figure S5. Representative Gating Strategy for Intracellularly-stained Monocytes. This supplemental figure relates to Figure 3. PBMCs cultured either alone or co-incubated with hADMs were collected, stained extracellularly for CD14, and intracellularly for IL-10 & TNF before undergoing flow cytometry. Quantifications of functional cytokines can be seen in Figure 3.

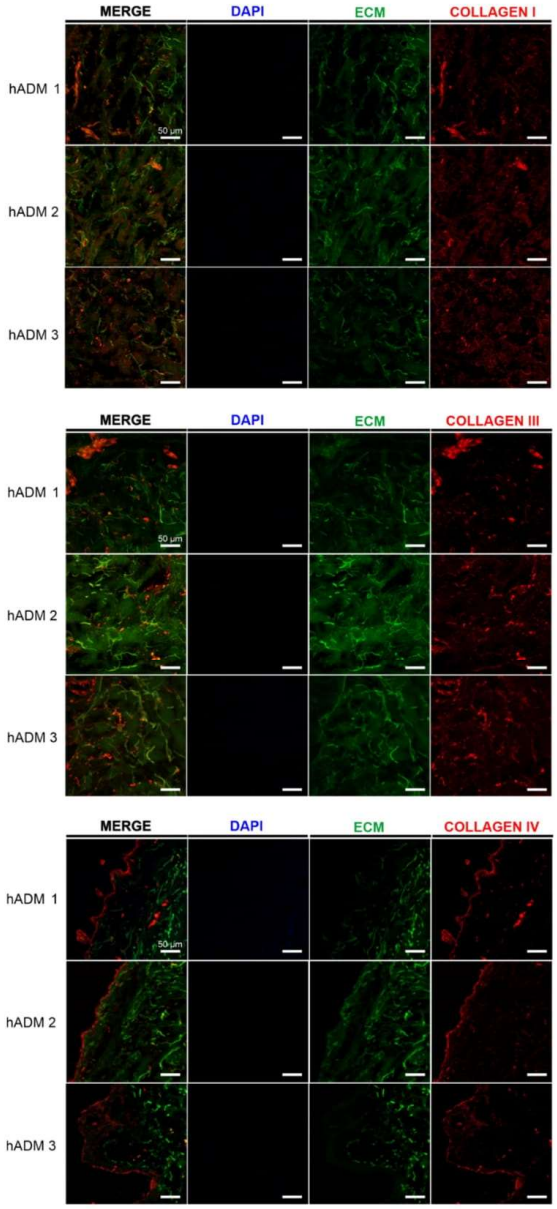

Figure S6. Representative Photos of ADMs without PBMC co-incubation. hADMs were examined confocally after fluorescence-conjugated antibody staining for collagens I, III and IV. Images for collagens I, III and IV can be seen for each hADM and in 3 separate channels as well as merged together. MERGE: Combined representation of channels 1–3, detailed below. Channel 1 (DAPI): cell nuclei staining via DAPI using a 405 nm laser. Channel 2 (ECM): Autofluorescent collagen fibers and ECM using a 488 nm laser. Channel 3 (Specific Collagen): Specific collagen fibers were stained using a primary and subsequently a secondary fluorescence-conjugated antibody using a 647 nm laser. All scale bars represent 50 µm.

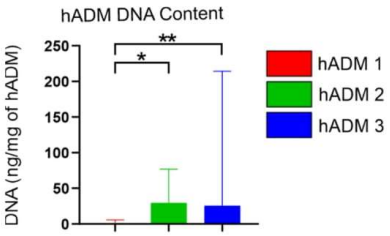

Figure S7. Quantification of residual DNA. hADMs were examined for their residual DNA content after processing. hADMs were entirely digested by proteinase K followed by DNA isolation and quantification. Results expressed as medians +/− interquartile ranges. One-tailed U Mann-Whitney test was used. $n = 4$ with 2 technical replicates; * $p < 0.05$, ** $p < 0.01$.

VIII. Summary of Results

a. English

Chronic ulcerative and hardly healing wounds are a significant clinical problem worldwide. Despite the development of new treatment strategies, their efficacy is unsatisfactory. One of the proposed methods aims to use an acellular dermal matrix (ADM) of animal or human origin. Notably, the source of the skin, the way it is processing, and the sterilization of the final product may affect the therapeutic effectiveness of ADM. An innovative approach is the use of human skin from living donors harvested from a skin fold obtained during the abdominoplasty procedure. However, the impact of different processing methods on the immunomodulatory properties of the novel human ADMs (hADMs) remains elusive. Therefore, the aim of the study was: 1. to analyze the effect of different methods of human abdominoplasty skin processing on acellular dermal matrix immunogenicity; 2. to evaluate immune-modulatory properties of differentially prepared human abdominoplasty skin-derived acellular dermal matrix; and 3. to evaluate the effects of immune cell on the structure of human abdominoplasty skin-derived acellular dermal matrix.

The skin was processed using three different decellularization methods, including the use of anionic detergent (sodium dodecyl sulfate; SDS, in hADM 1) or a non-ionic detergent (Triton X-100 in hADM 2) or a combination of recombinant trypsin and Triton X-100 (in hADM 3). Peripheral Blood Mononuclear Cells (PBMC) were isolated from the blood of healthy donors by density gradient centrifugation. Freshly isolated cells were incubated in the presence of the prepared hADM. The immunogenicity of novel dermal matrices was assessed based on a T cell proliferation assay using flow cytometry. The in vitro immunomodulatory potential of the new hADMs was evaluated using the multicolor flow cytometry method and immunoassays. The influence of the immune cells on the collagen structure of the matrices was analyzed with confocal microscopy.

First, I found that differentially prepared matrices possess different immunogenicity. The hADM 1 induced low T cell proliferation without significant changes in the cytokine profile. In contrast, hADM 2 and 3 were characterized by higher immunogenicity than hADM1. Next, the activation and phenotype of T cells and monocytes were assessed. Interestingly, analyzed hADMs did not affect T cell

phenotype and composition after 3-day incubation. However, significant changes in the composition of different monocyte subsets were observed, namely increased maturation towards cells with anti-inflammatory and pro-angiogenic potential. These changes were particularly significant after incubating in the presence of hADM1. Finally, the collagen structure of hADM was examined after incubation with immune cells. I observed collagen IV degradation and its collocation with PBMC.

In conclusion, abdominoplasty skin is suitable for the production of hADM. In addition, using anionic detergent in the processing of abdominoplasty skin allows the manufacturing of hADM with low immunogenicity and immunomodulatory properties, indicating a high therapeutic potential. However, further research is needed to assess the therapeutic utility of hADM 1 in treating chronic and hardly healing wounds.

b. Polish

Przewlekłe wrzodziejące i trudno gojące się rany, stanowią znaczący problem kliniczny na świecie. Pomimo opracowywania nowych strategii leczenia ich skuteczność jest niezadawalająca. Jedną z wykorzystywanych metod jest zastosowanie bezkomórkowych macierzy skórnych (ADM, ang. acellular demal martix) pochodzenia zwierzęcego lub ludzkiego. Źródło skóry, sposób jej przetwarzania oraz sterylizacja końcowego produktu może wpływać na skuteczność stosowanych ADM w terapii ran. Nowatorskim podejściem w produkcji ADM jest wykorzystanie skóry ludzkiej (hADM, ang. human acellular dermal matrix) pochodzącej od dawców żywych i pozyskanej z fałdu skórnego pobranego podczas zabiegu abdominoplastyki. Nieznany jest jednak wpływ w jakim różne sposoby przetwarzania tego materiału, wpływają na właściwości immunomodulacyjne końcowego produktu. Dlatego celem niniejszej pracy była: 1. Analiza wpływu różnych metod przetwarzania skóry pobranej podczas zabiegu abdominoplastyki na immunogenność hADM; 2. Ocena immunomodulacyjnych właściwości hADM powstałej ze skóry ludzkiej pobranej podczas zabiegu abdominoplastyki; oraz 3. Ocena wpływu komórek układu immunologicznego na strukturę hADM wytowrzonej ze skóry ludzkiej pobranej podczas zabiegu abdominoplastyki.

Skóra została przygotowana przy użyciu trzech różnych metod decelularyzacji obejmujących zastosowanie detergentu jonowego (dodecylosiarczan sodu; SDS, w hADM 1) lub detergentu niejonowego (Triton X-100 w hADM 2) lub kombinacji rekombinowanej trypsyny i Triton X-100 (w hADM 3). Komórki jednojądrzaste krwi obwodowej (PBMC, ang. Peripheral Blood Mononuclear Cells) izolowano z krwi zdrowych dawców metodą wirowania na gradiencie gęstości. Świeżo izolowane komórki inkubowano w obecności wytworzonych hADM. Immunogenność nowych matryc skórnych oceniono w oparciu o test proliferacji limfocytów T przy zastosowaniu cytometrii przepływowej. Potencjał immunomodulacyjny in vitro nowych hADM oceniono z wykorzystaniem metody wielokolorowej cytometrii przepływowej oraz testów immunoenzymatycznych. Wpływ komórek układu immunologicznego na strukturę kolagenową wytworzonych matryc analizowano w oparciu o technikę mikroskopii konfokalnej.

. Analizowane matryce wykazywały zróżnicowaną immunogenność. hADM 1 indukował niską proliferację limfocytów T bez znaczących zmian w profilu cytokin. Natomiast hADM 2 i 3 cechowały się wyższą immunogennością w porównaniu do hADM1. Następnie oceniono poziom aktywacji oraz fenotyp limfocytów T i monocytów. Co ciekawe, żaden z analizowanych nowych hADM nie wpływał na skład ilościowy wybranych populacji limfocytów T po 3 dniach inkubacji. Zaobserwowano natomiast istotne zmiany w składzie procentowym subpopulacji monocytów, mianowicie zwiększone dojrzewanie w kierunku komórek o potencjale przeciwzapalnym i proangiogennym. Zmiany te były w szczególności istotne po inkubacji komórek w obecności hADM1. Na koniec zbadano strukturę kolagenową hADM po inkubacji z komórkami układu immunologicznego. Zaobserwowano degradację kolagenu IV oraz jego kolokalizację z PBMC.

Podsumowując, wykazano, że skóra po abdominoplastyce jest odpowiednia do wytwarzania hADM. Ponadto wykorzystanie detergentu jonowego w procesie przetwarzania skóry pobranej podczas zabiegu abdominoplastyki tworzy hADM, który wykazuje niską immunogenność oraz posiada właściwości immunomodulujące, które mogą świadczyć o wysokim potencjale terapeutycznym. Potrzebne są jednak dalsze badania, aby ocenić przydatność terapeutyczną hADM 1 w leczeniu ran przewlekłych i ciężko gojących się.

IX. Author & Co-Author Contribution to Dissertation Publications

Holl, J.; Pawlukianiec, C.; Corton Ruiz, J.; Groth, D.; Grubczak, K.; Hady, H.R.; Dadan, J.; Reszec, J.; Czaban, S.; Kowalewski, C.; Moniuszko, M.; Eljaszewicz, A. Skin Substitute Preparation Method Induces Immunomodulatory Changes in Co-Incubated Cells through Collagen Modification. Pharmaceutics 2021, 13, 2164. https://doi.org/10.3390/pharmaceutics13122164

Author	Nature of Participation	Contribution (in %)
Jordan Michael Holl	**PERFORMED ALL EXPERIMENTS, PERFORMED ALL DATA ANALYSIS, DECELLULARZED AND BIO-BANKED HADMS, ORIGINAL DRAFT WRITING, FIGURES & TABLE CREATION, PROJECT ADMINISTRATION, & FUNDING ACQUISITION**	**65**
Andrzej Eljaszewicz	**EXPERIMENTAL CONCEPTUALIZATION & DESIGN, SKIN DECELLULARIZATION, ORIGINAL DRAFT WRITING, FIGURE & TABLE CREATION, MANUSCRIPT REVISION & EDITING, VALIDATION OF RESULTS, PROJECT ADMINISTRATION, & PROJECT SUPERVISION**	**22**
Cezary Pawlukianiec	**PERFORMING FLOW CYTOMETRY EXPERIMENTS**	**2**
Javier Corton Ruiz	**PERFORMING CONFOCAL MICROSCOPY EXPERIMENTS**	**2**
Marcin Moniuszko	**EXPERIMENTAL CONCEPTUALIZATION & DESIGN, REVISION & EDITING OF THE MANUSCRIPT, PROJECT ADMINISTRATION, PROJECT SUPERVISION, & FUNDING ACQUISITION**	**2**

Dawid Groth	**QUALIFICATION OF PATIENTS, ACQUISITION OF BIOBANKING & SKIN SAMPLES, & SKIN DECELLULARIZATION**	**1**
Kamil Grubczak	**PERFORMANCE OF FLOW CYTOMETRY EXPERIMENTS & VALIDATION OF EXPERIMENTAL RESULTS**	**1**
Hady Razak Hady	**THE QUALIFICATION OF PATIENTS, & THE ACQUISITION OF BIOBANKING & SKIN SAMPLES**	**1**
Jacek Dadan	**THE QUALIFICATION OF PATIENTS, & THE ACQUISITION OF BIOBANKING & SKIN SAMPLES**	**1**
Joanna Reszec	**THE QUALIFICATION OF PATIENTS, & THE ACQUISITION OF BIOBANKING & SKIN SAMPLES, SKIN DECELLULARIZATION, & VALIDATION OF EXPERIMENTAL RESULTS**	**1**
Slawomir Czaban	**THE QUALIFICATION OF PATIENTS, & THE ACQUISITION OF BIOBANKING & SKIN SAMPLES**	**1**
Cezary Kowalewski	**VALIDATION OF EXPERIMENTAL RESULTS, PROJECT ADMINISTRATION, & FUNDING ACQUISITION**	**1**

I hereby declare that all co-authors agreed to use these articles in this dissertation

Signature

Holl, J.; Kowalewski, C.; Zimek, Z.; Fiedor, P.; Kaminski, A.; Oldak, T.; Moniuszko, M.; Eljaszewicz, A. Chronic Diabetic Wounds and Their Treatment with Skin Substitutes. *Cells* **2021**, *10*, 655. https://doi.org/10.3390/cells10030655

Author	Nature of Participation	Contribution (in %)
Jordan Michael Holl	**CONCEPTUALIZATION, ORIGINAL DRAFT WRITING & PREPARATION, REVIEW & EDITING, VISUALIZATIONS, & FUNDING ACQUISITIION**	**70**
Andrzej Eljaszewicz	**CONCEPTUALIZATION, ORIGINAL DRAFT WRITING & PREPARATION, REVIEW & EDITING, & SUPERVISION**	**20**
Marcin Moniuszko	**CONCEPTUALIZATION, REVIEW & EDITING, SUPERVISION, & FUNDING ACQUISITION**	**5**
Cezary Kowalewski	**REVIEW & EDITING, & PROJECT FUNDING**	**1**
Zbigniew Zimek	**REVIEW & EDITING**	**1**
Piotr Fiedor	**REVIEW & EDITING**	**1**
Artur Kaminski	**REVIEW & EDITING**	**1**
Tomasz Oldak	**REVIEW & EDITING**	**1**

I hereby declare that all co-authors agreed to use these articles in this dissertation

Signature

X. Funding & Ethical Consent Disclosures

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b. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee at the Medical University of Bialystok. Permission number R-I-002/634/2018 authorized on 2019-02-28 applies to all *in vitro* data – namely the usage of PBMCs from healthy donor buffy coats acquired from the Bialystok Regional Blood Bank. Permission number R-I-002/140/2016 authorized on 2016-4-28 applies to the collection and processing of bariatric skin in the creation of hADMs utilized in all experiments.

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