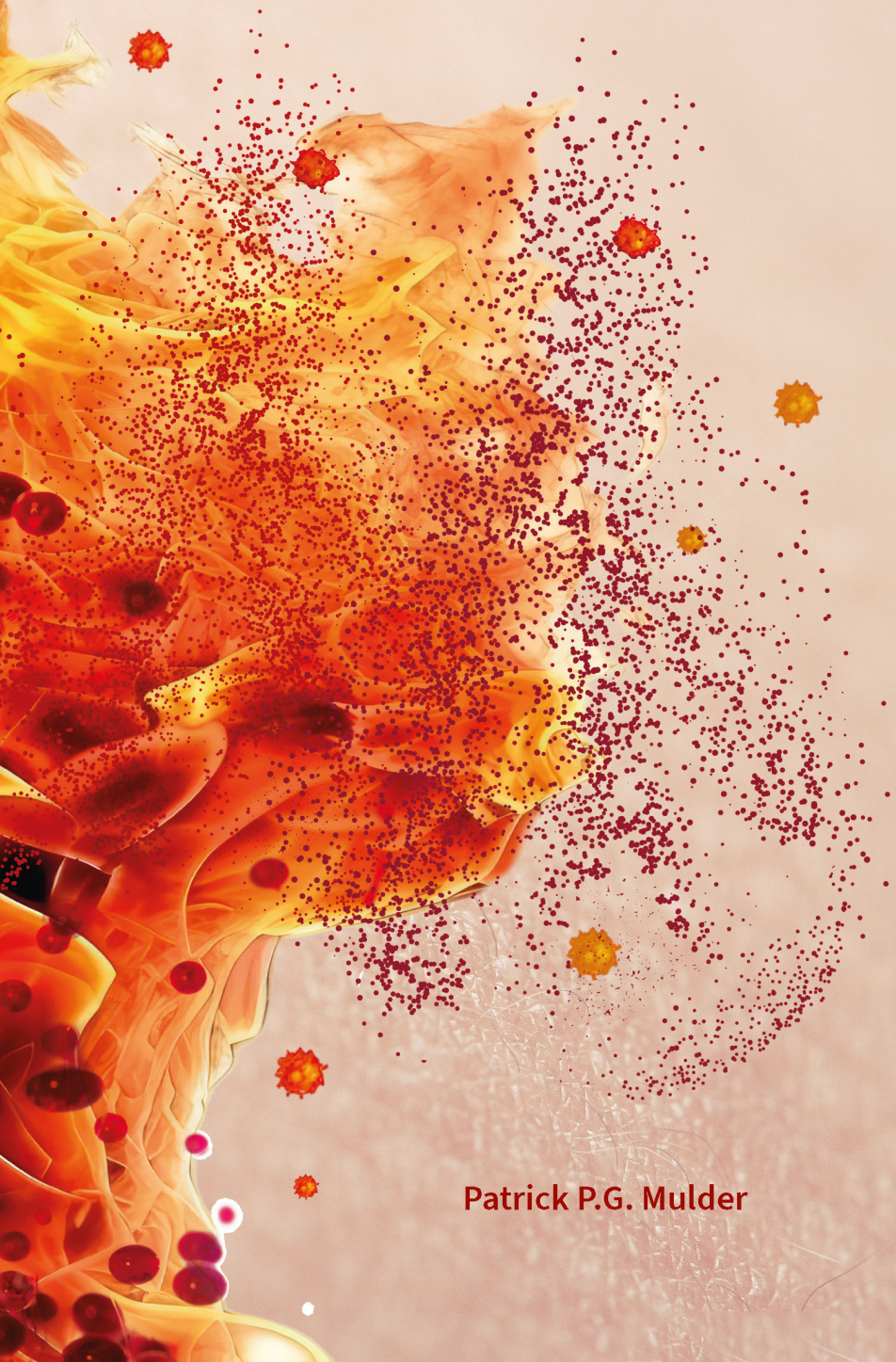
IMMUNE RESPONSE TO BURN INJURY:

From Animal and Patient Data Towards In Vitro Modeling



Patrick P.G. Mulder

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Colophon

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PhD thesis, Radboud University, the Netherlands

The research described in this thesis was performed at the Association of Dutch Burn Centres, Beverwijk, the Netherlands and at the Department of Laboratory Medicine, Laboratory of Medical Immunology, Radboud University Medical Center, Nijmegen, the Netherlands. The presented work was supported by a grant from the Dutch Burns Foundation. Distribution of this thesis was financially supported by Stichting Brandwonden Research Instituut, Radboud University, Stichting ETB-BISLIFE, Nederlandse Brandwonden Stichting, Stichting Proefdiervrij and Medskin Solutions dr. Suwelack.

Provided by thesis specialist Ridderprint, ridderprint.nl

Printing: Ridderprint
Layout and design: Katie McGonigal, persoonlijkproefschrift.nl
ISBN: 978-94-6483-447-5

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Proefschrift ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.M. Sanders,
volgens besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 11 december 2023
om 14.30 uur precies

door

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CHAPTER 1

General Introduction

Burn injury is a prevalent cause of disability and mortality throughout the world and its consequences affect patients both physically and mentally [1,2]. Depending on the severity of the injury and condition of the victim, healing of burns can be problematic, leading to secondary medical complications [3–5]. Health issues that often occur relatively early after burn injury are systemic inflammatory response syndrome (SIRS), hyper-metabolism, wound deepening, bacterial infection and hypovolemia due to a massive loss of fluids [6–9]. These extreme reactions in the body are likely to hinder and delay the wound healing process and have a major impact on morbidity and mortality of burn survivors [10,11].

Treatment of burn injuries is an intensive and time-consuming process. Complications of burn injury are generally present for the long-term or permanent and even years later new symptoms might occur, especially when patients are growing or when vital organs are irreversibly damaged [8,12]. Among long-term complications of burn injury are (hypertrophic) scar formation, loss of skin elasticity, contractions or diseases related to vital organs. Next to that, problems with mental well-being and reduced quality of life impact patients' overall health [13,14]. Over the years, it has become more and more evident that the immune system plays an indispensable role in most (patho-) physiological responses to burn injury [15,16]. It remains, however, largely unclear how specific immune reactions lead to burn-related diseases.

IMMUNE CELLS AND INFLAMMATORY FACTORS IN WOUND HEALING

Next to being a protective, physical barrier to the outside, the skin is an important regulator of homeostasis [17]. Cells in the skin continuously carry out immune surveillance to ensure early and effective defense mechanisms against both internal (e.g. oncogenesis) and external threats (e.g. bacteria or viruses) [18]. The immune system consists of two arms: the innate and the adaptive immune system. The innate immune system reacts in a generic, rapid and nonspecific way, while the adaptive arm is more specialized, organized and takes more time to develop [19]. Granulocytes, mast cells, monocytes, macrophages, dendritic cells and natural killers cells (NK cells) are cells of the innate immune system and react through stimulation of pathogen-recognition receptors (PRRs). Upon interaction with pathogens, they activate cascades and recruit other immune cells via cytokine release and antigen presentation [20]. The adaptive immune system consists of T cells and B cells which are lymphocyte subtypes with a unique repertoire of immune receptors to discriminate auto-antigens from allo-antigens. These cells can react strongly to pathogen antigens by secreting antibodies, toxins and cytokines and are able to build immunological memory that will establish a stronger and more rapid response after subsequent re-encounter [19,21]. Beside fibroblasts and

keratinocytes, healthy skin is inhabited mainly by lymphocytes and antigen presenting cells (dendritic cells, Langerhans cells and macrophages) that survey the skin and react to foreign structures and danger signals [17,18,22].

The immune response plays a central role during wound healing. It is essential for a proper host defense against invading microbes and coordinates healing processes during the different stages of skin regeneration [23]. The inflammatory response starts immediately after trauma. Injured skin will release damage associated molecular patterns (DAMPs) that emerge from ruptured cells [24,25]. DAMPs such as HMGB1, IL-1α or DNA are structures that act as danger signals and stimulate pattern PRRs on surrounding cells and skin-resident immune cells [26–28]. These cells respond to PRR stimulation by secreting effector molecules such as cytokines and chemokines that attract and navigate immune cells towards the wound site [29]. Immune cells that are active during tissue damage and regeneration include neutrophils, eosinophils, mast cells, monocytes, macrophages, T cells, B cells and NK cells (**Figure 1**).

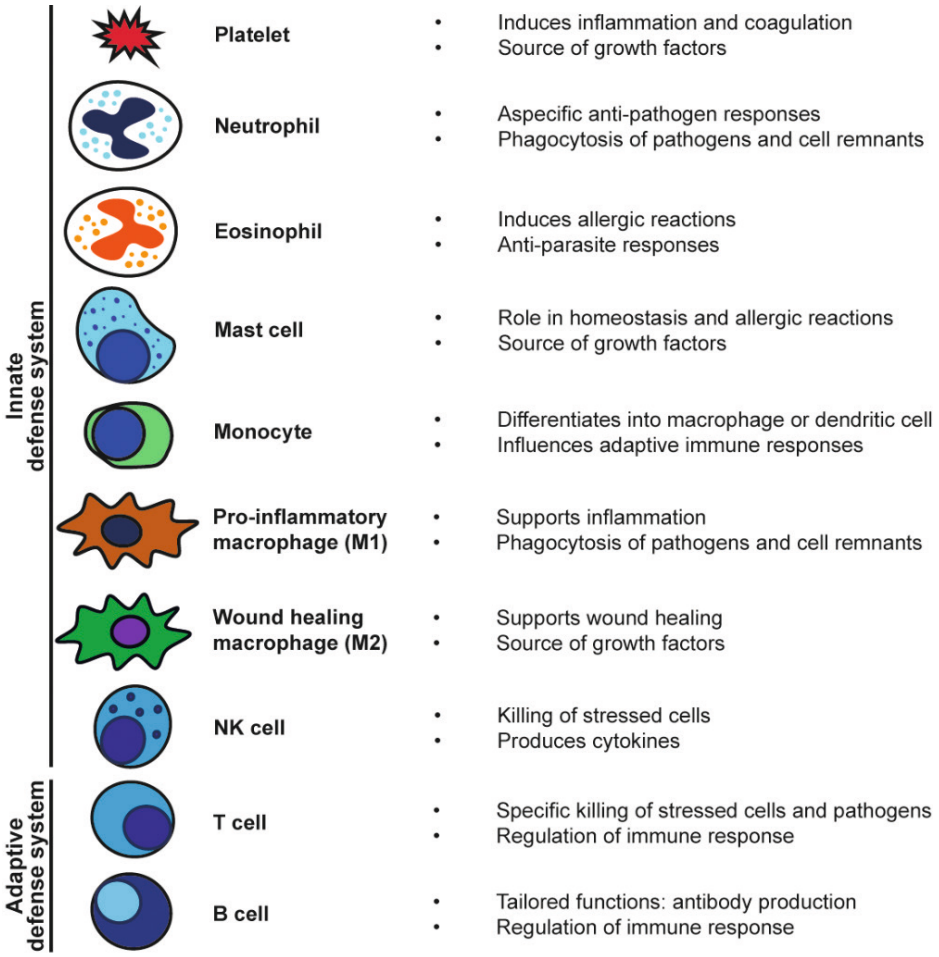

Figure 1. Immune cells involved in wound healing.

Next to the immune cells, there are platelets which are fragments of megakaryocytes that start coagulation to stop the bleeding and produce factors that initiate the inflammatory response [30]. Neutrophils have a short life-span and are primarily needed to phagocytose and destroy cell remnants and invading bacteria [31]. Neutrophils are released from the bone marrow into the blood and undergo different stages of maturity [32]. Neutrophils will accumulate in large numbers at the site of tissue injury and will eventually die via apoptosis [33]. Eosinophils are suggested to play roles during wound healing and might be involved in coagulation, vascular repair and inflammation, however, the exact mechanisms are yet to be discovered [34]. Mast cells proposedly enhance inflammation and vascular permeability through the secretion of histamines early after injury and can stimulate re-epithelization and angiogenesis later on by the release of

growth factors [35,36]. Monocytes reside in the blood until they migrate into tissues upon inflammatory stimuli [37]. In tissues, monocytes will differentiate into macrophages or dendritic cells to perform immune surveillance and protect against pathogens [37]. The most important macrophage subsets during wound healing are the pro-inflammatory macrophages (M1) and macrophages that support wound healing (M2) [38,39].

NK cells are cytotoxic lymphocytes that are involved in tissue homeostasis and killing of stressed or infected cells [40]. Based on their cytotoxicity and cytokine and marker expression they can be classified as either NK^{dim} cells (less cytokine production, more cytotoxic) and NK^{bright} cells (more cytokine production, less cytotoxic) [41]. T cells and B cells are part of the adaptive immune system and generate tailored responses to pathogens through specific effector T cells and antibody production [42]. After stimulation, naïve T cells can differentiate into specific subtypes with different effector functions: Th1, Th2, T9, Th17, Th22, Tfh cells or regulatory T cells (Tregs) [43]. The Th phenotype will influence other immune cells and the direction and duration of the overall immune response. These immune cells produce and are influenced by inflammatory mediators (**Table 1**).

Table 1. Inflammatory mediators involved in wound healing. Information derived from [44–48].

Category	Mediator	Source	Function
Chemokines	CCL2 (MCP-1)	Monocytes, macrophages, dendritic cells	Attracts monocytes, basophils and T cells
	CCL3 (MIP-1α)	Macrophages, eosinophils,	Attracts monocytes, macrophages and neutrophils, lymphocytes
	CCL4 (MIP-1β)	Monocytes, macrophages, epithelial cells, fibroblasts	Attracts monocytes, macrophages, lymphocytes, dendritic cells
	CCL11 (eotaxin)	Endothelial cells, eosinophils, monocytes, fibroblasts	Attracts eosinophils
	CXCL1 (GROα)	Macrophages, neutrophils, epithelial cells	Attracts neutrophils
	CXCL8 (IL-8)	Macrophages, epithelial cells, endothelial cells	Attracts neutrophils

Table 1. Continued.

Category	Mediator	Source	Function
Cytokines	IL-1(α/β)	Endothelial cells, keratinocytes, neutrophils, others	Pro-inflammatory, DAMP, stimulates cell extravasation, T cell activation
	IL-2	T cells	Th1 T cell response, T cell growth factor, activates immune cells
	IL-4	T cells, eosinophils, basophils, mast cells	Anti-inflammatory, Th2 T cell response, regulate allergic responses
	IL-6	Macrophages, keratinocytes, fibroblasts, lymphocytes, others	Pro-/anti-inflammatory, regulates acute phase proteins, stress response
	IL-10	T cells, B cells, mast cells, macrophages	Anti-inflammatory, downregulation of cells
	IL-12 (p40/p70)	Macrophages, dendritic cells, lymphocytes, neutrophils, others	Pro-inflammatory, Th1 T cell response, NK cell activation
	IL-17 (A/F)	T cells	Pro-inflammatory, Th17 T cell response, activates cells
	IL-18	Liver, other organs	Pro-inflammatory, Th1 T cell response, immune regulation
	IL-33	Endothelial cells, epithelial cells	Pro-inflammatory, induces production of type 2 cytokines, homeostasis
	IFN-γ	T cells, NK cells, macrophages	Activation of immune cells, antiviral activity
	TNF-α	Neutrophils, lymphocytes, endothelial cells, mast cells	Pro-inflammatory, neutrophil activation

Table 1. Continued.

Category	Mediator	Source	Function
Growth factors	EGF	Glands, platelets	Stimulates growth of epidermal and epithelial cells
	FGF (1-23)	Macrophages	Proliferation of fibroblasts, induce production of granulation tissue
	G-CSF	Endothelial cells, macrophages	Stimulates bone marrow to release granulocytes into blood
	GM-CSF	T cells, macrophages, endothelial cells, fibroblasts	Maturation of granulocytes and monocytes
	KGF (FGF7)	Mesenchymal cells, fibroblasts	Induces re-epithelization by keratinocytes
	PDGF	Platelets	Stimulates growth of mesenchymal cells, promotes wound healing
	PGE	Macrophages	Pro-inflammatory, vasodilator, inhibits aggregation of platelets
	TGF-β1	Platelets, fibroblasts, monocytes, T cells	Anti-inflammatory, regulates cell growth, inhibition of lymphocytes
	VEGF (A-F)	Macrophages, platelets, keratinocytes	Stimulates angiogenesis
Other mediators	CRP	Liver	Pro-inflammatory, acute phase protein, stress response
	Histamine	Mast cells, basophils	Anti-pathogen response, allergic reaction
	HMGB1	Damaged cells	Pro-inflammatory, DAMP, stress response

DISTORTED WOUND HEALING DURING BURN INJURY

During wound healing, the immune homeostasis and tissue repair processes are usually tightly controlled to avoid collateral damage and to ensure a timely recovery [49–51]. Because burn injury often destroys a large portion of skin, it creates a large area of necrotic tissue that can cause an overstimulation of the immune system [27,52]. Fibroblasts, keratinocytes and innate immune cells are highly responsive and release extremely high levels of cytokines that in turn attract massive amounts of inflammatory cells. Extreme influx of pro-inflammatory immune cells can lead to expansion of the wound area, thereby producing additional inflammatory signals [29,53]. Eventually, this can become in a vicious circle of inflammation that will impede tissue repair (**Figure 2**).

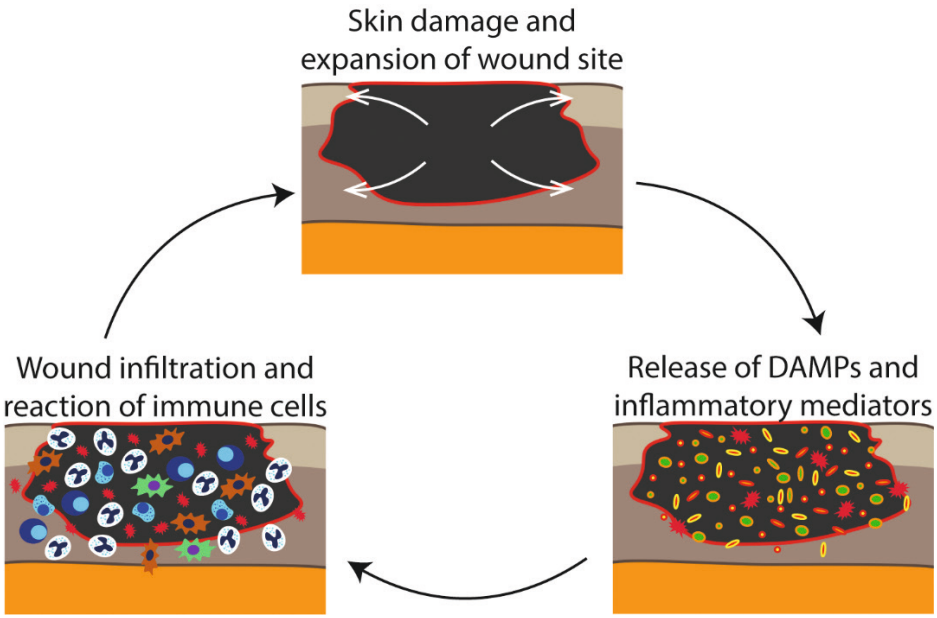

Figure 2. Vicious circle of inflammation and tissue damage that can establish after burn injury.

During the inflammatory phase after trauma, immune cells will migrate into the wounded skin to remove debris and prevent bacterial colonization [38]. Within days, a portion of these cells disappear through apoptosis while others differentiate into a state that supports wound healing [49]. Generally within one week after injury, lymphocytes will infiltrate the wound site to regulate any ongoing inflammation and, if required, orchestrate a tailored effector response to eliminate infiltrated pathogens [54]. Following the effector phase, reduction of the immune response is needed to establish a proper wound healing process. This will shift the focus from inflammation towards

proliferation of keratinocytes and production of collagen, which are required for tissue restoration [55]. After substantial burn injury, it is thought that processes in the immune response are derailed, leading to persistent inflammation. Ongoing inflammation and an aberrant wound healing process can lead to long-term sequalae such as excessive scar formation, hypertrophic scars and contractures [6,38,56]. Such functional and cosmetic impairments will also impact patients' mental well-being [14,57].

The processes in wound healing are connected to one another and inflammation proposedly plays a central role (**Figure 3**). Some of these processes are better elucidated than others. For instance, it is still poorly understood which immune reactions are distorted during burn injury or how bone marrow stress response leads to reduced lymphocyte activity and immune deficiency. Also, a great portion of the available evidence comes exclusively from animal studies [58]. It is therefore necessary to shine more light on the reactions in the burn-induced immune response and to bridge the gap between animal data and the human situation [59]. To limit complications and improve wound healing in patients, it is of utmost importance that the involved immune cells and inflammatory mediators are studied in more detail. More information on specific subsets and interplay between cells will help to design more effective ways to improve wound healing after major burn injuries.

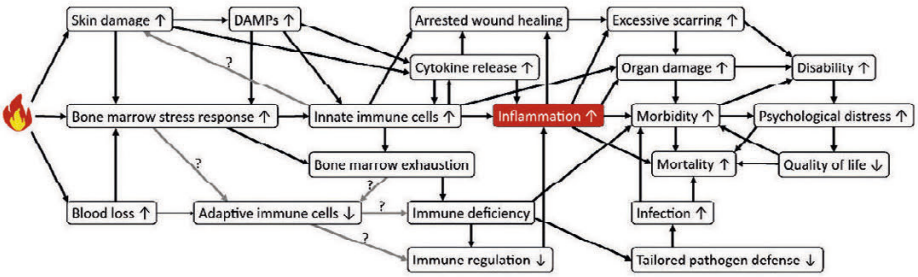

Figure 3. Scheme of reactions and consequences to burn injury. It highlights a central role of the immune response.

SYNTHESIS OF AVAILABLE LITERATURE

Researchers have previously investigated burn injury and have sought for ways to improve treatment. Most investigational or interventional studies in the field of burn injury have been performed on experimental animals [58,60,61]. Most of these studies focused on only a few aspects of wound healing and inflammation. Since it is difficult to keep up with all the information and because existing evidence is scattered, there is a strong need for an overview of the available literature [62]. Systematic reviewing

is a valuable method to synthesize an overview of empirical evidence from separate investigations. These overviews provide insights that will advance experimental design contributing to the reduction and refinement of animal experimentation and will support evidence-based clinical practice [63,64].

BLOOD AND WOUND TISSUE FROM BURN PATIENTS

Alternatively, valuable insights into the burn-induced immune response can be generated by investigating patient specimens. Phenotypic characterization and quantification of cells, and analysis of inflammatory mediators in blood and wound tissue inform us of the specific immune cells and factors that are actively involved in inflammation. Patient studies are limited by restrictions in sampling and absence of baseline values. However, valuable information can be generated by using leftover blood and burn tissue specimen originating from routine blood withdrawals and surgeries as part of clinical practice. Laboratory techniques such as flow cytometry, immunoassays and microscopy can uncover cellular activity and processes that are involved in the burn-induced immune response. Moreover, by analyzing patient samples from different time intervals after burn injury, time-dependent effects can be investigated.

MODELING THE POST-BURN IMMUNE RESPONSE

Growing ethical and scientific concerns drive scientists to search for animal-free approaches to study burn injury. An appealing alternative to animal experimentation is the use of in vitro skin models. Such skin models mimic the tissue architecture of native human skin. The information collected from literature and patient studies can be used to develop and adjust in vitro skin models. Such models can be used to study aspects of wound healing and inflammation after burn injury in a standardized and controlled setting. Since in vitro skin models are not connected to a blood circulation, the influx of immune cells and factors is missing and should be simulated. Therefore, there is a need for more sophisticated in vitro skin models to mimic defined aspects of the burn-induced immune response. Ultimately, the information from animal, patient and skin model studies can spark the design of therapeutic interventions that will improve recovery speed and reduce the side effects of a hyper-inflammatory response such as excessive scarring. Early safety and efficacy tests of promising therapeutic candidates can be performed using the in vitro skin models before progressing to burn patients.

AIM AND THESIS OUTLINE

To limit secondary complications and thereby improve patients' overall health and outcome, it is paramount to improve our understanding of the pathophysiological reactions to burn injury. The research aim of this thesis was to improve our understanding of the burn-induced immune response and to develop an in vitro skin model to study cellular reactions without the need for animal experimentation. This thesis is divided into four parts that describe the pursuit of this aim step by step.

In **Part 1**, the empirical evidence regarding burn-induced immune response in animal models is systematically reviewed. Two systematic reviews were performed that synthesize the available literature on the levels of immune cells (**Chapter 2**) and inflammatory factors (**Chapter 3**) after burn injury. Meta-analyses and subgroups analyses were performed to reveal time-depend effects and to identify factors of influence.

Part 2 of this thesis is focused on the immune response in burn patients. These data were generated using blood and post-operative burn tissue samples from patients. The systemic and local immune profile after burn injury in time was studied by analyzing immune cells and cytokines using flow cytometry and immunohistochemistry. For comparison, blood and skin from healthy subjects were used. **Chapter 4** describes the dynamics and phenotypic changes of immune cells and response levels of effector molecules in patient blood. In **Chapter 5** the effect of burn injury on immune cells and inflammatory mediators in burn wound tissue is displayed.

Part 3 of this thesis contains the experimental work with full skin equivalent models to simulate aspects of burn injury in vitro. **Chapter 6** shows the optimization and validation of our full skin equivalent model that can be used to study burn wound healing and the concomitant cytokine response. In **Chapter 7** the full skin equivalent model was supplemented with T cells or monocyte-derived macrophages to study their phenotype and reactions within this model of burn injury.

The findings in this thesis are put into a broader perspective in **Chapter 8**, **Chapter 9** and **Chapter 10**, which contain the General Discussion, English Summary and Dutch Summary (Nederlandse Samenvatting).

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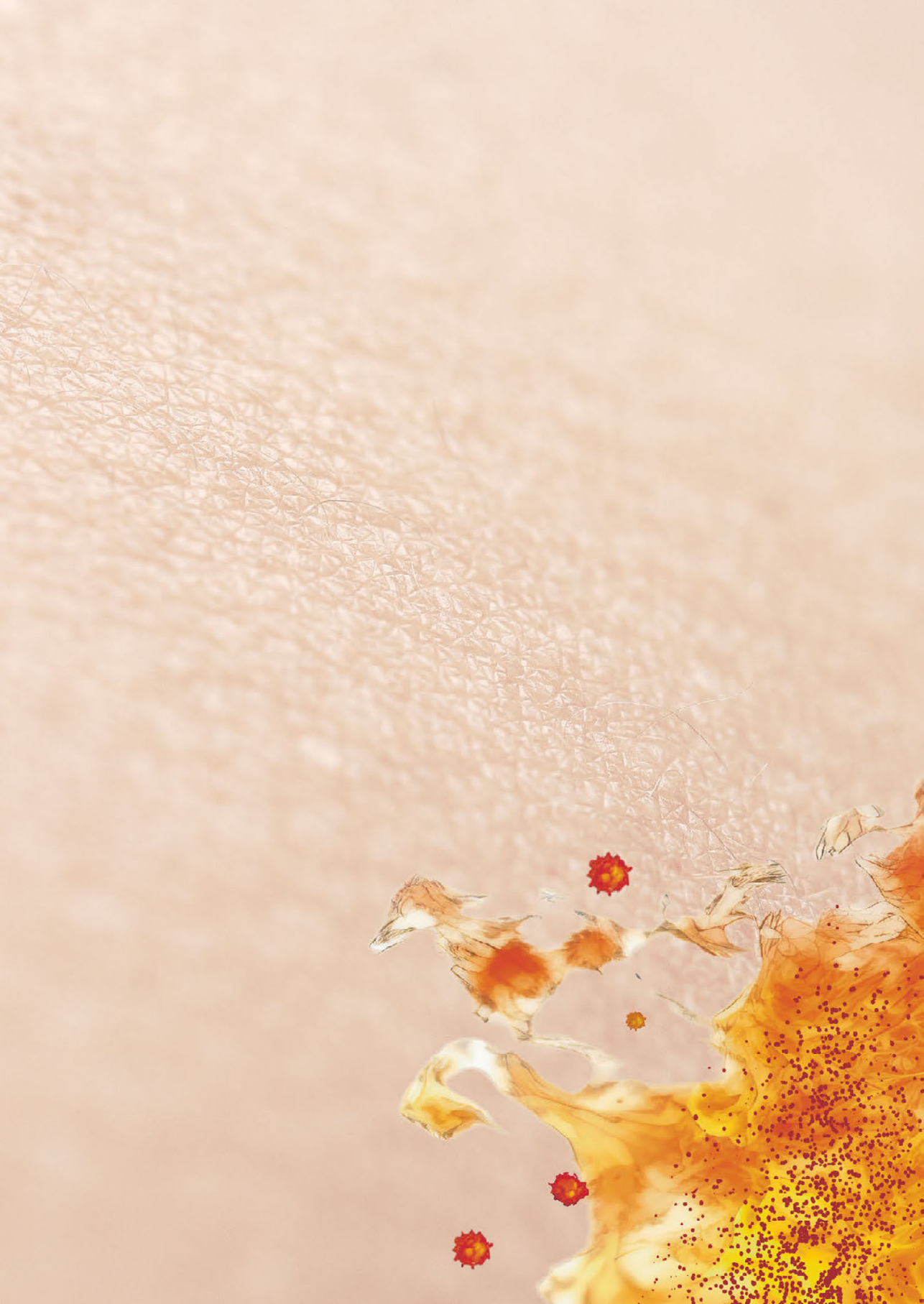
PART 1

Immune Response in Animal Burn Models



CHAPTER 2

Burn-Induced Local and Systemic Immune Response: Systematic Review and Meta-Analysis of Animal Studies

Published in Journal of Investigative Dermatology, **2022***, 142, 3093-3109*
DOI: 10.1016/j.jid.2022.05.004

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ABSTRACT

Because burn injuries are often followed by a derailed immune response and excessive inflammation, a thorough understanding of the occurring reactions is key to preventing secondary complications. This systematic review, which includes 247 animal studies, shows the post burn response of 14 different immune cell types involved in immediate and long-term effects in both wound tissue and circulation. Peripheral blood neutrophil and monocyte numbers increased directly after burns, whereas thrombocyte numbers increased near the end of the first week. However, lymphocyte numbers were decreased for at least 2 weeks. In burn wound tissue, neutrophil and macrophage numbers accumulated during the first 3 weeks. Burns also altered cellular functions because we found an increased migratory potential of leukocytes, impaired antibacterial activity of neutrophils, and enhanced inflammatory mediator production by macrophages. Neutrophil surges were positively associated with burn size and were highest in rats. Altogether, this comprehensive overview of the temporal immune cell dynamics shows that unlike normal wound healing, burn injury induces a long-lasting inflammatory response. It provides a fundamental research basis to improve experimental set-ups, burn care, and outcomes.

INTRODUCTION

Burn trauma often induces an overreaction of the immune system, known as systemic inflammatory response syndrome, which can cause damage to surrounding tissues and even distant organs [1,2]. Hyperactive inflammation and obstruction of wound healing can lead to excessive scarring [3] and psychological distress [4]. Information on the specific immune cells and inflammatory factors involved in the different phases of burn wound healing in humans is however scattered and incomplete.

Human studies are limited by the absence of baseline values, heterogeneity among cases, and restrictions in (the timing of) blood and wound sampling. Animal experiments, executed in controlled and standardized settings [5], could improve our understanding of the mechanisms underlying the burn-induced immune response in humans. Undoubtedly, various genomic and physiological processes of the human response to trauma differ from that of animals, such as signaling pathways, wound contraction, and scar formation [6–8]. Nevertheless, animal studies contain valuable information that will improve our understanding of the cellular immune response to burn trauma. In this study, we aimed to identify the immune cells involved in the local and systemic inflammatory response to burn injury in animal models. Ultimately, we anticipate that this review leads to new perspectives in burn care and will support the improvement of treatment for patients.

RESULTS

Study selection, characteristics, and quality

Our search generated 10,733 citations, of which 1,224 were considered relevant during title and abstract screening. From this selection, 111 studies were inaccessible, 247 were included in the systematic review (**Figure 1**), and 182 were used in meta-analyses (**Supplementary File 1**, **Supplementary File 2**). An overview of the study characteristics (**Figure 2A-G**) showed that most experiments were performed on young mice or rats. Full-thickness dorsal injury using hot water was the most common burn technique. It is worth noting that underreporting complicated the assessment of the overall study quality. Risk of bias (RoB) analysis showed that 33.5% of the included studies reported the use of randomization of animals before experimentation (**Figure 2H**). The majority of studies (94.0%) did not report the use of blinding, and a conflict-of-interest statement was present in 33.9% of the studies, in which four studies reported an actual conflict (**Figure 2I,J**). Overall, there was no significant indication of publication bias for the overall outcomes, but we did find a substantial risk of selection and performance bias.

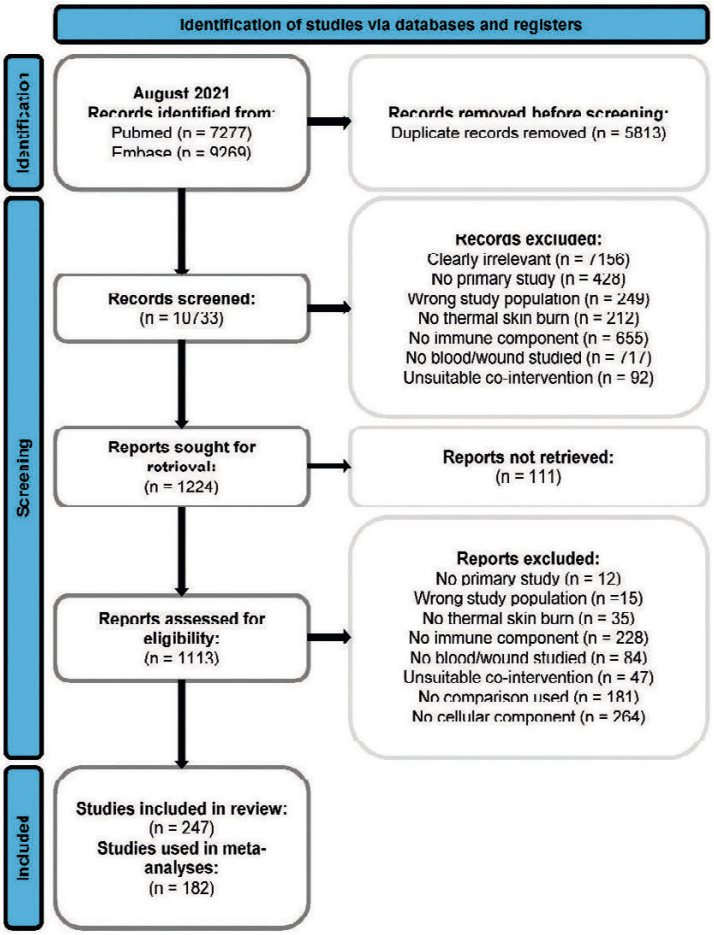


Figure 1. PRISMA flowchart of study identification, screening, and inclusion. Representation of the steps taken to select the relevant studies for the systematic review and meta-analyses [9]. PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

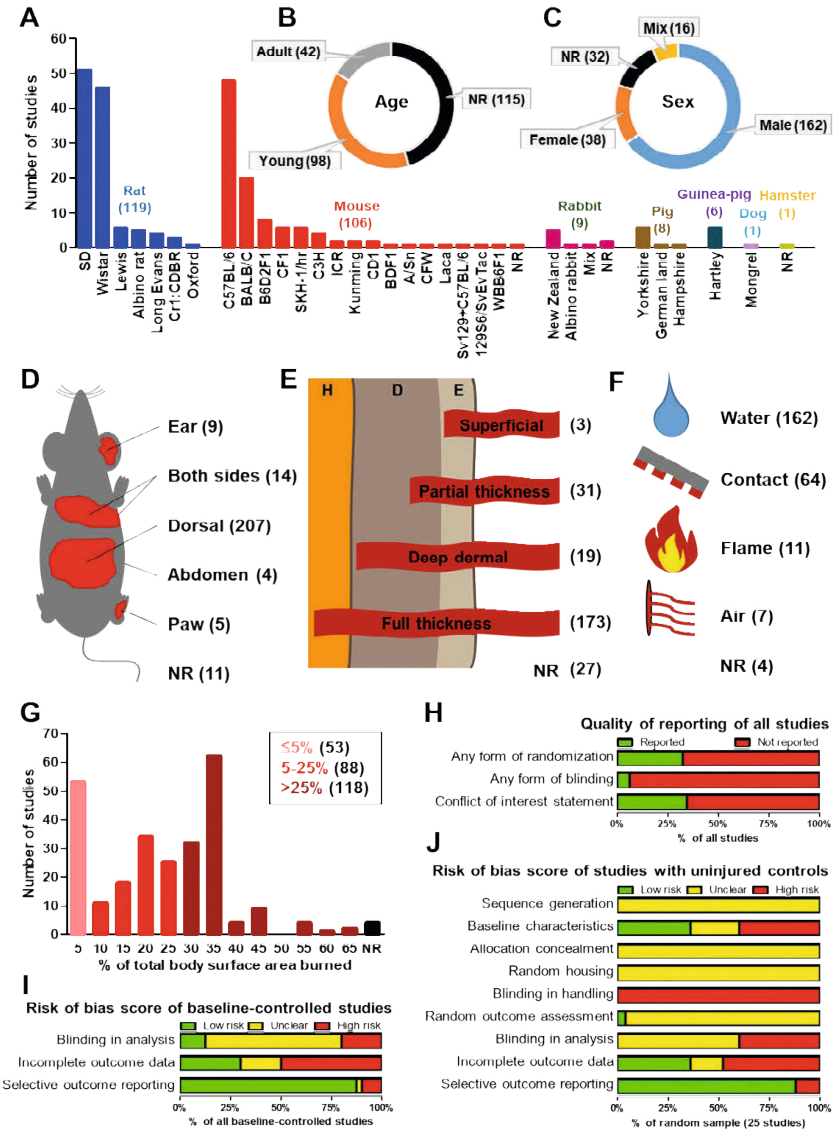


Figure 2. Characteristics of studies in systematic review and risk of bias assessment. Numbers indicate the number of studies. (**A**) Types of animal species and strains. (**B**) Age of study animals. (**C**) Sex of study animals. (**D**) Location of burn injury. (**E**) Depth of burn injury. (**F**) Type of burn agent. (**G**) TBSA that was burned as a percentage. (**H**) Quality of reporting of all included studies. (**I**) Risk of bias assessment of all baseline-controlled studies. (**J**) Complete risk of bias assessment of a random sample consisting of 25 of the included studies. D, dermis; E. Epidermis; H, hypodermis; NR, not reported; TBSA, total body surface area.

Burn-induced immune response is dominated by innate immune cells

Meta-analyses were performed on outcome measures for which at least five articles were available (**Supplementary Table 1**). Immune cell counts in blood or wound tissue from burn-injured animals were compared with immune cell counts in blood or skin from uninjured animals (baseline or control group). Overall, there was a significant increase in leukocytes in both peripheral blood and wound tissue (**Figure 3**). Systemically, the numbers of neutrophils and monocytes were significantly elevated, whereas lymphocyte numbers decreased. Total leukocyte counts were higher in baseline-controlled studies than in studies with separate uninjured controls. There was no significant change in overall eosinophil or thrombocyte counts. The higher standardized mean difference of neutrophils than of total leukocytes might be caused by the decrease in lymphocyte counts. Within the lymphocyte population, only B-cell counts were significantly decreased (**Figure 3B**).

In burn wound tissue, the numbers of neutrophils, macrophages, and mast cells were increased (**Figure 3C**). Cell migratory activity, mainly tested by adherence to endothelium or in vitro migration assays, was increased in total leukocytes but not in neutrophils (**Figure 3D**). Migratory activity of leukocytes was lower in baseline-controlled studies than in studies with separate uninjured controls. Antibacterial function of neutrophils was decreased after burn injury, whereas there was no significant effect on ROS production or inflammatory mediator secretion by neutrophils. The secretion of inflammatory mediators by macrophages was increased. There were not enough studies reporting total lymphocyte counts in wound tissue to be included in the meta-analysis.

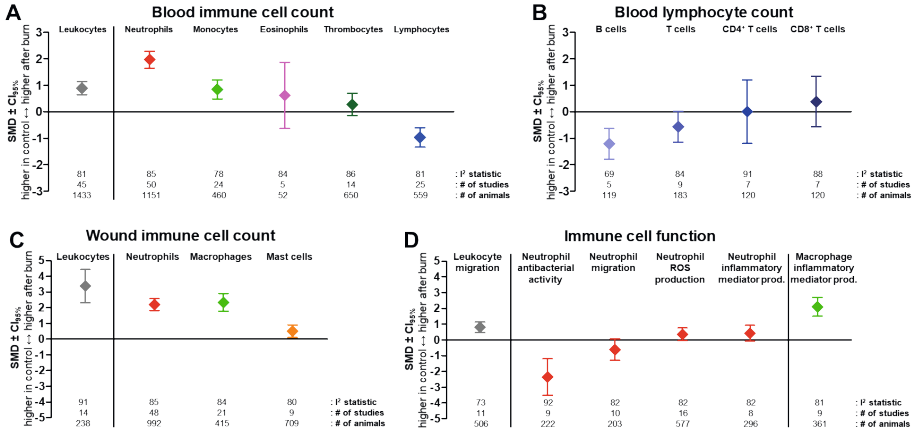


Figure 3. Overall outcome of immune cell counts and function after burn injury. Overall meta-analysis of(**A**) blood immune cell counts, (**B**) blood lymphocyte counts, (**C**) wound immune cell counts, and (**D**) immune cell functions. Results are shown as SMD of immune cell counts in the blood or wound tissue from burn-injured animals compared with immune cell counts in blood or skin from uninjured animals (baseline or control group) ± $CI_{95\%}$. The I^2 statistic, number of studies, and the total number of animals used in the burn group for each meta-analysis are shown below the graphs. $CI_{95\%}$, 95% confidence interval; SMD, standardized mean difference.

Blood innate response intensifies and is persistent

We performed longitudinal analysis on selected time intervals encompassing the four different biological phases of wound healing: hemostasis, inflammation, proliferation, and remodeling (**Figure 4A-G**). Meta-regression analyses were performed from post burn day (PBD) 0 until PBD 21 (**Figure 4H**). Blood leukocytes displayed a steady increase, with the highest counts from PBD 5 until PBD 28 (**Figure 4A**). Neutrophil counts were immediately increased during injury and remained elevated up to PBDs 15–21 (**Figure 4B**). Monocyte counts were increased from PBD 5 until PBD 14 (**Figure 4C**). Thrombocyte counts were decreased on PBDs 0–1 and later increased on PBDs 5–9 (**Figure 4D**). The decline of lymphocytes was most predominant directly after burn injury, whereas on PBDs 10–14, counts returned to control levels (**Figure 4E**). We detected a decrease in B-cell counts on PBDs 5–9 but found no significant differences in T-cell counts (**Figure 4F,G**). To further investigate the opposed dynamics of neutrophils and lymphocytes during burn injury, we calculated the neutrophil/lymphocyte ratio (NLR) for studies that reported both neutrophil and lymphocyte counts (**Supplementary Figure 1**). During the first 9 days, significantly higher NLRs were observed in burn-injured animals, which is an indication of systemic inflammatory response syndrome [10]. Overall, the temporal analysis revealed that whereas the increase in neutrophil counts was immediate, total

leukocyte, monocyte, and thrombocyte counts increased during the first week, whereas lymphocyte numbers decreased.

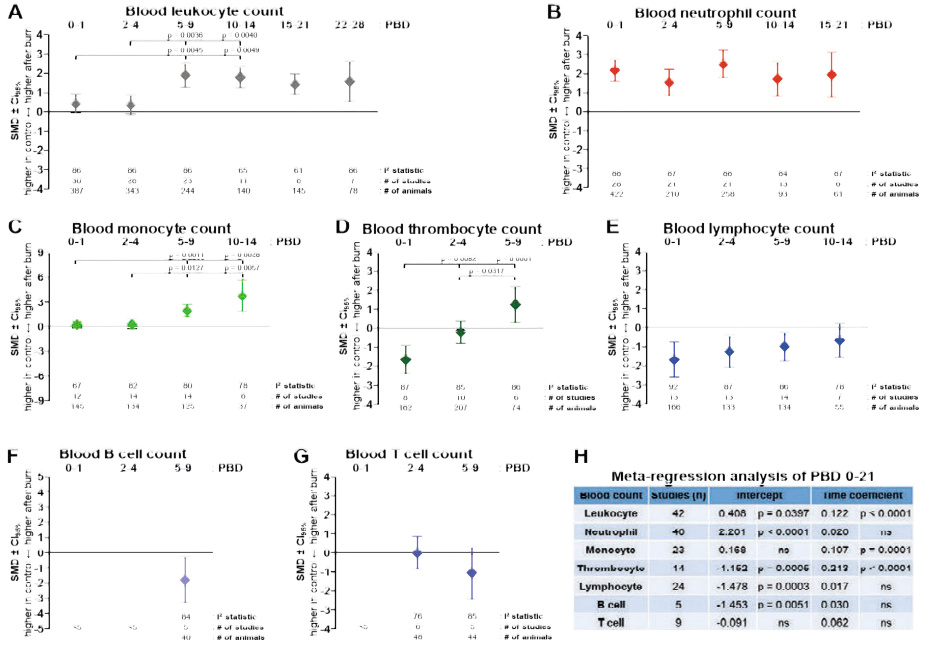
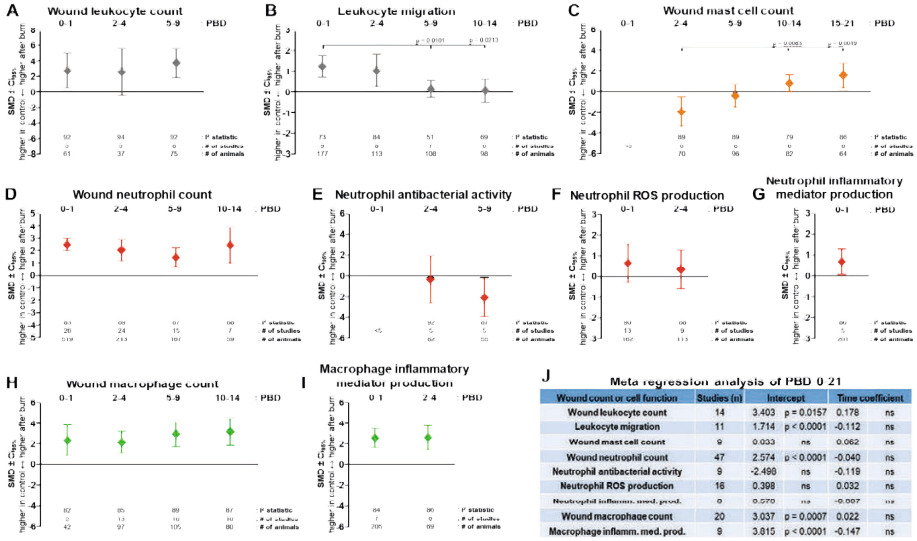


Figure 4. Longitudinal analyses of blood immune cell counts after burn injury. Longitudinal meta-analysis of blood cell counts: (**A**) leukocytes, (**B**) neutrophils, (**C**) monocytes, (**D**) thrombocytes, (**E**) lymphocytes, (**F**) B cells, and (**G**) T cells. (**H**) Meta-regression with immediate effect (intercept) and linear coefficient of time after burn (PBD 0 until PBD 21). Results are shown as SMD of immune cell counts in blood from burn-injured animals compared with immune cell counts in blood from uninjured animals (baseline or control group) ± $CI_{95\%}$. The I^2 statistic, number of studies, and the total number of animals used in the burn group for each interval are shown below the graphs. Bonferroni-corrected P-values of significant differences between time intervals are given in the graphs. $CI_{95\%}$, 95% confidence interval; PBD, post burn day; SMD, standardized mean difference.

Direct innate response in wound is accompanied by altered functions

Longitudinal analyses were performed on cell counts in wound tissue as well as on cell function (**Figure 5**) and revealed an instant increase in leukocyte migratory activity on PBDs 0–4 and an increase in wound leukocyte numbers on PBDs 0–1 and 5–9 (**Figure 5A,B**). Mast cell numbers showed a decrease around PBDs 2–4 and a subsequent increase from PBD 10 until PBD 21 (**Figure 5C**). On the other hand, neutrophil numbers increased instantly and remained elevated until at least PBD 14 (**Figure 5D**). Although the production of ROS by neutrophils was not significantly altered by burn injury, we did detect an increase in inflammatory mediator secretion by neutrophils on PBDs 0–1

and decreased neutrophil antibacterial activity on PBDs 5–9 (**Figure 5E-G**). Macrophage numbers increased immediately and remained elevated until PBD 14 (**Figure 5H**). Release of inflammatory mediators by macrophages was increased on PBDs 0–4 (**Figure 5I**). Altogether, the instant increase of innate immune cells in wound tissue persisted for at least 2 weeks, whereas certain functions were affected.



Wound count or cell function	Studies (n)	Intercept		Time coefficient
Wound leukocyte count	14	3.403	p = 0.0157	0.178	ns
Leukocyte migration	11	1.714	p < 0.0001	-0.112	ns
Wound mast cell count	9	0.033	ns	0.062	ns
Wound neutrophil count	47	2.574	p < 0.0001	-0.040	ns
Neutrophil antibacterial activity	9	-2.498	ns	-0.119	ns
Neutrophil ROS production	16	0.398	ns	0.032	ns
Neutrophil inflamm. med. prod.	6	0.570	ns	-0.007	ns
Wound macrophage count	20	3.037	p = 0.0007	0.022	ns
Macrophage inflamm. med. prod.	9	3.815	p < 0.0001	-0.147	ns

Figure 5. Longitudinal analyses of wound immune cell counts and cell function after burn injury. Longitudinal meta-analysis of (**A**) burn wound leukocyte counts, (**B**) leukocyte migration, (**C**) burn wound mast cell counts, (**D**) burn wound neutrophil counts, (**E**) neutrophil antibacterial activity, (**F**) neutrophil ROS production, (**G**) neutrophil inflammatory mediator production, (**H**)burn wound macrophage counts, and (**I**) macrophage inflammatory mediator production. (**J**) Meta-regression with the immediate effect (intercept) and linear coefficient of time after burn (PBD 0 until PBD 21). Results are shown as SMD of immune cell counts in wound tissue from burn-injured animals compared with immune cell counts in the skin from uninjured animals (baseline or control group) ± $CI_{95\%}$. The I^2 statistic, number of studies, and the total number of animals in the burn group for each interval are shown below the graphs. Bonferroni-corrected P-values of significant differences between intervals are given in the graphs. $CI_{95\%}$, 95% confidence interval; inflamm., inflammatory; med., mediator; NS, not significant; PBD, post burn day; prod., production; SMD, standardized mean difference.

Immune response depends on animal characteristics and burn technique

To investigate the differences between experimental models, subgroup analyses were performed (**Figure 6**). The highest blood leukocyte counts were found in rats or in adult animals. Sensitivity analyses confirmed that the interspecies effect was still present when only young animals were compared and that the difference from aging remained when only rats were analyzed. Neutrophil counts were higher in studies using >25%

total body surface area (TBSA) than in those using 5–25% TBSA and were highest in rats. Sensitivity analysis showed that the effect of TBSA was present in mice but not in rats. Surprisingly, neutrophil wound counts in studies using 5–25% TBSA were lower than in those using ≤5% TBSA, in both mice and rats. Blood neutrophil counts were higher in males than in females. Interestingly, both wound leukocyte and neutrophil counts were lower in scalds than in metal burns. Within TBSA groups, the difference in neutrophil counts between species was still present in wound tissue but not in blood, indicating that collinearity could play a role. The difference between sexes for blood counts and the effect of metal burns on wound neutrophil counts were not influenced by TBSA or species. Because the majority of the studies used full-thickness burns, subgroup analysis on wound depth could only be performed for wound neutrophil counts. Overall, the leukocyte response was affected by type of species, animal age, and burn agent, whereas the neutrophil counts depended on species, sex, wound size, and burn agent.

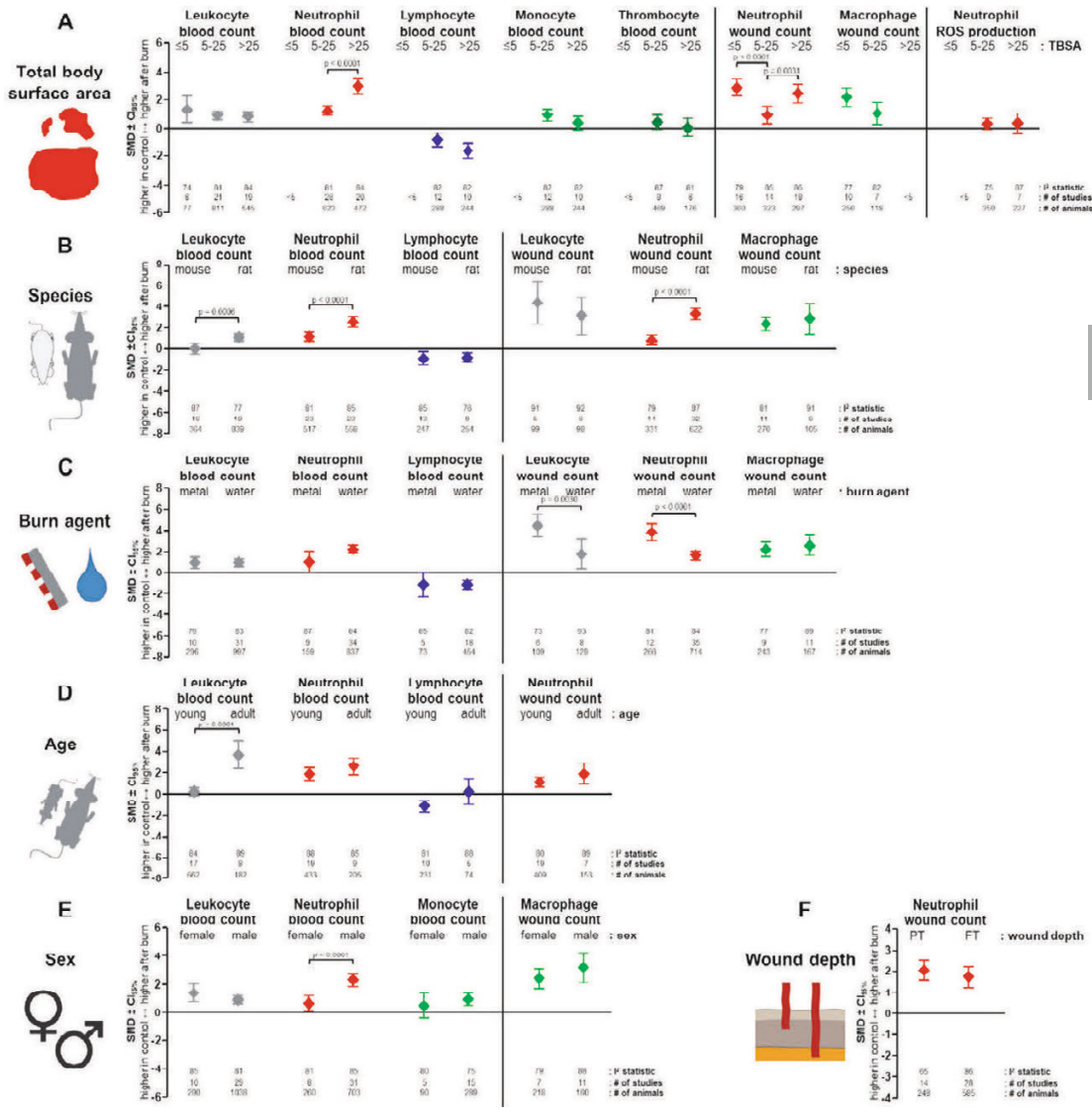


Figure 6. Subgroup analysis of immune cell counts after burn injury. Subgroup analysis of (**A**) burned TBSA, (**B**) species, (**C**) burn agent, (**D**) age, (**E**) sex, and (**F**) wound depth. Only subgroups for which at least five articles were available were used in the analysis. Results are shown as SMD of immune cell counts in blood or wound tissue from burn-injured animals compared with immune cell counts in blood or skin from uninjured animals (baseline or control group) ± $CI_{95\%}$. The I^2 statistic, number of studies, and the total number of animals in the burn group for each subgroup are shown below the graphs. Bonferroni-corrected P-values of significant differences between subgroups are given in the graphs. $CI_{95\%}$, 95% confidence interval; FT, full-thickness; PT, partial-thickness; SMD, standardized mean difference; TBSA, total body surface area.

DISCUSSION

An improved understanding of the burn-induced immune response is necessary to prevent secondary pathologies in patients with burns as much as possible. In this study, we synthesized available literature on the post burn immune response in animals into a comprehensive systematic overview. Even though there was great heterogeneity and variation among the studies, the meta-analyses clearly displayed the dynamics of innate and adaptive immune cells after burn injury. In peripheral blood, the numbers of neutrophils, monocytes, and thrombocytes increased shortly or within 1 week after burn injury and remained increased over the first month. In contrast, lymphocyte numbers were reduced during the first 2 weeks, indicating that the response is driven by the innate arm of the immune system and that resolution of inflammation is delayed. In wound tissue, we observed an immediate surge of neutrophils and macrophages during the first 2 weeks, whereas for mast cells, a time-dependent response was observed because numbers decreased near the end of the first week and steadily increased from PBD 10 onward. Although several studies investigated the specific subsets of lymphocytes in wound tissue, there were not enough data available on total lymphocyte counts. Furthermore, burn injury affected cell function because we showed that migration of leukocytes and inflammatory mediator production by neutrophils and macrophages were increased earlier on and that antibacterial activity of neutrophils was reduced on PBDs 5–9.

In general, wound healing entails four biological phases, namely hemostasis, inflammation, proliferation, and remodeling. The immediate increase in thrombocyte and neutrophil numbers during the inflammation phase is attenuated within the first week [8,11,12]. Macrophage numbers, which are important for the transition from inflammation to proliferation [13], normalize later on, whereas lymphocyte numbers increase from the second week onward [14]. In this study, we show that at least in animals, these processes are derailed and that high numbers of circulatory thrombocytes, neutrophils, and monocytes are persistent, whereas lymphocyte numbers are actually reduced. This suggests that the timing in typical schematic depictions of the cellular immune response during wound healing does not hold true for burn injury. Unlike in humans, B-cell counts in uninjured rodents are higher than their T-cell counts[15], which could explain the larger effect of burn injury on B cells than on T cells that we found in animals. A relative increase in innate immune cells and a decrease in lymphocytes have also been detected in patients with burns [16,17]. Danger-associated molecular patterns that are released by wounded tissues are suggested to cause a continuous activation of the immune system [18,19]. In turn, a hyperactive immune system can cause damage to

surrounding tissues, thereby producing additional danger-associated molecular patterns and cytokines that uphold the inflammation.

The time-dependent response of thrombocytes is similar to the early thrombocyte response in burn patients [20]. The typical early trauma-induced leukopenia in patients with burn wounds that is caused by exsanguination, resuscitation, and emigration of immune cells from the blood circulation was in our meta-analysis only visible when the early time points were analyzed per day. Leukopenia is naturally restored by the bone marrow [21,22]. During acute inflammation, predominantly, neutrophils and monocytes are replenished by the bone marrow, which can lead to reduced lymphopoiesis and overrepresentation of innate immune cells in the circulation [23]. Moreover, the NLR, a marker for systemic inflammatory response syndrome in humans, was in animals also highly increased during the first 9 days after burns. In patients with burns, persistent leukocytosis in combination with lymphopenia is associated with persistent inflammation, arrested wound healing, increased susceptibility to opportunistic infection, and increased mortality [2,24,25]. Because the thrombocyte count and NLR correspond with systemic inflammatory response syndrome and septic events, they are of prognostic and diagnostic value [10,26].

In wound tissue of animals, increased levels of neutrophils, macrophages, and mast cells were detected until at least PBD 14. The transition of macrophages from an M1 phenotype toward an M2 phenotype is essential to facilitate proper wound healing [27,28]. Although monocyte or macrophage subtypes could not be investigated, we found that total wound macrophage numbers were increased and that the production of inflammatory mediators by macrophages was enhanced. The activity of neutrophils is altered after severe trauma in animals [29–32], but it remains unclear whether trauma, in general, enhances or weakens neutrophil activity (**Figure 5**). Presumably, the emergency release of neutrophils into the circulation is responsible for reduced chemotactic activity owing to the inflexibility of the banded nucleus of immature neutrophils [33], whereas rapid activation can lead to impaired antibacterial activity [31]. On the other hand, the immaturity of neutrophils could amplify the granule content and increase the release of inflammatory factors [34,35]. Mast cells have also been proposed to play an active role during wound healing in both animals and humans. They might enhance inflammation and vascular permeability through the secretion of histamines early after injury and stimulate re-epithelization and angiogenesis later on by the release of GFs [36,37]. This coincides with increased numbers of mast cells on PBDs 0–1 and on PBDs 15–21.

Only a minority of studies used porcine or canine models, and therefore it was unfeasible to study the differences between species other than mice and rats. Although pigs come

close to the human condition in terms of similar skin characteristics and physiology, porcine models are less attractive because of ethical concerns, higher expenses, and advanced operating requirements [38]. Subgroup analyses revealed that blood leukocyte and neutrophil counts were more abundant in rats than in mice. Because rats are larger animals, require a longer healing time, and are immunologically more similar to humans than mice [39], they might exhibit a more severe immune response than mice. In addition, murine studies generally analyzed the effects shortly after burn injury, thereby causing an overrepresentation of early sampling times. The severity of leukocytosis seemed to increase with animal age and may be explained by the fact that a young, underdeveloped immune system is supposedly tolerant and becomes gradually more active during maturity [40]. Interestingly, neutrophil responses appeared to depend on burn size and agent. The relationships between the burn size and inflammatory response in humans have been proposed before by others [35,41,42]. Metal burns induced a greater total leukocyte and neutrophil response in wound tissue than scalds. Water, mostly used at 100 °C, loses heat more rapidly and might therefore cause a less severe injury than metal. It was hardly possible to explore the differences related to wound depth because the majority of studies applied a full-thickness burn wound. Although most studies reported full-thickness injuries, only a limited number of studies actually investigated the wound depth. In addition, wound depth is more prone to subjectivity and depends on many factors such as skin thickness, burn temperature, and duration. Therefore, wound depth was a less useful parameter in these studies.

Numerous studies failed to adhere to the Animal Research: Reporting of In Vivo Experiments guidelines [43] and did not provide important experimental details or information on the number of animals or SDs, which are crucial to performing meta-analyses. The inability to apply blinding might have influenced the data acquisition, and owing to the poor reporting of studies, the general RoB was largely unclear. The improper design, conduct, and reporting in many animal studies have already been described in recent reviews [44–46], and future research will surely benefit from more standardized design and reporting [47]. Researchers have shown that resuscitation and pain treatment can influence immune reactions after thermal injury [48,49]. Owing to large variation in the type of anesthetic, resuscitation procedure, and pain management, specific effects on the immune response could not be investigated. Likewise, subgroup analysis of the different methods used to identify cell types was not possible. The overall cell counts showed substantial heterogeneity (I^2 = 68–92), which can be expected for animal studies [50]. In a few subgroup analyses, a trivial reduction of the I^2 statistic could be detected.

Although animal studies provide valuable insight into the post burn immune response and wound repair, appropriate translation of these findings to the human situation remains

crucial to predicting and treating consequential complications effectively. There are several considerable (physiological) differences that make it difficult to convert treatment opportunities directly to patients. Rodents, unlike humans, have more lymphocytes than innate cells, and receptor binding and cytokine responses differ owing to evolution and distinct history of microbial exposure [51,52]. In addition, there are important genomic and evolutionary differences that cause mouse models to poorly reflect certain aspects of human disease [7]. Furthermore, the ultrahygienic environment of laboratory animals makes the immune system, in general, less tolerant [52,53]. Still, important aspects of the burn-induced human immune response were also present in our meta-analyses, exemplified by the response of thrombocytes, neutrophils, and monocytes [16,17].

Altogether, this review of the burn-induced immune response in animals using meta-analyses puts in perspective the uncontrolled, hyperactive response of immune cells that persists for weeks after burn trauma. Although numerous physiological processes are distinct, many aspects of the human immune response to burns were found in our meta-analyses, including the innate and lymphocyte response and the dynamics of mast cells and thrombocytes. We anticipate that this knowledge will guide the design of future experimental models while supporting the reduction, refinement, and replacement of animal experimentation. It will lead, to our knowledge, to previously unreported insights in clinical research on burn trauma that can ultimately improve burn care and outcome.

MATERIALS AND METHODS

Study protocol and eligibility criteria

A review protocol was established beforehand and is registered at the International Prospective Register of Systematic Reviews (CRD42019136270; http://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=136270). We amended this protocol once to further specify the meta-analyses. The 10-article requirement was changed to five to enable the inclusion of additional cell types.

Search strategy

The search was performed using PubMed and Embase [54] (**Supplementary File 1**), with a final update on August 6, 2021. Briefly, we searched for articles with primary data on the immune response in animals with burn injury (search components: burn wound, immune response, and animal). No language or publication date restrictions were applied. Search results were combined, and duplicates were removed using EndNote software (X9, Clarivate Analytics, London, United Kingdom).

Study selection

Studies were selected independently by PPGM and BKHLB using Rayyan software (Rayyan Systems Inc. [55]) in three phases: title screening, abstract screening and full text screening. In the title screening, clearly irrelevant articles (not about burn injury) were excluded. During the abstract screening, studies involving animal skin burns that contained primary data were selected and reviews, posters and conference abstracts were excluded. In the full text screening, we selected articles involving animal thermal burns with outcome measures related to immune cells, and without co-interventions that interfere with the function of the immune system, such as infection or anti-inflammatory medication. Also, the presence of an appropriate control group (either healthy animals, baseline measures or sham controls) was verified. Discrepancies between the two reviewers were carefully checked and in case of doubt, references were included. Inaccessible articles were noted (**Supplementary File 2**) and excluded from the review.

Study characteristics

Independently, PPGM and BKHLB extracted the study characteristics (animal species and strain, age, sex, weight, burn size, burn time, burn agent, burn temperature, burn depth, anatomical location, type of control, cell type, detection method), each from half of the included studies. A random sample of 10% of the extracted data was checked by the other reviewer.

Study quality and RoB assessment

The reporting of any form of randomization or blinding and the presence of a conflict-of-interest statement was scored for all included studies by PPGM and BKHLB who both assessed half of the studies and checked at least 10% of the other reviewer. Full RoB assessment was conducted using SYRCLE's tool [45] on 25 randomly selected studies (random number generator in Excel). We evaluated the reporting of the following baseline characteristics: animal sex, age or weight (reporting of a range of < 10% was considered as low risk of bias). To check the completeness of outcome reporting, we evaluated the number of animals in the method and the results section for each experiment and outcome. The RoB was evaluated independently by PPGM and BKHLB. In the case of discrepancies, a third reviewer was consulted. This assessment provided an indication of the RoB of all included studies. Because only items 7, 8 and 9 from the RoB tool apply to baseline controlled studies, we evaluated those studies separately.

Outcome data extraction

All quantitative outcome measures related to immune cells, such as immune cell counts and cell function were collected in a database, which is available upon request. PPGM and BKHLB independently extracted the outcome measures (mean outcome and standard

deviation, unit of measurement, number of animals), each from half of the included studies and checked at least 10% of the other reviewer. The following outcome measures in either blood or wound tissue were included: immune cell counts, immune cell migration assays, antibacterial activity, production of inflammatory mediators or reactive oxygen species by specific cell types and apoptosis. Data from graphs was extracted using the digital ruler feature in ImageJ (version 1.53j; NIH [56]). In case of missing data, such as the number of animals or standard deviation, we contacted corresponding authors via email and ResearchGate (including a reminder after two weeks) (response rate 17%). Data presented as standard error of mean (SEM) were transformed to standard deviation (SD) with the following formula: $SD = SEM * \sqrt{number\ of\ animals}$.

Synthesis of results and meta-analysis

Meta-analyses were only performed on outcome measures of at least five studies. Data were analyzed using Comprehensive Meta-Analysis (version 3; Biostat, Englewood, NJ), and the effect sizes were expressed as standardized mean difference of immune cell counts in blood or wound tissue from burn-injured animals compared with counts in blood or skin from uninjured animals (baseline or uninjured control) with 95% confidence interval. A random-effects model was used in the analyses, and I^2 statistic was used as a measure for statistical heterogeneity. Cell types that were considered the same entity were pooled (**Supplementary Table 1**). Possible publication bias was explored using Duval and Tweedie's trim and fill methodology (**Supplementary File 2**). NLRs were calculated using absolute data from studies that measured both blood neutrophil and lymphocyte counts.

Subgroup analysis

Predefined subgroup analyses were performed on: time post burn (divided into categories 0-1, 2-4, 5-9, 10-14, 15-21, 22-28 or >29 days), burned total body surface area (TBSA, ≤5%, 5%-25% or >25%), wound depth (superficial, partial-thickness, deep dermal or full-thickness), burn agent (flame, water or metal), animal species (mouse, rat or pig), sex and age (young or adult). In the case of repeated measures within a time interval, the maximum effect size per time interval was chosen. When required, TBSA was calculated using the reported area of the burn, weight (W) of the animals and Meeh-Rubner's formula () (Gouma et al. 2012). The following K values were used: 9 (mouse), 9,83 (rat), 12 (rabbit), 10,5 (guinea pig), 10,1 (dog) and 10 (pig). When TBSA was missing in the articles, it was estimated based on the reported age and weight information available at Animal Resources Centre (https://www.arc.wa.gov.au/), The Jackson Laboratory (https://www.jax.org/), and Roysfarm (https://www.roysfarm.com/). Using the weight of animal, the animal age was estimated when this was not reported. Animal age subgroups, young or adult, was based on social maturity of animals: adults were >3 months (mouse), >6

months (rat), >6 months (pig), >12 weeks (hamster), >12 months (rabbit), >6 months (Guinea-pig), >1 year (dog) of age. For wound depth the following categories were used: superficial ("first degree"), partial-thickness ("second degree"), deep dermal ("deep second degree") and full-thickness ("third degree", "fourth degree", "severe burn injury"). P values were based on the $CI_{95\%}$ of the difference between subgroups. For both longitudinal and subgroup analyses, Bonferroni correction was applied, i.e., the p values were multiplied by the number of comparisons within each subgroup analysis. Differences between baseline controlled studies and studies that used a separate control group were assessed.

Meta-regression
Meta-regression analyses were performed posthoc on the standardized mean difference of cell counts and cell function using time after burn injury as a continuous variable, including PBD 0 until PBD 21 (**Supplementary File 2**). Random effects–restricted maximum likelihood model was used, and repeated measures (same animal, multiple sampling times) of studies were included.

Studies included in meta-analysis
[29,48,57–236]

Studies included in systematic review
[29,48,57–302]

Baseline-controlled studies that were used for risk of bias assessment
[48,65,71,73,79,80,85,86,98,113,114,134,146,157,158,160,180,187–189,190,201,210,220,224,227,241,247–249, 254,255,260–262,264,272,280,295,303]

Studies with uninjured controls that were used for risk of bias assessment
[61,63,66,67,74,92,103,104,109,122,131,133,136,149,175,177,186,204,205,236,239,251,252,257,304]

ACKNOWLEDGMENTS

We want to thank Alice Tillema of the Radboud University Medical Center Library for helping to design the search strategy; Carlijn Hooijmans of SYstematic Review Centre for Laboratory animal Experimentation for her assistance with the data analysis; and Anouk Elgersma, Rosa Rentenaar, and Myrthe Witbaard of the Association of Dutch Burn Centres for their assistance during data extraction. This research was funded by the ZonMw More

program Knowledge with Less Animals under grant number 114024139 (PPGM) and by the Dutch Burn Foundation under grant number WO/17.108 (BKHLB).

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SUPPLEMENTARY MATERIAL

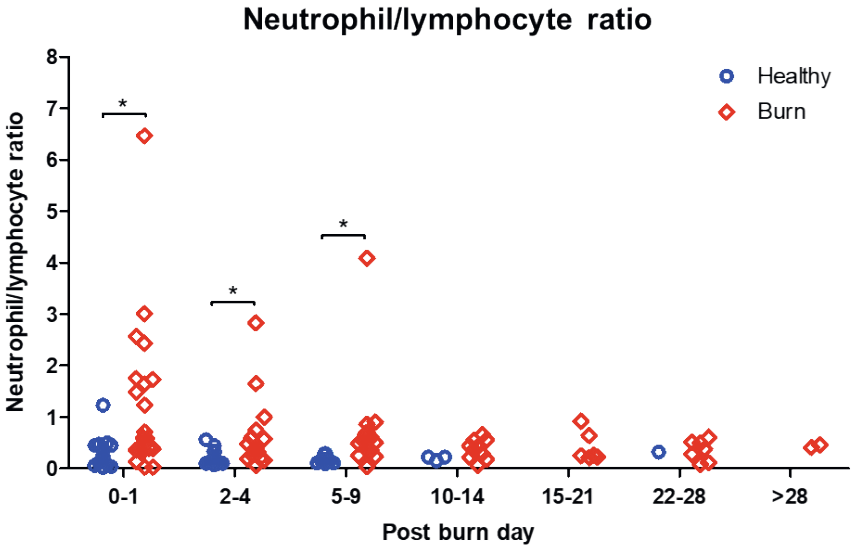
Supplementary material is linked to the online version of the article at: https://www.jidonline.org/article/S0022-202X(22)00397-9/fulltext.

Supplementary Table 1. Outcome measures and references used in systematic review and meta-analysis. A minimum of 5 articles was required for inclusion of a defined outcome measure in the meta-analysis. For cell function apoptosis, no cell type reached this minimum.

Cell type	Outcome data in meta-analysis (number of studies)	References in systematic review
Neutrophils (granulocytes, polymorphonuclear cells)	• Blood immune cell count (50) • Wound immune cell count (48) • Migration (10) • Antibacterial function (9) • ROS production (16) • Inflammatory mediator production (8)	29, 48, 76, 218–222, 227, 229–231, 235, 77, 259, 281, 305, 78–81, 83, 85–87, 57, 88, 89, 91, 93, 95, 96, 98–101, 60, 102, 107, 108, 110, 112, 114–118, 61, 121, 123, 126, 128, 129, 131–135, 62, 136, 138–140, 142, 144, 145, 147, 148, 150, 63, 151, 155–158, 161–163, 165, 166, 67, 170–177, 181, 182, 70, 183, 184, 186, 191–193, 196, 198–200, 73, 203, 204, 206, 207, 210–213, 215, 217
Leukocytes (white blood cells, inflammatory cells)	• Blood immune cell count (45) • Wound immune cell count (14) • Migration (11)	59, 60, 75, 76, 78, 89, 91, 92, 95, 100, 101, 104, 61, 106, 107, 111, 113, 118, 140, 141, 143, 151, 154, 62, 156–158, 160, 162–164, 168, 169, 178, 63, 179–181, 186–190, 192, 194, 65, 205, 208, 214, 216, 219, 223, 224, 227, 228, 232, 69, 233, 258, 305, 306, 71, 72, 74
Lymphocytes	• Blood immune cell count (25)	48, 60, 106, 118, 128, 142–144, 151–153, 157, 61, 158, 163, 170, 181, 184, 186, 195, 209, 219, 262, 62, 266, 286, 288, 290, 67, 69, 75, 76, 91, 95]
Monocytes	• Blood immune cell count (24)	48, 58, 111, 118, 128, 140, 142, 144, 151, 156, 157, 163, 60, 181, 186, 217, 230, 236, 243, 61, 64, 67, 69, 76, 89, 95
Macrophages (monocytes in wound tissue)	• Wound immune cell count (21) • Inflammatory mediator production (9)	61, 73, 109, 120, 124, 125, 127, 130, 159, 165, 202, 208, 75, 210, 219–221, 225, 229, 231, 233, 234, 262, 79, 274, 281, 282, 296, 307, 84, 94, 97, 98, 103, 108
Thrombocytes (platelets)	• Blood immune cell count (14)	59, 67, 219, 223, 227, 230, 273, 74, 92, 101, 121, 157, 158, 181, 201
Mast cells	• Wound immune cell count (9)	66, 105, 109, 119, 167, 208, 215, 225, 226

Supplementary Table 1. Continued.

Cell type	Outcome data in meta-analysis (number of studies)	References in systematic review
T cells (T lymphocytes)	• Blood immune cell count (9)	58, 96, 170, 195, 197, 209, 236, 270, 276, 278, 279, 281, 106, 292, 294, 295, 111, 118, 129, 152, 153, 157, 159
CD4$^+$ T cells	• Blood immune cell count (7)	106, 111, 129, 153, 157, 170, 236
CD8$^+$ T cells	• Blood immune cell count (7)	106, 111, 129, 153, 157, 170, 236
B cells	• Blood immune cell count (5)	58, 111, 197, 209, 236
Eosinophils	• Blood immune cell count (5)	60, 108, 128, 140, 219, 228, 244, 253, 287
Basophils		128]
Undefined cells		84, 98, 159, 208, 241, 253, 261, 272
Phagocytes (neutrophils + monocytes)		67, 84, 195, 249
Dendritic cells		225, 253, 258
NK cells		111, 253
Langerhans cells		225, 247, 249
NKT cells		253
PBMNCs (monocytes + lymphocytes)		58, 84, 155, 236, 255, 271, 284



Supplementary Figure 1. **Neutrophil/lymphocyte ratio.** For studies that measured both neutrophil and lymphocyte numbers, the neutrophil/lymphocyte ratio was calculated for animals with burn (red) and for control animals (blue). Statistical differences between animals with burn and their control are indicated by black asterisks (Wilcoxon signed rank test: $p < 0.05$).

CHAPTER 3

Kinetics of Inflammatory Mediators in the Immune Response to Burns: Systematic Review and Meta-Analysis of Animal Studies

In Press at Journal of Investigative Dermatology, **2023**
DOI: 10.1016/j.jid.2023.09.269

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ABSTRACT

Burns are often accompanied by a dysfunctional immune response, which can lead to systemic inflammation, shock and excessive scarring. Because detailed information on the underlying immune reactions is scattered, we systematically reviewed animal experimental data for all reported inflammatory mediators. Meta-analyses of 352 studies revealed a strong increase in cytokines, chemokines and growth factors, particularly 19 mediators in blood and 12 in burn tissue. Temporal kinetics showed long-lasting surges of pro-inflammatory cytokines in blood and burn tissue. Significant time-dependent effects were seen for IL-1β, IL-6, TGF-β1 and CCL2. The response of anti-inflammatory mediators was limited. Burn technique had a profound impact on systemic response levels. Large burn size and scalds further increased systemic, but not local inflammation. Animal characteristics greatly impacted inflammation, e.g., IL-1β, IL-6 and TNF-α levels were highest in young, male rats. Collectively, this review provides guidance for experimental set-ups to advance burn research and exposes inflammatory pathways that could be targeted through immunotherapy for burn patients.

INTRODUCTION

Burn injury is among the most challenging types of trauma in clinical practice, as it often causes life-threatening complications that include systemic inflammation, hypermetabolism and excessive scarring [1–4]. Overstimulation of the immune system can lead to a persistent surge of non-specific, innate immune cells that escalate inflammation and, paradoxically, can increase susceptibility to infection and reduce vaccine response [5,6]. The burn-induced immune response is characterized by a local and a systemic increase in inflammatory mediators, neutrophils, monocyte/macrophages and shifts in lymphocyte subsets [7–9]. The inflammatory mediators are important coordinators of cellular traffic and direct the immune response [10]. Patient's recovery and overall outcome might be improved by immune modulation to limit tissue damage caused by a derailed and overactive immune system [11]. Detailed information on the involvement of specific cytokines, chemokines and growth factors during the post-burn immune response is warranted to re-balance profound immune dysregulation in patients.

The absence of baseline values, variation among injuries and restrictions in the collection of blood and wound samples seriously hamper human burn research. Experimental animal models, which are more standardized and controllable, are an appealing alternative approach to study the underlying mechanisms of the burn-induced immune response [12]. However, our society strives to reduce, replace and refine animal experimentation because of ethical concerns and important genetic and physiological differences [13–15]. Systemic reviewing is a valuable method to synthesize an overview of empirical evidence from separate investigations, while contributing to reduction and refinement of animal experimentation and providing insights that might be relevant for clinical practice [16,17].

Previously, the burn-induced response of 14 different immune cell types in blood and wound tissue from experimental animals were analyzed in meta-analyses [8]. This review demonstrated a persistent presence of mainly neutrophils, monocytes and macrophages with altered functions, such as enhanced inflammatory mediator production. Immune cells produce inflammatory mediators to influence the intensity and direction of the immune response. In this systematic review, we generated an overview of the cytokines, chemokines and growth factors studied in experimental animal burn models. Overall study quality was assessed, and meta-analyses were performed to reveal the effect of burn injury on the level of inflammatory mediators present in blood and wound tissue. Furthermore, the temporal dynamics and role of wound severity, type of burn agent and differences in study models are demonstrated. This overview provides an excellent

research basis that is useful to refine experimental set-ups and design (targeted) interventions to improve treatment of burn patients.

RESULTS

Study selection and study characteristics

The study protocol was published on PROSPERO and was amended once to further specify the meta-analyses. Our search generated 11,375 records, of which 1,387 were considered relevant during title and abstract screening (**Figure 1**). From this selection, the full-text versions of 115 studies were inaccessible and a total of 424 studies were included in the systematic review from which 352 were used for meta-analyses (**Supplementary Table** 1 and **Supplementary Table 2**). **Supplementary Figure 1** displays an overview of the characteristics of the included studies. The vast majority of studies used rats or mice, mostly young and male. Most studies produced a full-thickness injury using hot water or contact with hot objects on the dorsal area. Size of burn injuries varied from 80 studies with ≤5%, 132 with 5-25% and 189 with >25% of total body surface area (TBSA).

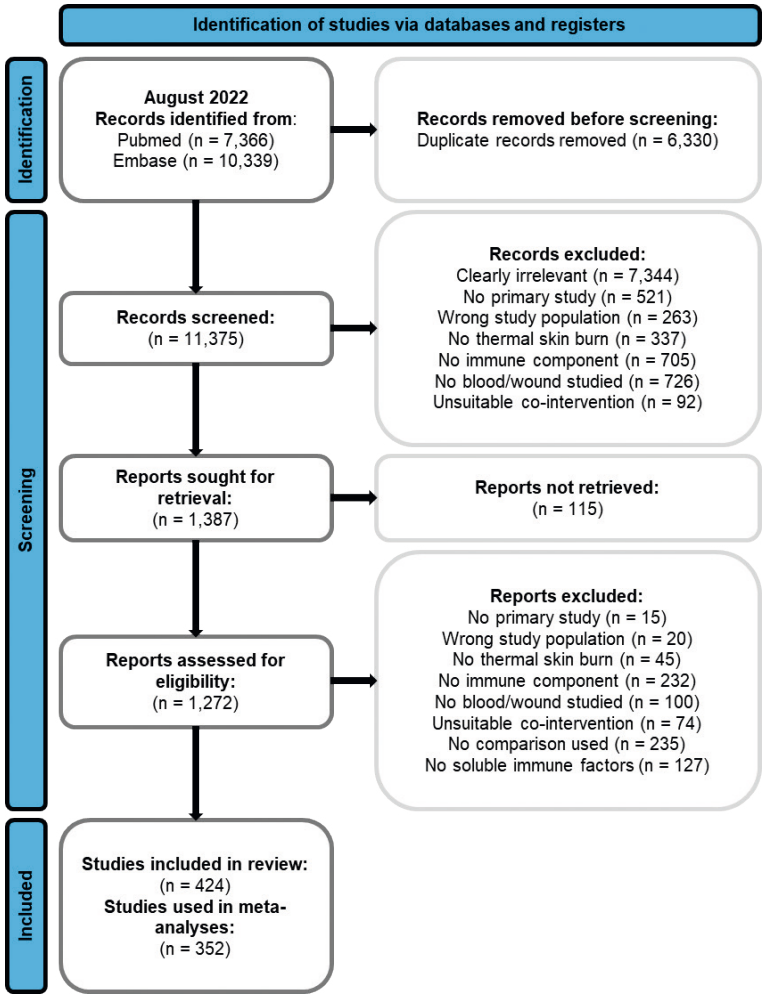

Figure 1. PRISMA flowchart showing study identification, screening and inclusion. Representation of the steps to select relevant studies for the systematic review and meta-analyses [18].

Study quality and risk of bias assessment

Underreporting in studies complicated the assessment of overall study quality drastically. Only 43.7% of the studies in this systematic review reported the use of animal randomization prior to burn induction (**Figure 2A**). The vast majority of studies (98.1%) did not report the use of any form of blinding during experimentation or analysis. Moreover, a conflict-of-interest statement was present in only 45.1% of the studies. In 25 randomly selected studies that used uninjured controls the risk of bias (RoB) was unclear for at least 4 of the 10 scored items owing to underreporting (**Figure 2B**). A considerably

high RoB was found for differences between animal groups at baseline, mainly due to large ranges in the reported weight or age. Obviously, blinding in animal handling is impossible in studies of burn injury and led to a high RoB in all of the studies. In 36% of the studies there was suspicion of incomplete outcome data (attrition bias) and 12% of the studies scored a high RoB for selective reporting of outcome data. Separately, we scored baseline-controlled studies and found an even higher risk of attrition bias in these studies (**Figure 2C**).

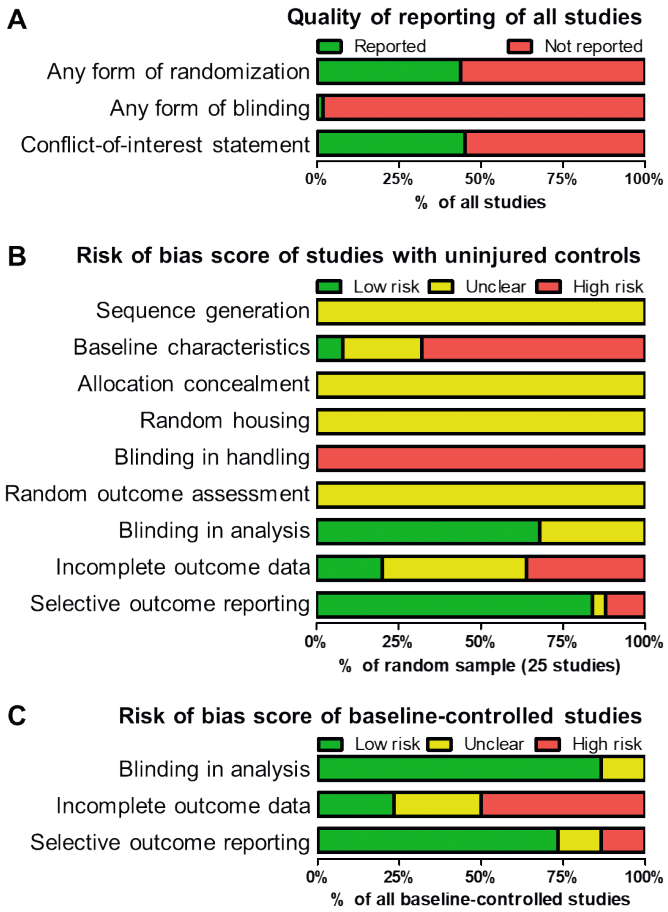

Figure 2. Quality of reporting and risk of bias assessment. (**A**) Quality of reporting of all included studies (n = 424). (**B**) Complete risk of bias assessment of a random sample consisting of 25 studies. (**C**) Risk of bias assessment of all of the included baseline-controlled studies (n = 29).

Healing rate of burns is associated with wound depth

Healing rate of the animal burn models was assessed over time by means of evaluating the percentage of remaining wound area (**Figure 3**). As burn injury is an acute type of trauma that generally heals within 4 weeks in rodents [19], we decided (post hoc) to categorize short time intervals early after burn followed by longer time intervals up to post burn day (PBD) 21, encompassing the different biological phases of wound healing: hemostasis, inflammation and proliferation. Categorization by wound depth showed a difference in wound healing rate. As expected, for deep dermal and especially full-thickness burn wounds the healing rate was slower than for partial-thickness burns. There was insufficient data available to assess the effect of variables such as TBSA, age or species on wound closure time.

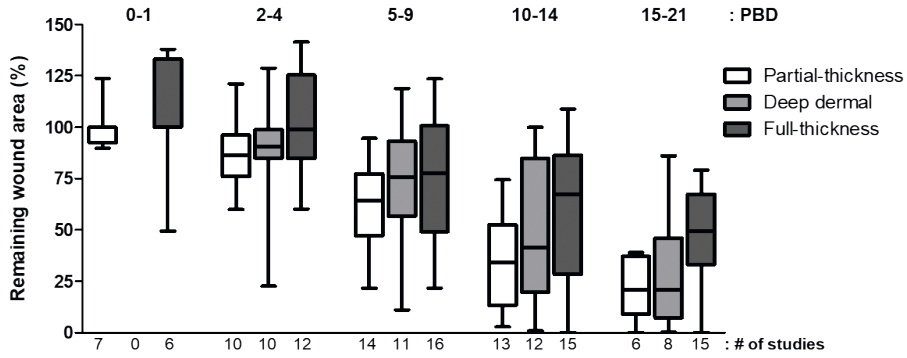

Figure 3. Healing rate of partial-thickness, deep dermal and full-thickness burn wounds in animal models. Measurements of re-epithelization and contraction were converted into remaining wound area. Data is presented as boxplots with median and quartiles. The number of studies is shown below the graph. Standardized mean difference could not be calculated for this type of measurement because control values are set at 100% without standard deviation.

Burn injury increases levels of pro-inflammatory cytokines in blood and wound tissue

Meta-analyses were performed on the effect of burn injury on the overall level of inflammatory mediators (**Figure 4** and **Supplementary Table 1**). There was no significant indication of publication bias for these outcomes. Burn injury significantly increased systemic levels of IL-1β, IL-6, IL-10, IL-12p70, IL-17, IFN-γ, TNF-α, CXCL1 (GROα), CXCL2 (MIP-2), CXCL8 (IL-8), CCL2 (monocyte chemoattractant protein 1, MCP-1), granulocyte colony-stimulating factor (G-CSF), prostaglandin E_2 (PGE_2), TGF-β1, vascular endothelial growth factor (VEGF)-A, c-reactive protein (CRP), histamine, high mobility group box 1 (HMGB1) and nitric oxide (NO). In contrast to subunit IL-12p70, the level of IL-12 in blood was decreased after burn. Systemic levels of IL-2, IL-3, IL-4, IL-5, IL-13, CCL3 (macrophage

inflammatory protein (MIP)-1α), CCL4 (MIP-1β), CCL11 (eotaxin) and GM-CSF were not significantly different from uninjured animals. In burn wound tissue the levels of IL-1β, IL-6, IL-10, TNF-α, CXCL1, CXCL2, CCL2, CCL3 (MIP-1α), epidermal growth factor (EGF), fibroblast growth factor (FGF)2, TGF-β1, VEGF-A and NO were significantly increased. None of the tested inflammatory mediators was decreased in burn wound tissue. The level of IL-1α, histamine and inducible nitric oxide synthase (iNOS) in wound tissue were not significantly different from uninjured animals. Distinct patterns between blood and burn tissue levels were observed: IL-1α and histamine were only increased in blood and CCL3 was only increased in burn tissue.

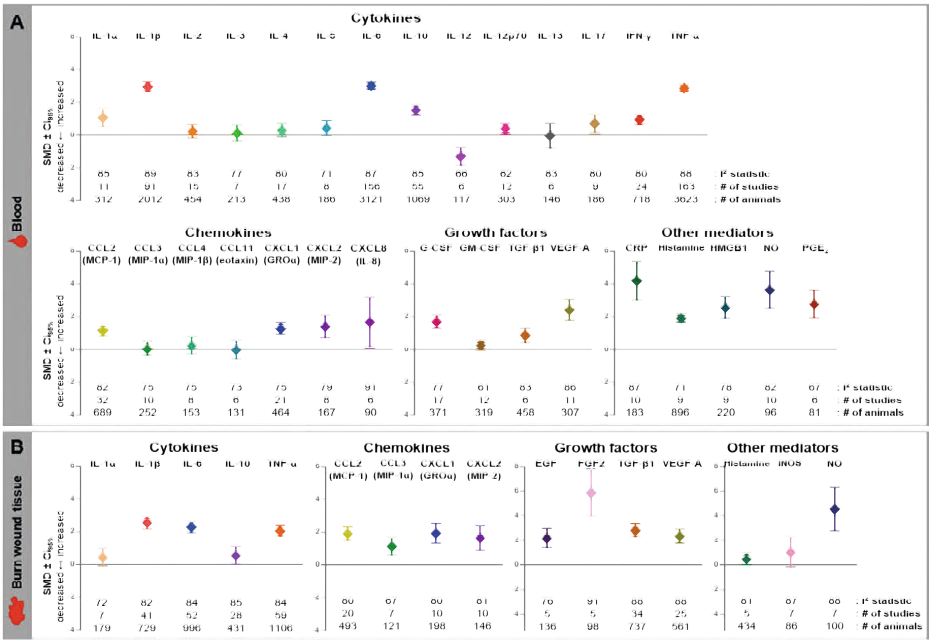

Figure 4. Overall level of inflammatory mediator levels after burn injury. Meta-analysis of the overall levels of cytokines, chemokines, growth factors and other mediators in (**A**) blood or (**B**) burn wound tissue at any time after burn injury. Only mediators for which at least 5 studies were available are shown. Results are shown as SMD of levels of inflammatory mediators from burn-injured animals compared to uninjured animals ± $CI_{95\%}$. There is a statistically significant difference with uninjured animals when the $CI_{95\%}$ does not cross the x-axis. The I^2 statistic, number of studies and total number of animals used in the burn-injured group for each meta-analysis are shown below the graphs. $CI_{95\%}$, 95% confidence interval; SMD, standardized mean difference.

The inflammatory response to burn injury is prolonged in blood and wound tissue

To study the effects of burn injury on the levels of inflammatory mediators over time, subgroup analyses were conducted for time intervals after burn (**Figure 5**). Systemic levels of IL-2 were increased at PBD5-9, while IL-4 was increased directly after injury (PBD

0-1), but not at PBD 5-9. Blood IFN-γ and G-CSF on the other hand, were increased from PBD 0-1 up to at least PBD 5-9. In both blood and wound tissue, IL-1β was increased from PBD 0-1 up to PBD 10-14. As opposed to wound tissue, the blood IL-1β levels decreased towards the level of uninjured animals at PBD 5-14. IL-6 was also increased directly after burn in both blood and wound tissue up to at least PBD 10-14 and wound tissue IL-6 even showed a sharp increase from PBD 0-1 up to 2-4. While IL-10 was increased in blood up to PBD 5-9, the wound tissue levels were similar to controls, except at PBD 5-9. TNF-α levels were similar for blood and wound tissue and were increased up to PBD 10-14 or PBD 15-21, respectively. CCL2 (MCP-1) levels in both blood and wound tissue were increased directly after burn, and appeared to gradually increase over time up to at least PBD 5-9. Wound TGF-β1 levels were increased from PBD 2-4 up to PBD 15-21. Increased levels of wound VEGF-A were found at PBD 2-4 up to PBD 10-14.

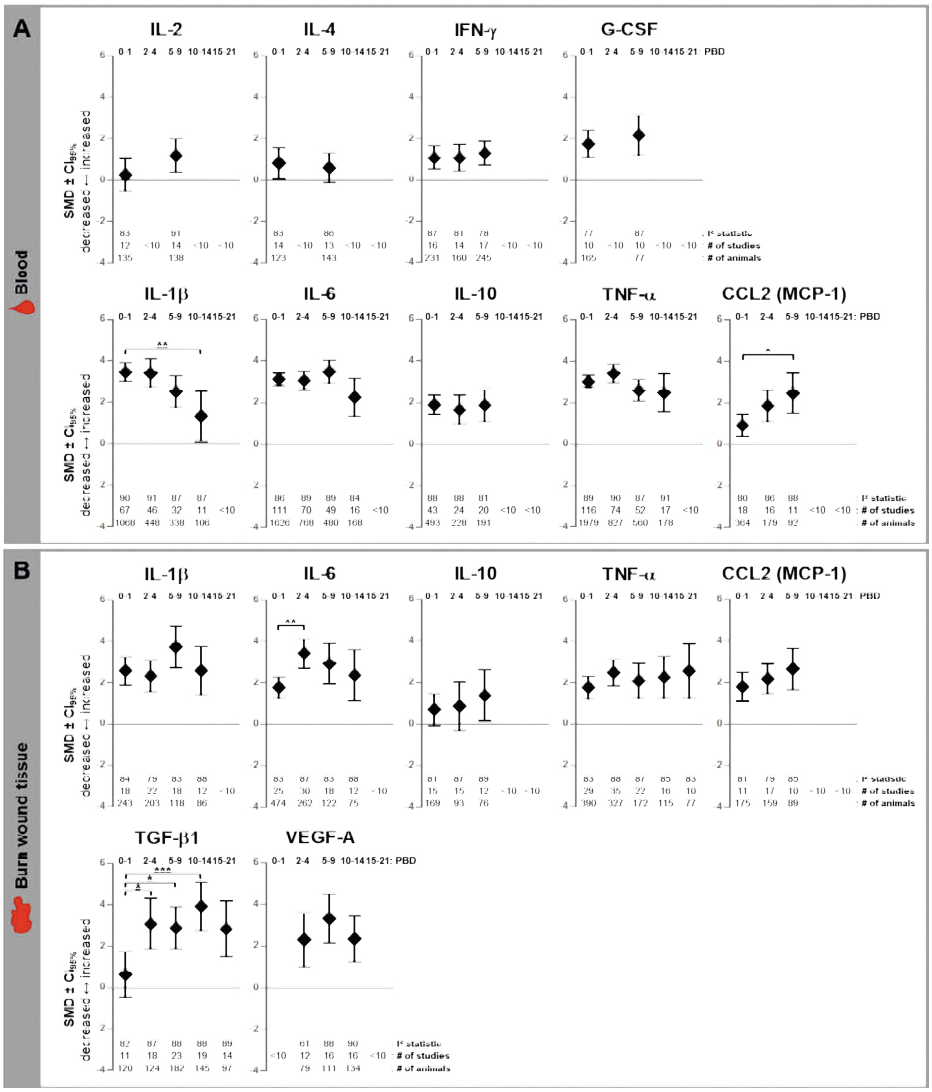

Figure 5. Longitudinal analysis of inflammatory mediator levels after burn injury. Subgroup analysis of different time intervals after burn injury on the inflammatory mediators levels in (**A**) blood or (**B**) burn wound tissue. Only time intervals containing at least 10 studies are shown. Results are shown as SMD of levels of inflammatory mediators in burn-injured animals compared to uninjured animals ± $CI_{95\%}$. There is a statistically significant difference with uninjured animals when the $CI_{95\%}$ does not cross the x-axis. The I^2 statistic, number of studies and total number of animals used in the burn-injured group for each meta-analysis are shown below the graphs. Bonferroni-corrected p-values based on the $CI_{95\%}$ of the difference between time intervals are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001. $CI_{95\%}$, 95% confidence interval; SMD, standardized mean difference.

Cytokine response levels are associated with burn size and burn agent

Next, we investigated differences in inflammatory mediator levels caused by type and severity of burn injury through subgroup analyses on percentage of TBSA, wound depth, injury site and burn agent (**Figure 6**). Burn size was positively associated with blood levels for IL-1β, IL-6, IL-10 and TNF-α and wound levels of IL-6 in case of >25% burns as compared to 5-25% burns. In post-hoc meta-regression analysis, blood IL-6 and IL-10 positively correlated with the reported percentage of TBSA with a p value of 0.0452 and 0.0231, respectively. Surprisingly, blood TNF-α and wound IL-6 levels were higher in burns affecting an area of ≤5% as compared to burns affecting a larger area (5-25%). Wound depth was less significantly associated with cytokine responses than burn size. In full-thickness burns only the level of TNF-α in wounds was higher compared to partial-thickness burns. Blood IL-6 levels were higher in animals that were burned on both sides as compared to animals with only dorsal burns, which was likely related to the difference in burn size. The type of burn agent affected the level of inflammatory mediators in blood, but not in burn wound tissue. Blood IL-1β, IL-6 and IFN-γ levels were higher in burns caused by hot water as compared to burns created by contact with hot objects.

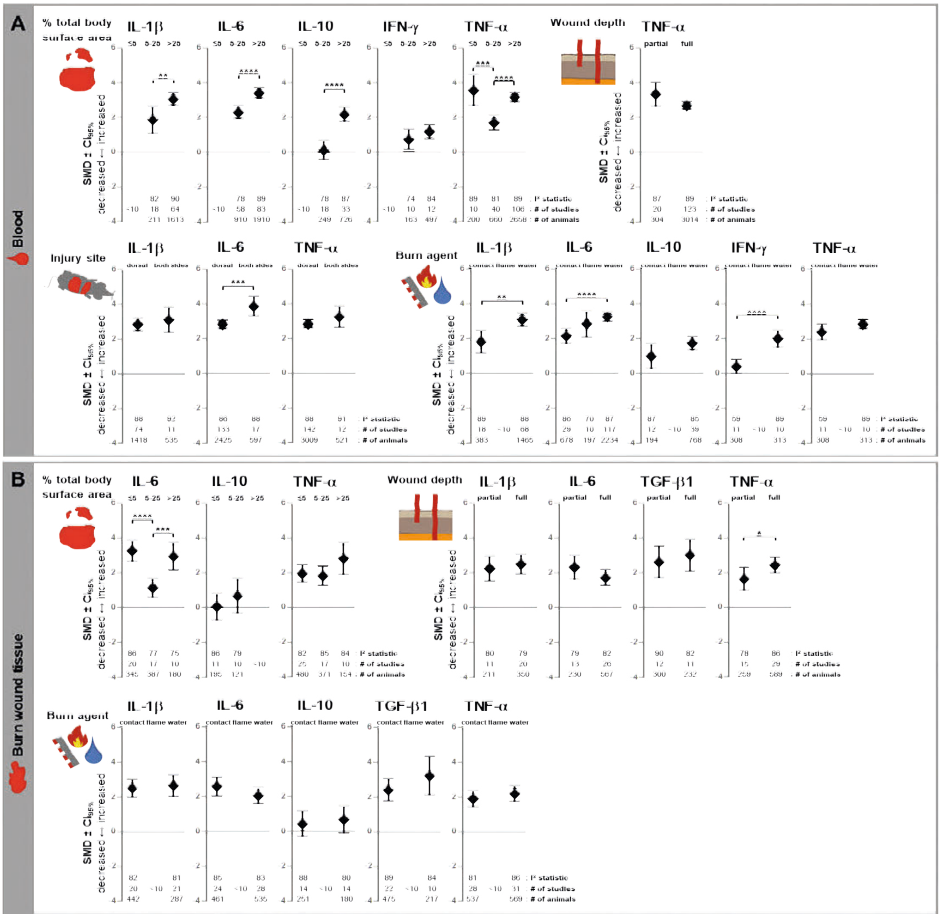

Figure 6. Subgroup analysis of inflammatory mediator levels based on type and severity of burn injury. Subgroup analysis of percentage of total body surface area, wound depth, injury site and type of burn agent on the inflammatory mediator levels in (**A**) blood or (**B**) burn wound tissue. Only subgroups containing at least 10 studies are shown. Results are shown as SMD of levels of inflammatory mediators from burn-injured animals compared to uninjured animals ± $CI_{95\%}$. There is a statistically significant difference with uninjured animals when the $CI_{95\%}$ does not cross the x-axis. The I^2 statistic, number of studies and total number of animals used in the burn-injured group for each meta-analysis are shown below the graphs. Bonferroni-corrected p-values based on the $CI_{95\%}$ of the difference between subgroups are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. $CI_{95\%}$, 95% confidence interval; SMD, standardized mean difference.

The level of inflammatory mediators is dependent on species, sex, age and detected form

There was great variation among the experimental models used to study the burn-induced immune response. As animal characteristics can impact outcome data, we explored differences in the levels of inflammatory mediators between diverse

experimental models. For this, we performed subgroup analyses based on animal species (mouse and rat only), sex, age and detected form (mRNA or protein) (**Figure 7**). Blood IL-1β, IL-6, IL-10, TNF-α and wound IL-6 levels were all higher in rats compared to mice. In contrast, higher levels of IL-β, IL-10 and TNF-α were found in wound tissue of mouse models over rat models. IL-6 and blood TNF-α were highest in male animals. Differences caused by age could only be assessed for blood levels. Blood IL-1β and TNF-α were higher in young animals than in adult animals. In wound tissue, mRNA and protein levels differed for IL-10 and TNF-α. Upon burn injury there was a significant increase in IL-10 mRNA, but not in IL-10 protein. For TNF-α, both mRNA and especially protein levels increased.

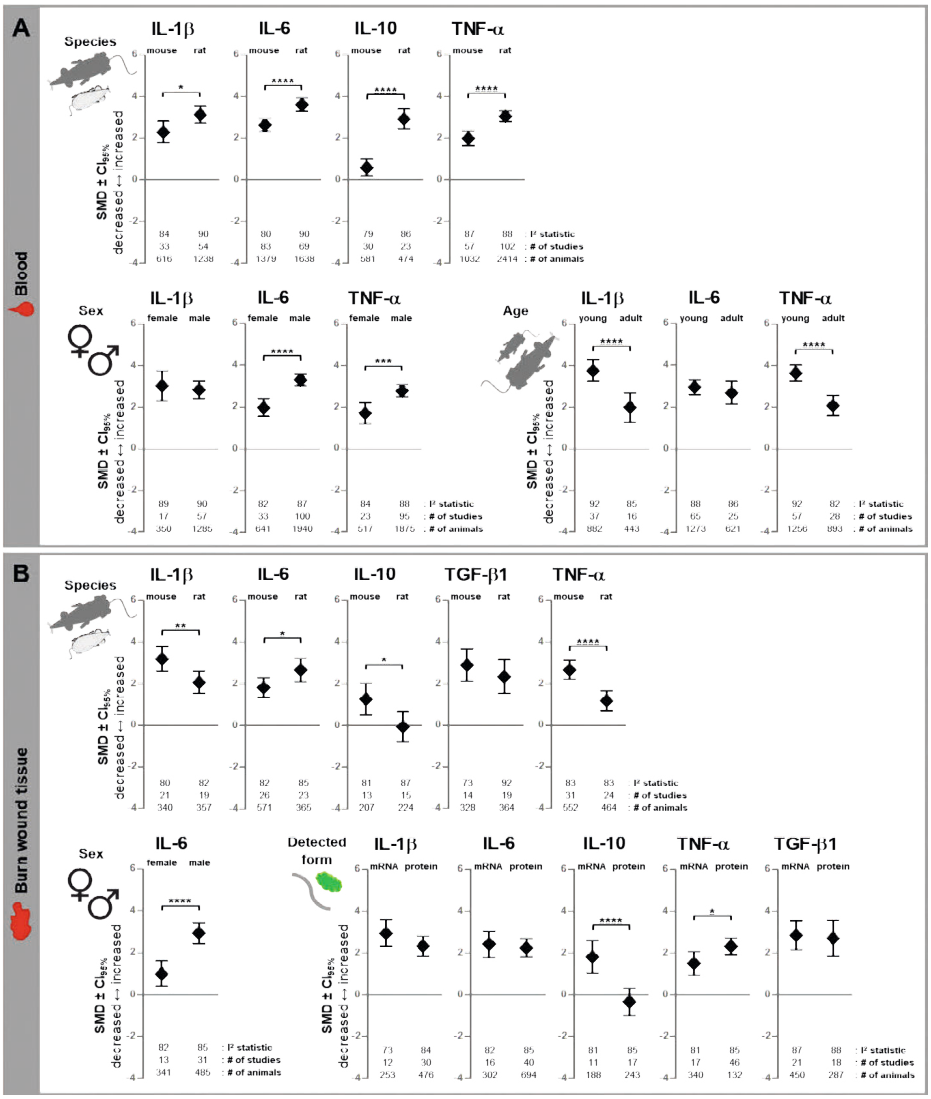

Figure 7. Subgroup analysis of inflammatory mediator levels after burn injury based on model characteristics. Subgroup analysis of animal species, sex, age and detected form on the inflammatory mediator levels in (**A**) blood or (**B**) burn wound tissue. Only subgroups containing at least 10 studies are shown. Results are shown as SMD of levels of inflammatory mediators from burn-injured animals compared to uninjured animals ± $CI_{95\%}$. There is a statistically significant difference with uninjured animals when the $CI_{95\%}$ does not cross the x-axis. The I^2 statistic, number of studies and total number of animals used in the burn-injured group for each meta-analysis are shown below the graphs. Bonferroni-corrected p-values based on the $CI_{95\%}$ of the difference between subgroups. Significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. $CI_{95\%}$, 95% confidence interval; SMD, standardized mean difference.

DISCUSSION

Burn injury induces a multitude of reactions in the body that can be harmful to healthy tissues. Detailed information on burn-induced immune reactions and related inflammatory mediators is needed to support the design of targeted interventions to treat derailed inflammation. Here, we clearly showed that burn injury stimulates the immune system, thereby enhancing the production of numerous mediators. There was one exception: the level of IL-12 was reduced. Burn severity and various model parameters influenced the response levels and expression pattern of IL-1β, IL-6, IL-10, IFN-γ and TNF-α significantly, which might have implications for both experimental set-ups and management of human burn injuries.

To improve our understanding of the burn-induced immune mechanisms, it is paramount to link cellular reactions to cytokine profiles. Previously, we reported massive recruitment of neutrophils to the blood and burn tissue until at least PBD 21 in animal burn models [8]. This phenomenon is caused by an emergency response whereby surges of immature neutrophils are released from bone marrow (left-shift response) [20,21]. Here, we found a concurrent increase in circulatory levels of IL-1β, IL-6, TNF-α and G-CSF (**Figure 5**). This finding emphasizes that IL-6 and G-CSF are important regulators of neutrophil traffic during inflammation [21–23]. Next to that, IL-1β and TNF-α are known to increase neutrophil migration and activation [24–26]. Moreover, we showed increased levels of CXCL1 and CXCL8, which are also important chemoattractants for neutrophils [27,28]. HMGB1was more prevalent after burn injury and is known to promote inflammation and increase neutrophil extracellular trap formation, thereby increasing the risk of secondary tissue damage and necrosis [29,30]. Although neutrophil migration and inflammatory mediator production were increased after burn, antibacterial activity was actually decreased [8]. Neutrophils might therefore be highly activated, yet less efficient at bacterial killing, which in turn is detrimental for wound healing and increases susceptibility to infection. Intervening with the overrepresentation or hyperactivity of neutrophils through inhibition of neutrophil-related inflammatory mediators might be an interesting approach to reduce inflammation in burn patients.

Monocytes play an active role in the immune response and differentiate into macrophages or dendritic cells as they migrate to the skin to remove damaged structures and invading bacteria [31]. The increase in CCL2 in blood and wound tissue we found here coincided with a gradual increase in blood monocytes from PBD 2-4 up to PBD 10-14 that we reported before [8]. CCL2 is an important chemoattractant for monocytes and is secreted by endothelial cells and fibroblasts in response to tissue damage [32]. Beyond chemotaxis, CCL2 plays a pivotal role in immune cell activation, differentiation

of monocytes to (M1) macrophages and degranulation responses of myeloid cells [33]. This is in line with increased macrophage activity after burn injury that we reported previously [8]. IFN-γ and TNF-α were also increased after burn injury and are known to stimulate macrophage M1 polarization as well (**Figure 4** and **Figure 5**). As a result of M1 polarization, the expression of IL-1α, IL-1β, IL-6 and TNF-α will increase [34,35], which was also the case for wound tissue levels shown here (**Figure 4**). M2 polarization on the other hand is induced by IL-4, IL-10, and IL-13, and is characterized by increased secretion of IL-10 and TGF-β1 [36,37]. After burn injury the levels of IL-4 and IL-13 were indifferent from healthy control animals, but the levels of TGF-β1 and IL-10 mRNA were increased, suggesting that there is M2 macrophage activity to some degree, but it might be limited. As M2 macrophage activity is essential during the proliferation and remodeling phase of healing [38], therapy directed to enhance this activity in burn patients could be beneficial for wound healing.

T cells and B cells have a protective role and can resolve inflammation, generate tailored pathogen defense responses and support wound healing together with M2 macrophages [39]. Information on lymphocyte responses and related mediators after burn injury is, however, limited. While the numbers of neutrophils and monocytes were high, the number of lymphocytes was actually decreased after burn injury [3,8,40,41]. Reduced lymphocyte activity can cause immune paralysis leading to poor immune regulation and increased risk of nosocomial infections [42]. IL-10 and TGF-β1, factors that typify regulatory T cell (Treg) activity, have inhibitory effects on pro-inflammatory cytokine production and support tissue restoration [43–45]. Although IL-10 and TGF-β1 were increased upon burn injury, Treg function and levels of anti-inflammatory factors may be insufficient to effectively control aberrant inflammation. For example, other cytokines typically associated with anti-inflammatory and tissue repair responses such as IL-2, IL-4, IL-5 and IL-13, were not increased after burn injury (**Figure 4**). These cytokines are produced by Th2 cells to drive B cell proliferation and M2 macrophage polarization [46]. After burn injury, Treg and Th2 responses might be delayed or insufficient. To treat uncontrolled inflammation successfully, patients might benefit from interventions that stimulate lymphocyte activity [47–49], e.g., by enhancing Th2 or Treg responses.

The levels of IL-6, blood IL-1β, IL-10 and TNF-α were higher in large burns as compared to moderate burns. Similarly, blood IL-6 were increased when animals were burned on both sides as compared to sole dorsal burns. This relation between the burn size and the intensity of the immune response has been demonstrated before in individual studies [50–52]. Scalds induced higher blood levels of IL-1β, IL-6 and IFN-γ than contact burns. Striking differences in the levels of IL-1β, IL-6, IL-10 and TNF-α were related to study model characteristics such as species (mouse or rat), sex and age. Circulatory levels of IL-1β,

IL-6, IL-10 and TNF-α were higher in rats than in mice, possibly because rats are larger animals, require a longer healing time and are immunologically more similar to humans than mice [19]. On the contrary, wound tissue levels of IL-1β, IL-10 and TNF-α were higher in mice, demonstrating that blood levels do not necessarily represent or predict levels in wound tissue. IL-6 and blood TNF-α were higher in male animals than in females. Sex chromosome genes and differences in hormonal systems proposedly contribute to differential regulation of immune responses between sexes [53]. For this reason male animals are often preferred over females, which creates bias in the available data [54].

Blood levels of IL-1β and TNF-α were higher in young animals than adults. Other researchers reported reduced levels of TNF-α when macrophages from adult mice were stimulated with LPS, due to reduced toll-like receptor expression and function [55,56]. Contrastingly, induced levels of immune cells are generally higher in adult animals [8]. This could mean that although induced levels of inflammatory mediators are higher in young animals, the cellular immune response can be more intense in adults. The young immune system might be underdeveloped and more tolerant, while the adult immune system is apparently more experienced and differently regulated, possibly due to long-term exposure to pathogens [57,58]. Higher levels of wound IL-10 were found by mRNA analysis than by protein analysis. This finding might be related to delayed or troubled protein synthesis or immediate uptake of IL-10 by immune cells. There is still much uncertainty about the relation between mRNA expression and protein levels, therefore more research is needed to clarify this [59,60]. Collectively, these insights demonstrate the importance of appropriate study model design and accurate interpretation of data [61] and might also be relevant in clinical practice.

Although our search strategy led to the inclusion of a large number of studies, this review, like many other reviews, was hurdled by underreporting of relevant study details (intervention and experimental procedures) and omission of data that is essential for meta-analysis (e.g., number of animals, standard deviation) (Osborne et al., 2018; du Sert et al., 2020). Authors were contacted for access to raw data sets or information on study details, but the response rate was low. Furthermore, in several studies the investigated subtype or form of IL-1 (α or β), IL-12 (subunit p40 or p70), IL-17 (A-F), VEGF (A-D) and TGF-β (1-3, latent or free [62]) was not specified, stressing the need for correct use of nomenclature in reporting. Failure to adhere to the ARRIVE guidelines makes a large portion of data unsuitable for reuse in advanced analyses and complicates the assessment of study quality and risk of bias. To improve research quality and reusability, there is a strong demand for sharpened reporting standards and effortless access to raw data [17,63]. In turn, this will lead to reduction, refinement and replacement of animal models and can support healthcare developments [13,64].

Translation of animal experimental data towards the human situation is challenging owing to genomic and physiological differences [14,15,65]. In addition, burn injury is known to cause immune-related complications on the long-term [66], therefore a limitation in most animals studies is the relatively short timeframe in which measurements take place. Nevertheless, a link between increased levels of inflammatory mediators IL-6, CCL2, CXCL1, CXCL8, G-CSF, IFN-γ and TNF-α and surges of innate immune cells (neutrophils and monocytes/macrophages) was also reported in blood and wound tissue from burn patients [2,3,7,66,67]. Moreover, disease severity in burn patients was associated with higher levels of CCL2, CXCL8, IL-6 and IL-10 [68]. Levels of these inflammatory mediators might therefore be useful predictors of disease progression and could be relevant for clinical decision making. Therapy of burn injury is primarily aimed at wound closure, preventing infection, relieving pain and limiting fibrosis [69]. As a malfunctioning immune system affects these aspects, future burn therapy should be more focused on the timely restoration of immune balance by modulating the intensity and duration of inflammatory responses [11]. Removal of damage associated molecular patterns such as HMGB1, could be targeted to remove inflammatory triggers [70]. This could be performed as general therapeutic approach, e.g., by early eschar debridement, or more specifically by a targeted intervention.

In line with therapeutic possibilities that were developed to counteract 'cytokine storm' associated with SARS-Cov-2 infection and complications, anti-inflammatory therapies such as Tocilizumab, Infliximab or other cytokine-inhibitors [71] might be helpful in reducing burn-induced inflammatory reactions as well. Cytokine removal might also be achieved through hemadsorption and hemodialysis [72,73]. Neutralization of specific cytokines such as IL-6 or TNF-α might reduce immune cell infiltration and secondary tissue necrosis, as was demonstrated in rat models [74,75]. Next to such specific actions, general anti-inflammatory therapies such as corticosteroid therapy could be beneficial. Furthermore, suppression of the extreme pro-inflammatory immune cell response can possibly be achieved by anti-oxidant therapy to protect tissues from oxidative stress caused by neutrophil and macrophage activity [76,77].

Altogether, this review provides an extensive overview of the empirical evidence on the burn-induced response of inflammatory mediators. It highlights factors that influence response levels, which are useful for refinement of experimental set-ups and evidence-based clinical practice. We demonstrated that burn injury causes severe inflammation, accompanied by increased levels of predominantly pro-inflammatory mediators both systemically and locally. Understanding the temporal dynamics is essential for the design of targeted immunotherapy to re-balance the malfunctioning immune system. This review will help improve future burn research into the post-burn immune response

and can stimulate the design of targeted interventions such as removal of inflammatory triggers, cytokine blockade or regulation of immune cells to improve treatment for burn patients.

MATERIALS AND METHODS

Study protocol and eligibility criteria

The associated review protocol was established beforehand and registered at the International Prospective Register of Systematic Reviews (PROSPERO) [78] under number CRD42019136270 (http://www.crd.york.ac.uk/PROSPERO/display_record.php?RecordID=136270). We amended this protocol once to further specify the meta-analyses.

Search strategy

The search was performed using PubMed and Embase according to the guidelines of Leenaars et al. [79], with a final update on August 17, 2022. The search strategy (search string) from Mulder et al. [8] was used. Briefly, articles with primary data on the immune response in animals with burn injury were searched using the following search components: burn injury, immune response, and animal. No restrictions were applied regarding language or publication date. Search results were combined and duplicate records were removed using EndNote software (X9, Clarivate Analytics, London, United Kingdom).

Study selection

Studies were selected by PPGM and BKHLB in blinded fashion using Rayyan software (Rayyan Systems, Cambridge, MA) [80] divided over three rounds: title screening, abstract screening, and full-text screening. During the title screening, articles clearly unrelated to burn injury were excluded. In the abstract screening, we selected studies that involved animal skin burns that contained primary data. Reviews, posters, and conference abstracts were excluded in this round. Selected studies for which the full text was inaccessible were excluded. During the full-text screening, studies involving animal thermal injuries with outcome measures related to inflammatory mediators were selected. Studies that used other burn types such as frostbite, chemical or electrical injuries were excluded. Furthermore, studies with co-interventions that obviously interfered with the function of the immune system, such as infection or administration of pro- or anti-inflammatory therapeutics were excluded. Additionally, the use of an appropriate control group (i.e. sham controls, uninjured animals, baseline values, or samples of uninjured tissue from same animals) was verified. At each screening round

(title, abstract and full text), discrepancies between PPGM and BKHLB were reviewed, and studies were included in case of disagreement.

Study characteristics

Independently, PPGM, MV and BKHLB extracted the study characteristics, including animal details (species, strain, age, sex and weight), burn method (burn agent, burn size, burn temperature, burn time, burn depth and injury site) and experimental set up (type of control, anesthesia, pain medication, resuscitation, inflammatory mediators and detection method). All of the extracted data was checked by at least one of the other reviewers.

Study quality and risk of bias assessment

The reporting of any form of randomization or blinding and the presence of a conflict-of-interest statement was scored for all of the included studies by PPGM and BKHLB who both assessed half of the studies and checked at least 10% of those of the other reviewer. Full risk of bias (RoB) assessment was conducted independently by PPGM and BKHLB using SYRCLE's tool [81] on 25 randomly selected studies (random number generator in Excel, Microsoft, Redmond, WA). We evaluated the reporting of the following baseline characteristics: animal sex, age, or weight (reporting of a range <10% was considered as a low RoB). To check the completeness of outcome reporting, we evaluated the number of animals in the method and results section for each experiment and outcome. In the case of discrepancies between the two reviewers that were not dismissible, MV was consulted as decisive third reviewer. This assessment provided an indication of the RoB of all included studies. All baseline-controlled studies (n = 29) were scored separately, as only items 7, 8, and 9 of the SYRCLE's RoB tool applied these studies.

Outcome data extraction

All quantitative outcome measures related to inflammatory mediators, such as cytokines, chemokines and growth factors, in either blood or wound tissue were collected in a database, which is available upon request. PPGM, MV and BKHLB independently extracted the outcome measures (mean outcome and SD, unit of measurement, number of animals). Extracted data was checked by at least one of the other reviewers. As a secondary outcome measure, data on the wound area (re-epithelization rate, wound closure time) was extracted and recalculated to percentage of remaining wound area. Data from graphs were extracted using the digital ruler feature in ImageJ (version 1.53j, National Institutes of Health, Bethesda, MD) [82]. Data presented as SEM were transformed to SD with the following formula: SD = SEM * . When studies used a relative expression (e.g., protein or mRNA expression level compared to uninjured animals) and no SD/SEM was available, the SD/SEM of the burn-injured group was used as imputation

for the uninjured animal group. In case of missing data, such as the number of animals or SD, we contacted corresponding authors by email and ResearchGate, including a reminder after 2 weeks.

Synthesis of results and meta-analysis

Meta-analyses were performed on overall outcome measures from which at least 5 studies were available. Data were analyzed using Comprehensive Meta-Analysis software (version 4; Biostat, Englewood, NJ), and the effect sizes were expressed as standardized mean difference (Hegdes's g) of inflammatory mediator levels in blood or wound tissue from burn-injured animals compared to levels in blood or skin from uninjured animals (baseline or uninjured controls) with 95% confidence interval. A random-effects model was used in the analyses and the I^2 statistic was used as a measure for statistical heterogeneity. Inflammatory mediators that were considered as the same entity were pooled (**Supplementary Table 1** and **Supplementary Table 2**). Remaining wound area was calculated using outcome data on wound closure, re-epithelization and contraction. Possible publication bias was explored using Duval and Tweedie's trim and fill methodology for overall outcomes with at least 10 studies [83]. Data was visualized using GraphPad version 5.01 (PRISM, Ja Jolla, USA).

Subgroup analyses

Predefined subgroup meta-analyses were performed on subgroups that consisted of at least 10 studies. Comprehensive Meta-Analysis software was used to determine differences based on time interval after burn (PBD 0-1, 2-4, 5-9, 10-14, 15-21, 22-28, or >29), percentage of total body surface area (≤5, 5-25, or >25), burn depth (superficial, partial-thickness, deep dermal, or full-thickness), injury site (abdominal, dorsal, both sides or paw), burn agent (water, contact, flame, or air), animal species (mouse, rat, or pig), animal sex, animal age (young or adult), and detection method (mRNA or protein analysis). In addition, differences between baseline-controlled studies and studies that used a separate control group were assessed. Common among-study variance across subgroups (pool within-group estimates of tau-squared) was assumed and subgroups were combined using fixed effects model. Effect was compared at different levels of the subgroups. Reported SMDs are based on the random effects model. P-values were based on the 95% confidence interval of the difference between subgroups. Bonferroni correction was applied, that is, the P-values were multiplied by the number of comparisons within each subgroup analysis.

In the case of repeated measures of an experimental group within a time interval, the maximum effect size within that time interval was selected. When required, total body surface area was calculated using the reported area of the burn, weight (W) of

the animals, and Meeh-Rubner's formula () [84]. The following K values were used: 9.0 (mouse), 9.8 (rat), 10.0 (pig), 10.1 (dog), 10.4 (cat) 10.5 (guinea pig), and 12.0 (rabbit). When total body surface area was missing in included articles, it was estimated on the basis of the reported age and weight information available at Animal Resources Centre (https://www.arc.wa.gov.au/), The Jackson Laboratory (https://www.jax.org/), and Roysfarm (https://www.roysfarm.com/). Using the weight of the animal, the animal's age was estimated when this was not reported. Animal age subgroups, young or adult, were based on the social maturity of the animals: adults were aged >12 weeks (hamster), >3 months (mouse), >6 months (rat, cat, pig or Guinea pig), and >12 months (rabbit or dog). For wound depth, the following categories were used: superficial (first degree), partial-thickness (second degree), deep dermal (deep second degree), and full-thickness (third degree, fourth degree, severe burn injury).

Meta-regression

Meta-regression analyses were performed post-hoc on the standardized mean difference of inflammatory mediator levels using the reported percentage of TBSA as a continuous variable. Random effects–restricted maximum likelihood model was used, and repeated measures (same animal, multiple sampling times) of studies were included.

Studies included in meta-analysis

[50,85–435]

Studies included in systematic review

[50,85–507]

Studies with uninjured controls that were used for risk of bias assessment

[114,116,130,131,154,171,198,221,224,234,244,259,263,288,290,301,304,310,325,345,397,434,444,454,508]

Baseline-controlled studies that were used for risk of bias assessment

[92,102,106,121,125,207,241,283,307,311,314,369,386,411,415–419,440,441,450,467,468,476,477,482,484,486,509]

ACKNOWLEDGMENTS

We want to thank Alice Tillema of the Radboud Medical Library for her help in designing the search strategy, dr. Rob de Vries of SYRCLE for his advice and dr. Kim Schilders, Anouk Elgersma, Rosa Rentenaar and Myrthe Witbaard of the ADBC for their assistance during data extraction. This research was funded by the ZonMw program More Knowledge with

Less Animals under grant 114024146 (PPGM) and the Dutch Burn Foundation under grant WO/17.108 (BKHLB).

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SUPPLEMENTARY

Supplementary Table 1. Outcome measures and associated references used in meta-analysis. A minimum of 5 articles was required for inclusion of a defined outcome measure in the meta-analysis. References that did not include a quantitative outcome measure or did not report essential study characteristics such as number of animals and standard deviation/error are also shown in "References in systematic review but not used in Meta-Analysis".

Immune Factor	References used in Meta-Analysis	References in systematic review but not used in Meta-Analysis
Blood CCL11 (eotaxin)	88, 108, 112, 118, 143, 305	
Blood CCL2 (MCP-1)	86, 88, 95, 98, 99, 105, 111, 112, 118, 119, 142, 150, 185, 198, 212, 214, 215, 221, 225, 245, 266, 270, 305, 314, 351, 358, 399, 400, 407, 423, 424, 510	452, 473, 493
Blood CCL3 (MIP-1α)	88, 89, 98, 108, 112, 118, 130, 266, 302, 305	
Blood CCL4 (MIP-1β)	88, 98, 108, 112, 118, 266, 302, 305	
Blood CRP (c-reactive protein)	180, 213, 292, 330, 349, 358, 384, 386, 401, 418	165, 496
Blood CXCL1 (GROα)	88, 89, 98, 105, 108, 112, 118, 119, 128, 130, 142, 185, 245, 266, 270, 302, 305, 365, 401, 407, 511	99, 170, 185, 473, 499
Blood CXCL2 (MIP-2)	98, 216, 266, 365, 379, 511	99, 143, 170, 185
Blood CXCL8 (IL-8)	86, 98, 185, 216, 266, 365, 379, 511	113, 146, 454
Blood G-CSF	88, 89, 98, 108, 112, 118, 119, 130, 142, 240, 245, 268, 282, 305, 367, 368, 407	143
Blood GM-CSF	88, 89, 97, 98, 102, 108, 112, 118, 119, 130, 266, 352	
Blood Histamine	91, 121, 172, 220, 281, 345, 372, 415, 416	415, 476, 481
Blood HMGB1	126, 193, 258, 297, 344, 348, 373, 404, 421	499
Blood IFN-γ	86, 88, 89, 97, 98, 102, 108, 112, 118, 130, 131, 193, 224, 229, 230, 251, 264, 266, 314, 322, 331, 349, 375, 423	474, 512

Supplementary Table 1. Continued.

Immune Factor	References used in Meta-Analysis	References in systematic review but not used in Meta-Analysis
Blood IL-1α	88, 98, 108, 112, 118, 130, 160, 245, 357, 358, 378	146
Blood IL-1β	50, 86, 88, 89, 93, 97–99, 102, 108, 110, 112, 118, 119, 122, 124, 125, 130, 135, 141, 147–149, 152, 154, 160, 162, 166, 169, 170, 180–183, 185, 187, 188, 191, 194, 195, 197, 200, 203, 205, 210, 212, 214–216, 222, 225, 238, 260–262, 266, 272–275, 277, 280, 290, 292, 294, 297, 299, 310, 325, 336, 339, 340, 344, 351, 352, 354, 357, 362, 374, 375, 378, 383, 387, 389, 399, 400, 404, 418, 428, 431, 509	146, 393, 470, 490, 493
Blood IL-2	88, 89, 97, 98, 108, 112, 118, 119, 130, 145, 147, 166, 337, 348, 375	
Blood IL-3	88, 89, 108, 112, 118, 130, 305	
Blood IL-4	88, 97, 98, 108, 110, 112, 118, 119, 130, 147, 224, 305, 309, 314, 331, 375, 386	89, 452, 454, 461, 474
Blood IL-5	88, 89, 97, 112, 118, 142, 225, 305	
Blood IL-6	50, 85, 86, 88–90, 93–99, 101–104, 108, 111, 112, 114, 118, 119, 122, 124, 127–130, 135, 138, 146–149, 152, 154, 157, 159, 160, 162, 163, 167, 170, 180–182, 185, 191, 193, 197, 198, 200, 203–206, 210, 212, 214–217, 221, 222, 225–227, 234, 236, 238, 240, 243, 256, 258, 261, 262, 264, 266, 268–270, 285–288, 290, 292, 295, 296, 299, 303, 305, 309, 313, 314, 317, 318, 322, 325, 332, 333, 335–337, 339–341, 343, 346, 349, 351–354, 358, 362, 363, 365, 366, 368, 373–375, 377–381, 390, 391, 393, 394, 398–403, 406–409, 418, 420–423, 425, 431, 433, 434, 509–512	113, 236, 441, 454, 459, 470, 471, 483, 490, 493, 507
Blood IL-10	50, 86, 88, 89, 93, 96–99, 108, 112, 118, 119, 122, 128, 130, 145, 147, 150, 159, 163, 166, 169, 170, 183, 193, 195, 198, 203, 216, 224, 225, 245, 256, 258, 264, 268, 270, 290, 299, 305, 310–312, 320, 332, 340, 343, 346, 349, 374, 407, 421, 423, 509, 511	113, 305, 311, 441, 470
Blood IL-12	145, 225, 229, 304, 375, 509	

Supplementary Table 1. Continued.

Immune Factor	References used in Meta-Analysis	References in systematic review but not used in Meta-Analysis
Blood IL-12p70	88, 89, 98, 108, 112, 118, 119, 130, 245, 266, 305, 423	
Blood IL-13	88, 108, 112, 118, 130, 305	
Blood IL-17 (IL-17A)	88, 89, 112, 118, 145, 266, 305, 343, 511	471
Blood NO	120, 123, 129, 155, 174, 197, 288, 292, 364, 388	454
Blood PGE2	113, 124, 165, 197, 269, 401	449
Blood TGF-β1 (TGF-β)	95, 145, 173, 239, 265, 314	
Blood TNF-α	50, 86, 88, 89, 93, 95–98, 107, 108, 110, 112, 114, 118–120, 122, 124, 125, 128–130, 138–141, 146–150, 152, 154, 156, 157, 159, 160, 162, 163, 166, 169, 170, 180–183, 185, 191–193, 195, 197, 198, 200–206, 210, 212–215, 217, 218, 223, 228, 234–236, 238, 247, 251–253, 256, 258, 260–262, 264, 266, 268–270, 272–277, 279, 280, 286, 288, 290–292, 294, 295, 299, 301, 305, 309–311, 313–315, 322, 327, 328, 332–335, 337, 339–342, 344, 346, 349, 351–354, 358, 360, 362–365, 374–376, 378, 382, 383, 387, 389, 390, 394, 396, 398, 400, 405, 408, 409, 414, 418, 421–423, 428–431, 513	393, 438, 441, 454, 464, 470, 474, 483, 490, 493, 507, 512
Blood VEGF-A (VEGF)	86, 98, 106, 108, 119, 130, 175, 194, 222, 266, 288	
Burn wound tissue CCL2 (MCP-1)	92, 116, 168, 176, 198, 207, 209, 237, 244, 257, 271, 278, 283, 289, 306, 323, 351, 365, 410, 411	469
Burn wound tissue CCL3 (MIP-1α)	92, 177, 244, 255, 257, 365, 411	437, 483, 499
Burn wound tissue CXCL1	87, 92, 168, 244, 255, 257, 271, 283, 411, 435	117, 469, 499
Burn wound tissue CXCL2	87, 92, 100, 136, 137, 176, 319, 365, 379, 411	117, 143, 469, 479

Supplementary Table 1. Continued.

Immune Factor	References used in Meta-Analysis	References in systematic review but not used in Meta-Analysis
Burn wound tissue EGF	92, 190, 207, 316, 361	143, 446, 468
Burn wound tissue FGF2	153, 177, 306, 321, 506	
Burn wound tissue Histamine	121, 241, 298, 412, 413	413, 476, 481
Burn wound tissue IL-1α	92, 153, 160, 161, 177, 255, 365	437, 440
Burn wound tissue IL-1β	92, 100, 109, 117, 133, 135, 136, 151, 160, 168, 176, 177, 186, 190, 196, 209, 211, 219, 232, 237, 244, 255, 257, 259, 289, 293, 294, 306, 321, 323, 351, 355, 359, 361, 365, 371, 379, 395, 400, 419, 432	306, 437, 440, 469, 479, 490, 492, 499, 501
Burn wound tissue IL-6	92, 109, 117, 134–137, 144, 151, 153, 159–161, 164, 168, 176, 190, 196, 198, 199, 209, 232, 233, 242–244, 250, 255, 257, 267, 271, 284, 289, 293, 306, 316, 324, 329, 350, 351, 361, 365, 370, 379, 395, 400, 410, 411, 419, 426, 427, 432	371, 410, 444, 479, 490, 499
Burn wound tissue IL-10	92, 109, 117, 133, 134, 151, 153, 159, 168, 176, 196, 198, 199, 209, 232, 242, 254, 255, 257, 283, 289, 361, 365, 371, 397, 410, 411, 417	410, 499, 504
Burn wound tissue iNOS	208, 209, 242, 292, 365, 397, 426	
Burn wound tissue NO	137, 155, 174, 208, 219, 246, 292	
Burn wound tissue TGF-β1 (TGF-β)	100, 117, 132, 134, 153, 164, 168, 176, 179, 184, 189, 190, 207, 209, 211, 232, 242, 259, 263, 267, 283, 306–308, 316, 338, 339, 350, 356, 361, 370, 392, 395, 397	184, 443, 444, 469, 489, 504
Burn wound tissue TNF-α	87, 92, 100, 109, 117, 134, 136, 137, 144, 151, 158–160, 164, 168, 171, 176, 186, 190, 196, 198, 199, 209, 231, 232, 237, 244, 250, 254, 257, 259, 271, 289, 293, 294, 306, 307, 316, 319, 321, 323, 324, 329, 351, 359, 365, 369–371, 385, 395, 397, 400, 410, 411, 419, 426, 427, 432	437, 447, 469, 478, 490, 504

Supplementary Table 1. Continued.

Immune Factor	References used in Meta-Analysis	References in systematic review but not used in Meta-Analysis
Burn wound tissue VEGF-A (VEGF)	92, 100, 133, 153, 168, 176, 189, 190, 199, 208, 237, 242, 259, 283, 292, 306, 308, 316, 326, 341, 347, 355, 356, 361, 395	174, 444, 469, 475, 489
Remaining wound area (re-epithelization, contraction)	100, 108, 119, 130, 132–134, 153, 154, 158, 176, 177, 179, 184, 191, 197, 199, 207–209, 231, 263, 283, 284, 288, 307, 308, 312, 314, 316, 339, 349, 350, 355, 356, 361, 365, 371, 392, 408, 411, 417, 436, 437, 443, 444, 447, 467–469, 474, 475, 489, 500–502, 505, 506	

Supplementary Table 2. Outcome measures and associated references in systematic review that could not be used in meta-analysis. For these factors, the minimum of 5 articles was not reached or they did not include a quantitative outcome measure or did not report essential study characteristics such as number of animals and standard deviation/error.

Inflammatory Factor	Analysis in blood	Analysis in burn wound tissue
α1-antitrypsin (a1aTc)	384, 462	
α2-macroglopbulin	157	490
Activin BC		504
Alkaline phosphatase (AP)	436, 445	
Arg1		469
α-1-acid	146, 384	
BAFF	442	
BMP7		491
Bradykinin		241, 456
C3	165	
CCL4 (MIP-1β)		244, 257, 365, 411, 437, 483
CCL5 (RANTES)	89, 112, 118, 119, 222	207, 371, 437, 479
CCL7 (MCP-3)		411
CCL8 (MCP-2)		469
CCL11 (eotaxin)		143, 411
CCL20 (MIP-3α)		411, 499
CCL21 (6Ckine)		504
CCL28		411
CH50	488, 497	
COX-2		133, 158, 242, 292
CTGF		134
CXCL4 (PF4, platelet factor 4)		365
CXCL5 (ENA78)	222	242, 365
CXCL8 (IL-8)		385, 419, 500
CXCL10 (IP-10)	108, 119, 130, 225	207, 365, 371, 411
CXCL11 (I-TAC)		365
CXCL12 (SDF1)		326
CXCL13		411
EGF	143, 194	
ET (1, 2, ETL1)	388, 457	

Supplementary Table 2. Continued.

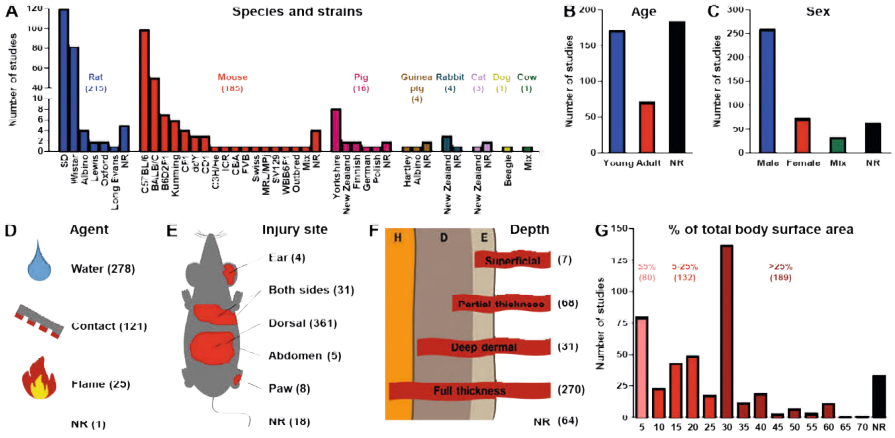
Inflammatory Factor	Analysis in blood	Analysis in burn wound tissue
FGF (1, 2, 7, 10)	470	100, 174, 397, 451, 468, 469, 489, 502
Flt3L	367	
Fractalkine		92
G-CSF		92, 143, 174, 244
GM-CSF		92
Haptoglobin (α-chain)	146, 157, 420	490
HGF (hepatocyte growth factor)	458	
HIF1α	498	326, 361
HMGB1		255
HVEM		504
IFN-β		319, 411
IFN-γ		92, 131, 151, 168, 410, 437
IGF-I	148, 149, 465	469, 502
IL-1	114, 157, 264, 337, 441, 448	199, 250, 254
IL-2		92
IL-1RA	102, 509	
IL-3		504
IL-4		92, 151, 490
IL-9	112, 118	504
IL-12 (p35, p40, p70, 12-23 complex)	97, 108, 112, 118, 130, 266, 304, 386	92, 410, 437, 447
IL-13		499
IL-15	108, 130	
IL-17A (IL-17)		151, 267, 504
IL-17C	266	
IL-17E	266	
IL-18	222, 229, 245, 274	92, 306, 411, 440
IL-22	471	267
IL-23		267, 319
IL-27	429	267
IL-33		480

Supplementary Table 2. Continued.

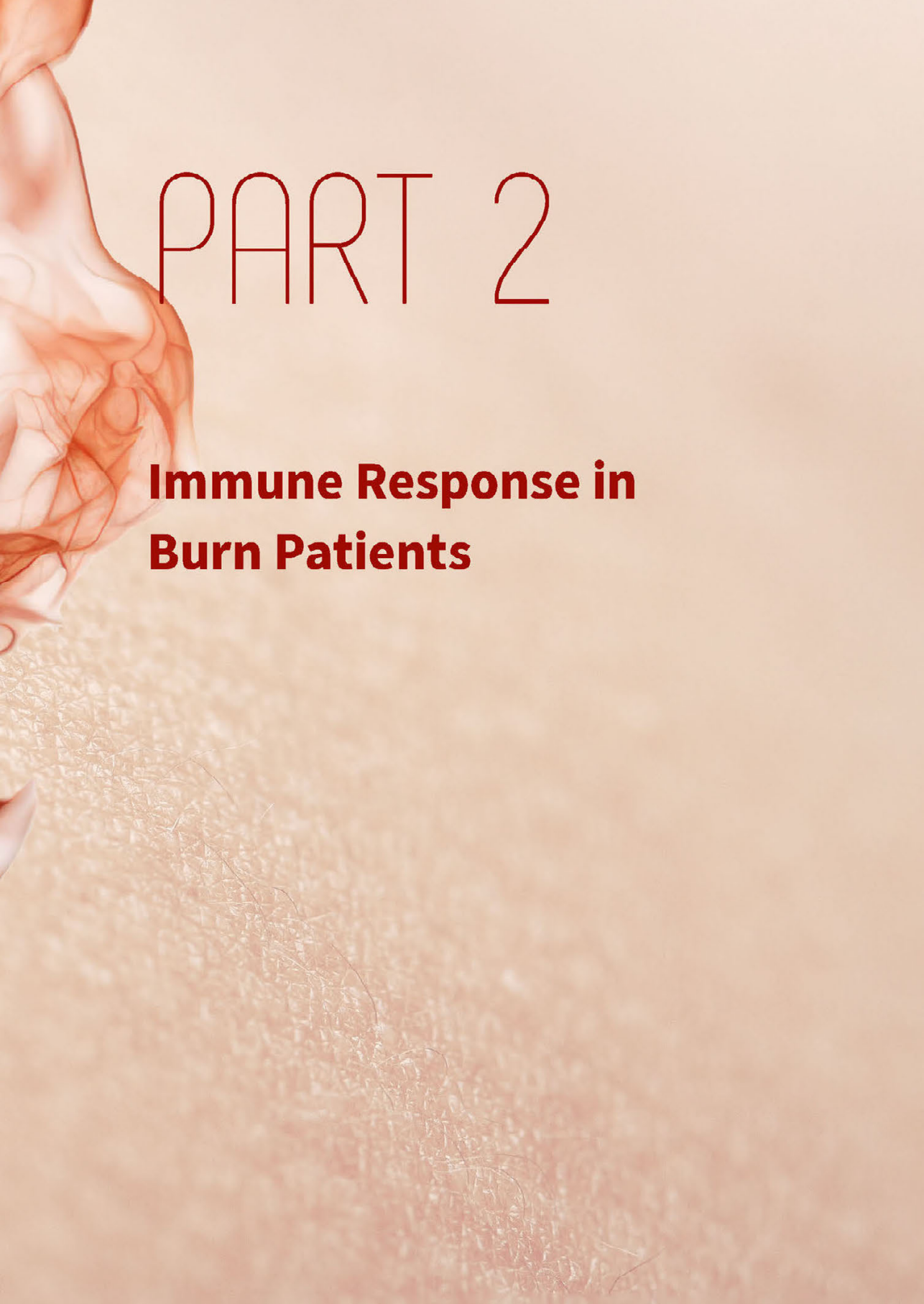
Inflammatory Factor	Analysis in blood	Analysis in burn wound tissue
KGF		361
Leptin		92
LTB4	197	495
M-CSF	240	
Macrophage inhibitory protein (MIF, MIF-1)	147, 166	365
NFkB (p65)	197	341
NLRP3		306, 323, 469, 501
NOS2		469
Nrf2		134
PDGF (A, BB, 6keto)		133, 174, 242, 450, 469, 494, 506
PGE (2, M)	467	219, 365, 411, 428, 485, 495
PgF2a		485, 503
PgI2		485
PLGF		397
RELMb		504
Retnla		469
ROS	454	
s100a8		306
sA100A9	143	143
SC5b-9	460, 484	
Selectin (E, P, L)	453	491
Serum amyloid (A-1, A-2, P)	420	
Substance P	477	477
TGF-α		446
TGF-β2	348	505
TGF-β3	314	153, 176, 392
TNF-β		490
Thrombomodulin		397
Thrombospondin		504
Tissue factor (TF)		246
TSG6	203	199
TxA		485
TXB2	113	450, 494, 495

Supplementary Table 2. Continued.

Inflammatory Factor	Analysis in blood	Analysis in burn wound tissue
VEGF-B		93, 153
VEGF-C		397
VEGF-D		397
Various factors (gene expression)	245, 439, 463, 472	92, 455, 466, 482, 486, 487

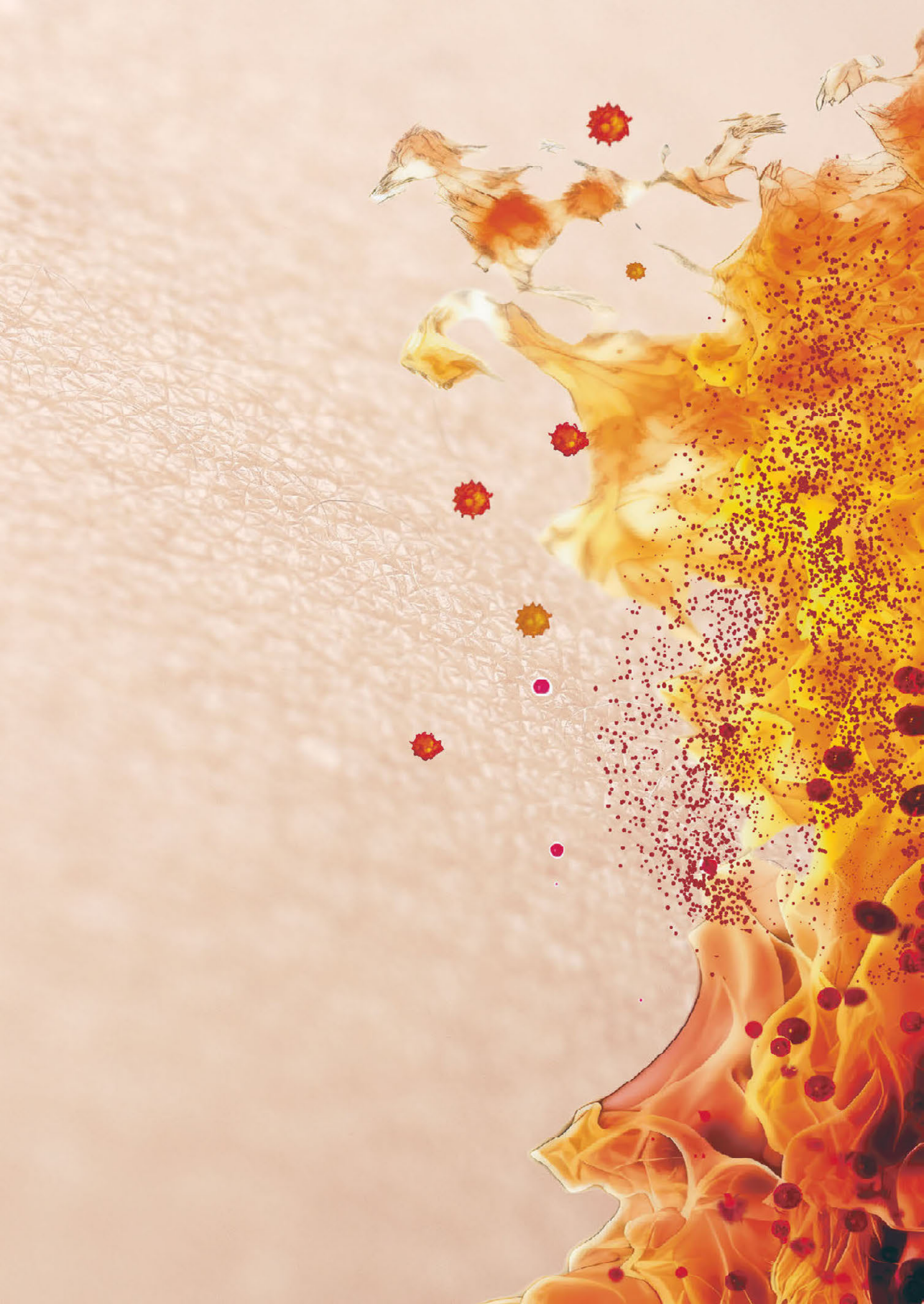


Supplementary Figure 1. Characteristics of animal models in systematic review. (**A**) Species and strains of study animals. (**B**) Sex of study animals. (**C**) Age of study animals. (**D**) Injury site. (**E**) Depth of burn injury. (**F**) Type of burn agent (burn cause). (**G**) Total body surface area that was burned as percentage. Numbers indicate the number of studies. D, dermis; E. Epidermis; H, hypodermis; NR, not reported.



PART 2

Immune Response in Burn Patients



CHAPTER 4

Persistent Systemic Inflammation in Patients With Severe Burn Injury Is Accompanied by Influx of Immature Neutrophils and Shifts in T Cell Subsets and Cytokine Profiles

Published in Frontiers in Immunology, **2021**,*11, 621222*
DOI: 10.3389/fimmu.2020.621222

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ABSTRACT

Severe burn injury causes local and systemic immune responses that can persist up to months, and can lead to systemic inflammatory response syndrome, organ damage and long-term sequalae such as hypertrophic scarring. To prevent these pathological conditions, a better understanding of the underlying mechanisms is essential. In this longitudinal study, we analyzed the temporal peripheral blood immune profile of 20 burn wound patients admitted to the intensive care by flow cytometry and secretome profiling, and compared this to data from 20 healthy subjects. The patient cohort showed signs of systemic inflammation and persistently high levels of pro-inflammatory soluble mediators, such as IL-6, IL-8, MCP-1, MIP-1β, and MIP-3α, were measured. Using both unsupervised and supervised flow cytometry techniques, we observed a continuous release of neutrophils and monocytes into the blood for at least 39 days. Increased numbers of immature neutrophils were present in peripheral blood in the first three weeks after injury (0.1-2.8 × 10^6/ml after burn vs. 5 × 10^3/ml in healthy controls). Total lymphocyte numbers did not increase, but numbers of effector T cells as well as regulatory T cells were increased from the second week onward. Within the $CD4^+$ T cell population, elevated numbers of $CCR4^+CCR6^-$ and $CCR4^+CCR6^+$ cells were found. Altogether, these data reveal that severe burn injury induced a persistent innate inflammatory response, including a release of immature neutrophils, and shifts in the T cell composition toward an overall more pro-inflammatory phenotype, thereby continuing systemic inflammation and increasing the risk of secondary complications.

INTRODUCTION

Burn injury and its consequences affect patients' overall health and quality of life because of long-term functional and cosmetic impairment [1]. Severe burn trauma induces pro-inflammatory immune responses in peripheral blood and affected tissues, regardless of infection [2,3]. This immune response can persist up to months and can lead to additional health problems, including systemic inflammatory response syndrome (SIRS), hypermetabolic state and damage to surrounding tissues and even distant organs [4–7]. Trauma instantly causes inflammation and produces damage associated molecular patterns (DAMPs) through necrotic and injured tissue, which stimulates the immune system to recruit acute phase immune cells [8,9]. A well-orchestrated immune response is essential for a proper healing process, as a persistent and dysregulated immune reaction can negatively affect wound closure and tissue repair. For example, an overactive immune system can cause tissue damage by proteases and oxygen radicals released by innate immune cells, and by hypercoagulation-induced ischemia [10,11]. Such collateral damage can even be linked to excessive scarring [12], which in turn cause debilitating deficiencies affecting physical, psychological and social aspects. So far, studies examining burn-induced systemic inflammation centered on data obtained from animal models [13]. The human response to trauma is however quite distinct from that of animals, which is exemplified by differences in wound healing and scar formation [14]. Data on the mechanisms behind the propagation and regulation of burn-induced immune response in humans is still very limited [15].

After initiation of the acute phase immune response due to burn injury, neutrophils and macrophages are the first immune cells homing to the wound area [1]. Neutrophils and macrophages originate from the blood circulation and are replenished by the bone marrow. These innate immune cells remove necrotic tissue and defend the body from pathogens by phagocytosis and the release of reactive oxygen species [16]. In this inflammatory phase, the innate immune cells enhance the inflammation and recruit other immune cells by secreting soluble mediators [3]. In the late phase of inflammation, T cells, originating from lymphoid tissues, and anti-inflammatory macrophages resolve the inflammation to limit ancillary damage to the tissue [17]. In trauma, T cell subtypes Th1 and Th17 cells play a role in the enhancement of inflammation, whereas Th2 and regulatory T cells (Tregs) are involved in its resolution [18]. A balance between these subtypes is essential for a proper transition from inflammation to wound healing [19]. In a normal wound healing situation, i.e. after minor injuries, neutrophils disappear from the wound area through apoptosis and macrophages differentiate from a pro-inflammatory state to a tissue remodeling state to re-establish homeostasis and initiate the proliferation phase wherein restoration of the skin can take place [20].

In burn trauma, the coordinated immune response is distorted and extended. A burn-induced hyperinflammatory state is accompanied by significant elevation of immune cells, cytokines, and acute phase proteins [9]. Particularly serum interleukin (IL)-6, IL-8, granulocyte colony-stimulating factor and monocyte chemoattractant protein (MCP)-1 revealed dramatic increases in a large set of severely burned (pediatric) patients [5,9]. These increases in cytokine levels were dependent on the size of the injury at 24–48 h after trauma [5]. In response to thermal injury, there is a rapid increase in bone marrow-derived endothelial progenitor cells in peripheral blood, which correlates with the extent of injury [21].

In order to improve wound healing and limit the formation of hypertrophic scars, an improved understanding of the immune response induced by severe trauma is needed. This knowledge, together with clinical perspectives, could be used to resolve an excessive immune response by therapy to restore the immune balance and optimize wound healing. Although the time-course of cytokines due to burns has been reported [5,9,22], data on immune cells were not included. Our aim was to characterize the inflammatory response by investigating peripheral blood changes in subsets of innate and adaptive immune cells in time (post burn day (PBD) 0–39) and 33 inflammatory mediators (PBD 0–48) in adult patients with severe burn injury.

RESULTS

Systemic Inflammation After Burn Injury is Associated With Prolonged Increase of Peripheral Blood Granulocytes and Monocytes

To examine the immune profile of burn wound patients in more detail, we performed multiparameter phenotyping by flow cytometry of peripheral blood of 20 burn wound patients up to 39 days after burn injury. All burn patients in the cohort showed signs of systemic inflammation (**Supplementary Figure 2**). Immediately after burn injury, total blood leukocyte counts were significantly increased compared to healthy controls. To analyze the response in time, a linear mixed model analysis was performed to determine the changes in comparison to time interval PBD 0–3. Data of PBD 0–3 was available for 15 patients. This analysis showed an additional increase in leukocyte counts until PBD 19–21 with the exception of PBD 13–15 (**Figure 1A**). Subtype analysis revealed that this increase of blood leukocytes could be ascribed to granulocyte and monocyte numbers, and not to lymphocyte counts (**Figures 1B-D**). In burn patients, granulocyte and monocyte counts rose significantly immediately after the injury compared to healthy controls but remained stable during the total time course (**Figure 1B,D**). Lymphocyte counts showed no increase compared to healthy controls. A small decrease around PBD 4–6 compared to PBD 0–3 was seen, followed by a non-significant tendency toward higher lymphocyte

counts (**Figure 1C**). The relative amounts of leukocyte subtypes are summarized in Figure 1E. We observed no confounding effect of TBSA (>26% vs. ≤26%) on the course of the inflammatory response (i.e. leukocytes, granulocytes, lymphocytes, and monocytes) in the mixed model analysis (data not shown).

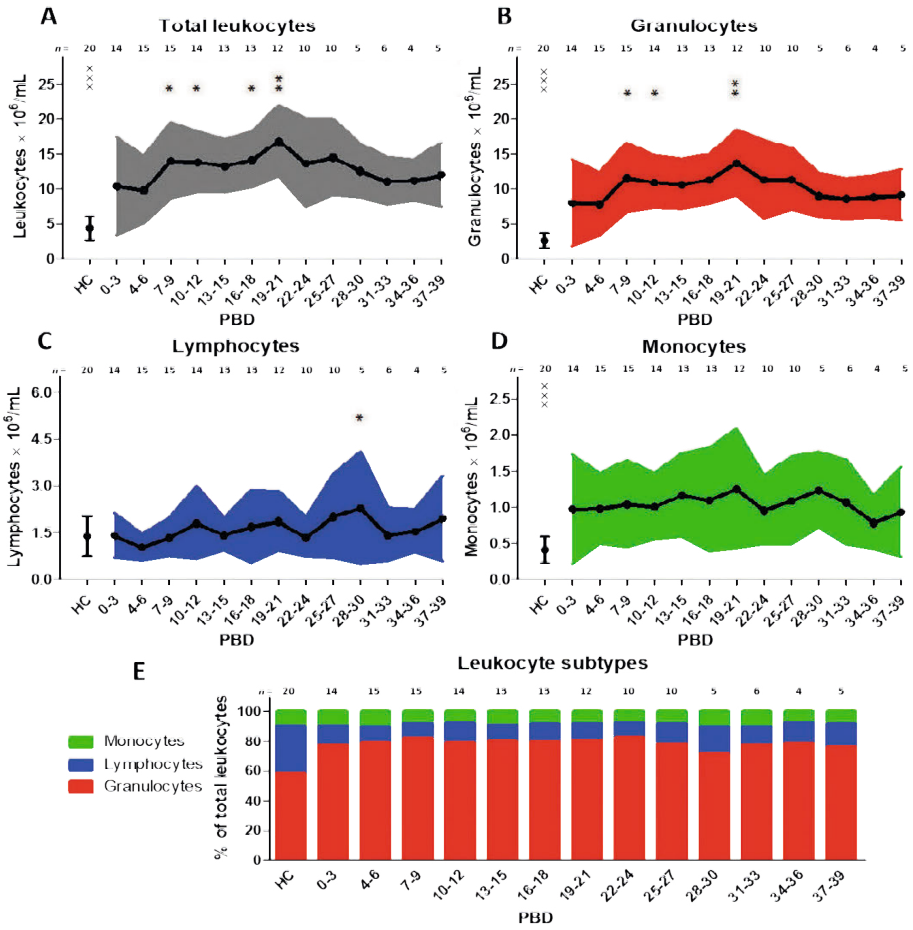

Figure 1. **Blood immune cells after severe burn injury.** Flow cytometry was used for phenotyping of leukocytes: (**A**) Total leukocyte numbers (gray). (**B**) Granulocyte numbers (red). (**C**) Lymphocyte numbers (blue). (**D**) Monocyte numbers (green). (**E**) Relative amount of leukocyte subtypes. Number of subjects per time interval is shown on top of the graphs. Values of burn wound patients and healthy controls (HC) are shown as mean (line and dots) ± standard deviation (colored band). Asterisks indicate significant differences in time within the burn patient group (linear mixed model analysis): *p < 0.05; **p < 0.01. Significant differences of outcomes in burn patients of PBD 0-3 compared to healthy controls are indicated by$^{\times}$ ($^{\times\times\times}$p < 0.001).

Burn Injury Is Associated With A Large, Continuous Surge of Immature Neutrophils, Classical and Non-Classical Monocytes

To further explore the effect of severe burn injury on systemic granulocyte and monocyte subsets in time, we performed an unsupervised analysis using Flow Self-Organizing Map clustering (FlowSOM) (**Figure 2**). We used data from flow cytometry stainings of 7 patients from which samples of all time points were available. The FlowSOM cluster structure was determined based on all data from these patients and 10 healthy controls. We could define 5 main cell clusters: $CD10^{dim}$ neutrophils (nodes 8–13), $CD10^{bright}$ neutrophils (nodes 2–7), $CD16^{-}$ granulocytes (including eosinophils; node 14), classical $CD14^{bright}CD16^{-}$ monocytes (node 16) and non-classical $CD14^{dim}CD16^{+}$ monocytes (node 1) (**Figure 2A**). Then, we analyzed the composition of these clusters in burn patients over time. CD10 was previously associated with the maturation stages of neutrophils [23–25]. In the first week post burn, the three mature ($CD10^{bright}$) neutrophil populations (nodes 3–5) were hardly present and the majority of neutrophils was immature ($CD10^{dim}$). From week 2 onward, mature $CD10^{bright}$ neutrophils reappeared, while immature $CD10^{dim}$ neutrophil numbers declined, but remained elevated for the remaining period of the study. The number of $CD16^{-}$ granulocytes slightly decreased in week 1 and returned to the level of healthy controls in week 2. Burn injury caused a shift toward more classical $CD14^{bright}CD16^{-}$ monocytes and the elevated level of this subtype persisted for the whole study period.

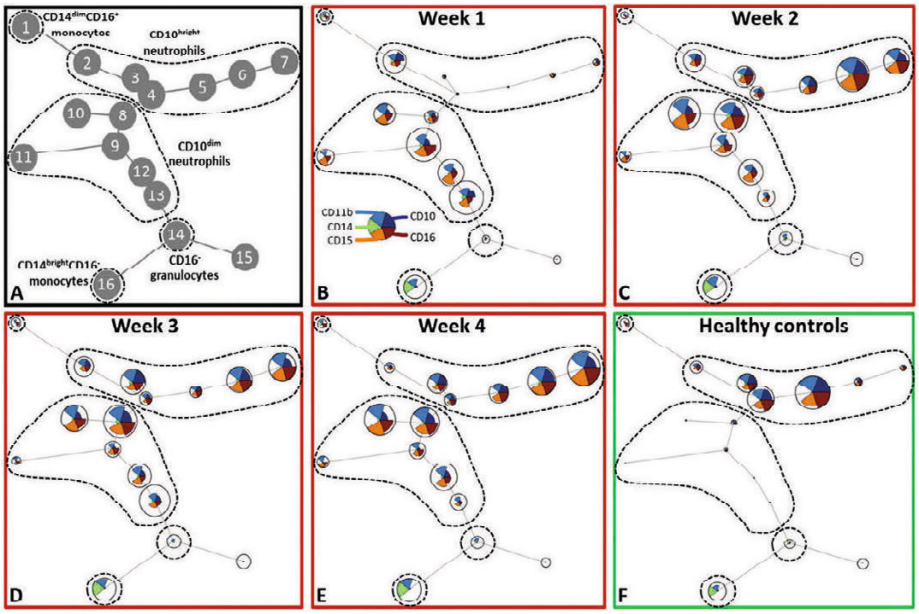

Figure 2. Unsupervised FlowSOM analysis of granulocyte and monocyte subtypes after severe burn injury. FlowSOM plots present proportions of populations and the expression of markers that were used in the innate flow cytometry panel (CD10, CD11b, CD14, CD15 and CD16). (**A**) Cluster structure based on flow cytometry data of 10 healthy controls and 7 burn wound patients that were observed for 4 weeks. The most pronounced subtypes are encircled by dashed lines: $CD16^{+}$ monocytes (node 1), $CD10^{bright}$ neutrophils (nodes 2-7), $CD10^{dim}$ neutrophils (nodes 8-13), $CD16^{-}$ granulocytes (node 14), $CD14^{bright}CD16^{-}$ monocytes (node 16). FlowSOM plots of: (**B**) Week 1; (**C**) Week 2; (**D**) Week 3; (**E**) Week 4 after burn; (**F**) Healthy controls.

We verified the unsupervised findings by supervised flow cytometry analysis of data from all patients. The leukocyte increase after burn injury was indeed due to a rise in neutrophil numbers and was associated with shifts in maturation stage (**Figure 3A**). Eosinophil numbers ($CD9^{+}CD15^{+}CD16^{-}$ granulocytes) increased over time but only to a small extent (**Figure 3B**). The high number of immature neutrophils at 0–3 days after injury decreased after PBD 6, but remained higher than in healthy controls until PBD 34–36. Mature neutrophil counts increased at PBD 4 and remained elevated from PBD 7 onward (**Figure 3C,D**). Supervised analysis confirmed the persistent increase in classical monocytes, but also revealed an increase in intermediate $CD14^{bright}CD16^{+}$ and non-classical $CD14^{dim}CD16^{+}$ monocytes. These data demonstrate that burn trauma induced a continuous release of (immature) neutrophils and monocyte subtypes.

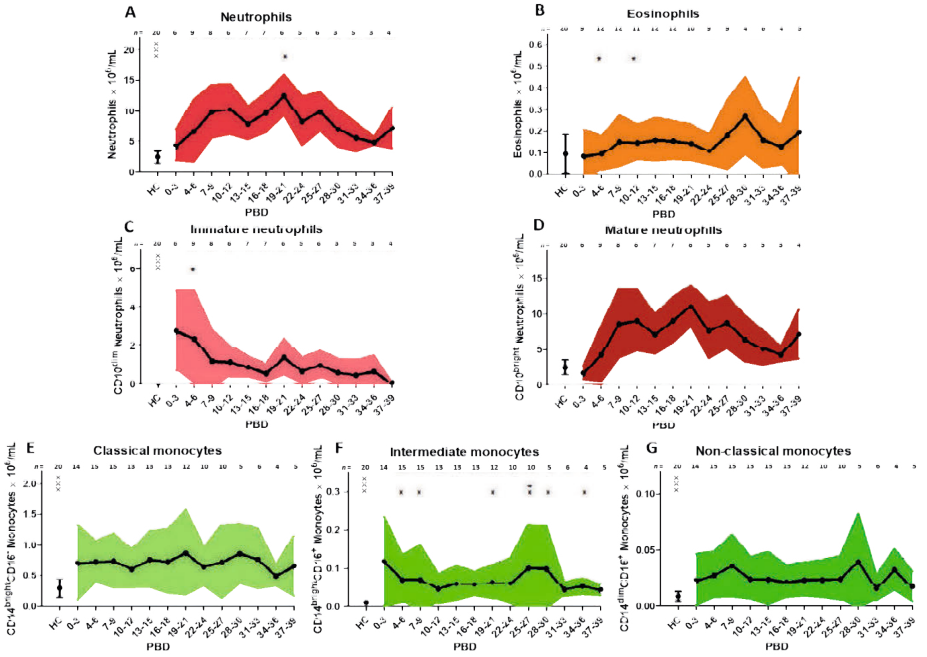

Figure 3. Supervised analysis of blood granulocyte and monocyte subsets after severe burn injury. Flow cytometry results of: (**A**) Neutrophils ($CD15^{+}CD16^{+}$ granulocytes). (**B**) Eosinophils ($CD15^{+}CD16^{-}CD9^{+}$ granulocytes). (**C**) Immature neutrophils ($CD10^{dim}$ neutrophils). (**D**) Mature neutrophils ($CD10^{bright}$ neutrophils). (**E**) Classical monocytes ($CD14^{bright}CD16^{-}$ monocytes). (**F**) Intermediate monocytes ($CD14^{bright}CD16^{+}$ monocytes). (**G**) Non-classical monocytes ($CD14^{dim}CD16^{+}$ monocytes). Number of subjects per time interval is shown on top of the graphs. Values of burn wound patients and healthy controls (HC) are shown as mean (line and dots) ± standard deviation (colored band). Asterisks indicate significant differences in time within the burn patient group (linear mixed model analysis): *$p < 0.05$; **$p < 0.01$. Significant differences of outcomes in burn patients on PBD 0-3 compared to healthy controls are indicated by ^{x} ($^{xxx}p < 0.001$).

Burn Injury Induces an Increase in CCR4 and CCR6 Expressing CD4+ T Cells and Tregs From the Second Week After Injury Onward

Although burn injury did not significantly alter the total number of lymphocytes, unsupervised analysis of the lymphocyte flow cytometry panel revealed changes in the T cell composition (**Figure 4**). Four main clusters of lymphocytes could be discriminated: $CD4^{+}$ T cells (nodes 1–7), Tregs (nodes 6, 7), $CD4^{-}$ T cells (nodes 8–12) and $CD3^{-}$ lymphocytes (nodes 13–16) (containing B cells and NK cells) (**Figure 4A**). In the $CD4^{+}$ T cell cluster, the $CCR4^{-}CCR6^{-}$ T cells (node 4), among which could be naïve T cells, decreased upon burn injury. $CCR4^{+}CCR6^{+}$ and $CCR4^{-}CCR6^{+}$ T cells (nodes 1 and 2, respectively) increased in week 2 and remained elevated in week 3 and 4. Two regulatory T cell populations were distinguished: $CCR4^{+}CCR6^{-}$ and $CCR4^{+}CCR6^{+}$ Tregs (nodes 6 and

7, respectively), which were both increased in week 2 and 3. In week 4, CCR4^{+}CCR6^{-} Treg numbers were comparable to healthy controls, while the numbers of CCR4^{+}CCR6^{+} Tregs were still increased. In the CD3^{-} lymphocyte cluster only small changes were observed.

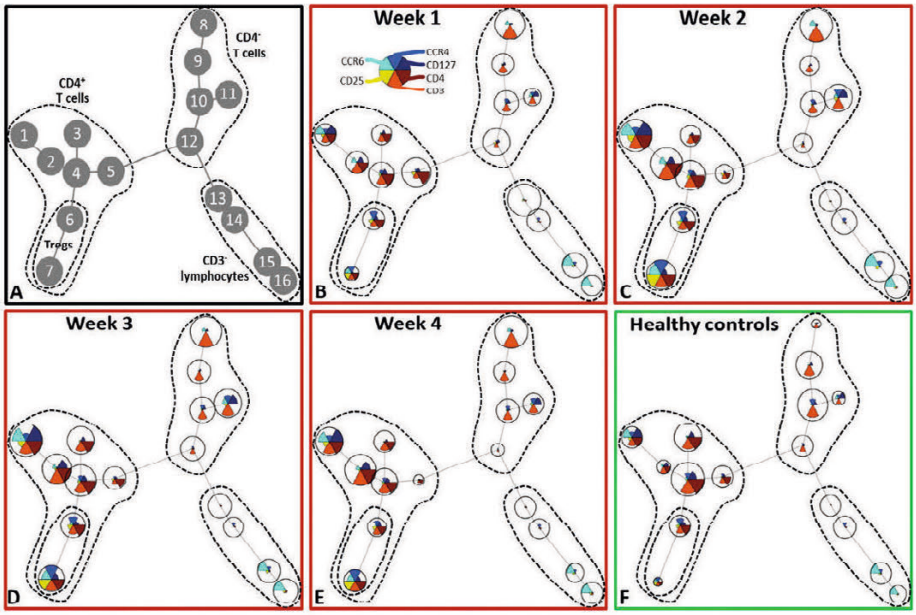

Figure 4. Unsupervised FlowSOM analysis of lymphocyte subtypes after severe burn injury. FlowSOM plots present proportions of populations and the expression of markers that were used in the lymphocyte flow cytometry panel (CD3, CD4, CD25, CD127, CCR4 and CCR6). (**A**) Cluster structure based on flow cytometry data of 10 healthy controls and 12 burn wound patients that were observed for 4 weeks. The most pronounced subtypes are encircled by dashed lines: CD4^{+} T cells (nodes 1-7), Tregs (nodes 6, 7), CD4^{-} T cells (nodes 8-12), CD3^{-} lymphocytes (nodes 13-16). FlowSOM plots of: (**B**) Week 1; (**C**) Week 2; (**D**) Week 3; (**E**) Week 4 after burn; (**F**) Healthy controls.

Similar to the analysis of the innate cells, we took a supervised approach on the lymphocyte flow cytometry data of all patients to verify the unsupervised findings (**Figure 5**). The increase in CD4^{+} T cells in the second week after burn injury was confirmed, while the number of CD4^{-} T cells did not change (**Figure 5A,B**). A more detailed analysis showed that Treg numbers were increased from PBD 7 until 39 (**Figure 5C**). Also, we confirmed the increase in chemokine receptors (CCR4 and CCR6) expressing CD4^{+} T cells and Tregs (**Figures 5F,I**). Furthermore, we could confirm the increase in CCR4^{+}CCR6^{-} Tregs (**Figure 5H**) after PBD 7, and observed a constant level of CCR4^{-}CCR6^{+} CD4^{+} T cells (**Figure 5D**). We found more CCR4^{+}CCR6^{+} CD4^{+} (non Treg) T cells than Tregs, suggesting that the balance might be tipped, enhancing the inflammation rather than resolving

it. Thus, within the lymphocyte population, there was an increase in effector cells and Tregs from week 2 onward that show a mixed pro- and anti-inflammatory phenotype.

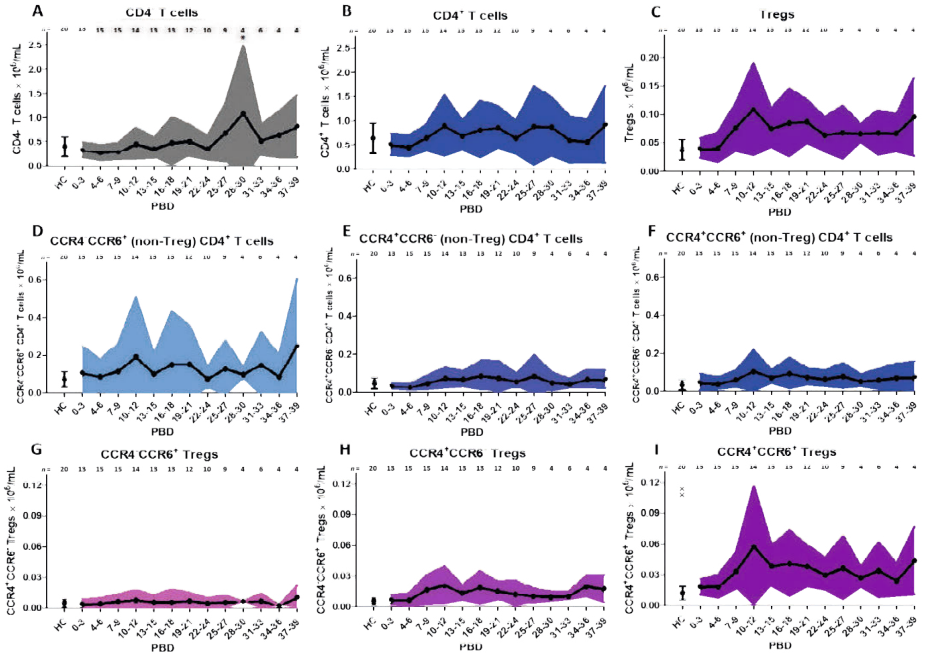

Figure 5. Supervised analysis of blood lymphocyte subsets after severe burn injury. Flow cytometry results of: (**A**) $CD4^-$ T cells ($CD3^+CD4^-$ lymphocytes). (**B**) $CD4^+$ T cells ($CD3^+CD4^+$ lymphocytes). (**C**) Tregs ($CD3^+CD4^+CD25^+CD127^-$ lymphocytes). (**D**) $CCR4^-CCR6^+$ $CD4^+$ (non-Treg) T cells; (**E**) $CCR4^+CCR6^-$ $CD4^+$ (non-Treg) T cells; (**F**) $CCR4^+CCR6^+$ $CD4^+$ (non-Treg) T cells; (**G**) $CCR4^-CCR6^+$ Tregs; (**H**) $CCR4^+CCR6^-$ Tregs; (**I**) $CCR4^+CCR6^+$ Tregs. Number of subjects per time interval is shown on top of the graphs. Cell subset concentrations of burn wound patients and healthy controls (HC) are shown as mean (line and dots) ± standard deviation (colored band). Asterisks indicate significant differences in time within the burn patient group (linear mixed model analysis): $^*p < 0.05$. Significant differences of outcomes in burn patients on PBD 0-3 compared to healthy controls are indicated by $^{\times}$ ($^{\times\times}p < 0.01$).

Burn Injury Induces High Levels of Circulating Pro-Inflammatory Immune Mediators

To study circulating immune mediators induced by burn injury, we screened a broad panel of 33 cytokines, chemokines and growth factors in plasma of burn wound patients from PBD 0 until 48. To highlight significant changes in burn wound patients, data was transformed to fold changes in relation to the levels detected in healthy controls and presented in volcano plots (**Figure 6A-F**). Pro-inflammatory cytokines IL-6 and IL-8 were increased at all time intervals. Furthermore, we found an increase in chemokines

MCP-1 (CCL2), MIP-1β (CCL4), RANTES (CCL5) and MIP-3α (CCL20), which are known chemoattractants for monocytes, granulocytes and T cells during inflammation [26]. IL-10 levels were only increased at PBD 0–3. RANTES and TGF-β2 were decreased at PBD 4–7 and increased at PBD12–28, following a similar pattern as the number of thrombocytes after burn injury (**Supplementary Figure 2C**). A summary of the significant increases and decreases in soluble factors is presented in a heatmap (**Figure 6G**).

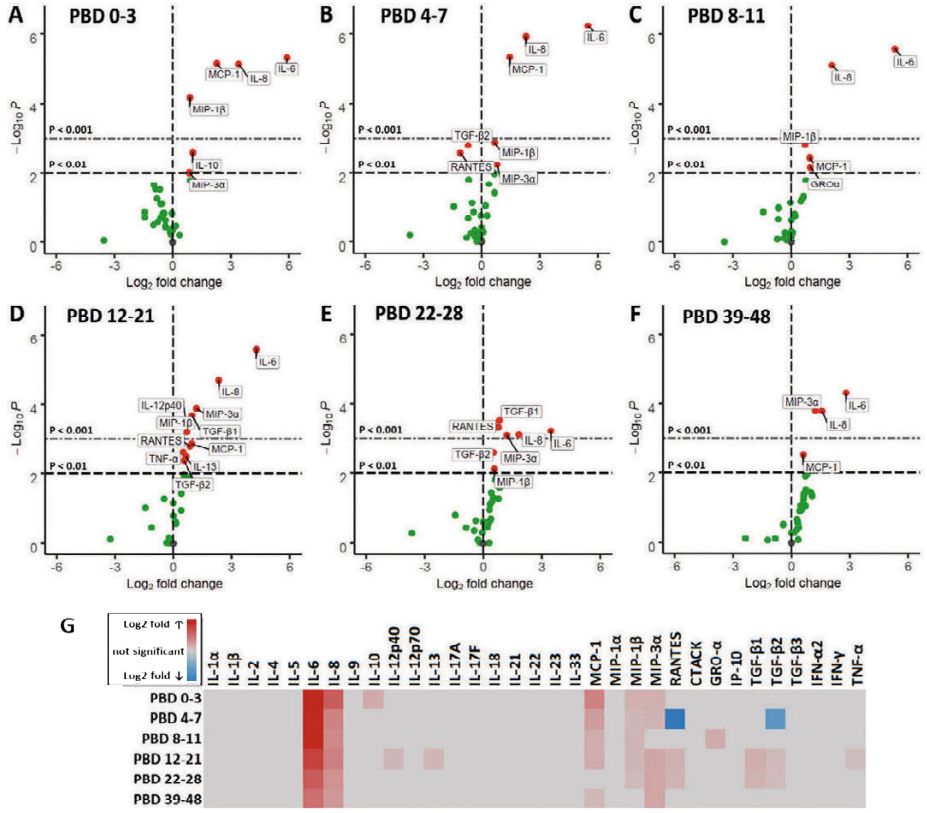

Figure 6. Volcano plots of 33 plasma immune factors after severe burn injury. Soluble mediators were analyzed in plasma of burn patients and healthy controls by Luminex immunoassay: MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), GRO-α (CXCL1), IP-10 (CXCL10), IFN-α2, IFN-γ, TNF-α, TGF-β1, TGF-β2, TGF-β3, CTACK (CCL27), RANTES (CCL5), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A (CTLA-8), IL-17F, IL-18, IL-21, IL-22, IL-23, and IL-33 (NF-HEV). Differences between burn and healthy group were expressed as (Log2) fold change of healthy group (n = 13) on the x-axis and the (-Log10) p value on the y-axis of various time intervals after burn. (**A**) PBD 0 to 3 (n = 10 patients). (**B**) PBD 4 to 7 (n = 14 patients). (**C**) PBD 8 to 11 (n = 13 patients). (**D**) PBD 12 to 21 (n = 15 patients). (**E**) PBD 22-28 (n = 13 patients). (**F**) PBD 39 to 48 (n = 8 patients). Because of multiple testing, we considered a p value of < 0.01 to be significant. Black dashed line shows p = 0.01, gray dashed line shows p = 0.001, green dots indicate non-significant changes and red dots show significant changes. (**G**) Heatmap of significant (p < 0.01) fold changes compared to healthy controls (Log2 fold). Fold changes are shown in gray (not significant), red (increase) or blue (decrease).

Next, we correlated immune cell subset numbers to the fold changes of soluble mediators by Pearson's correlation coefficient test and visualized the significant (p < 0.05) correlations in a heatmap (**Figure 7**). In the first week after injury, the most pronounced positive correlations were found between mature neutrophils and IL-1β,

IL-13 and IL-17A ($r > 0.7$; $p < 0.001$) and between non-classicalmonocytes and IL-4 ($r > 0.5$; $p < 0.001$). In the first and second week, there were negative correlations between classical monocytes and IL-6, IL-8, MCP-1 and GRO-α (r between -0.4 and -0.7; $p < 0.001$). Immature neutrophils showed a strong positive correlation in the third week with IL-6, IL-8, IL-10, MCP-1 and MIP-3α and in the fourth week with MCP-1 ($r > 0.8$; $p < 0.001$). In the first 2 weeks after injury, there were only weak positive correlations between T cells and soluble mediators, but this pattern changed in week 3 and 4, where strong correlations were predominantly found between T cells and immune mediators. In week 3, $CD4^+$ T cells and Tregs showed strong negative correlations with MIP-1β ($r < -0.8$; $p < 0.001$) and $CCR4^+CCR6^-$ Tregs showed a strong positive correlation with TGF-β1 and TGF-β2 ($r > 0.7$; $p < 0.001$). Four weeks after injury, we observed positive correlations between $CCR4^-CCR6^+$ $CD4^+$ T cells and MIP-1α, GRO-α and IFN-γ ($r > 0.8$; $p < 0.001$) and strong negative correlations between $CD4^+$ T cells and IL-4, IL-8, IL-12p40, IL-13, IL-17A, IFN-γ and TNF-α ($r < -0.9$; $p < 0.001$). Seemingly, in week 1–3 the presence of innate immune cells can be linked to pro-inflammatory cytokines, while in week 3–4 most pronounced correlations were found between $CD4^+$ T cell subsets and specific mediators (**Figure 7**).

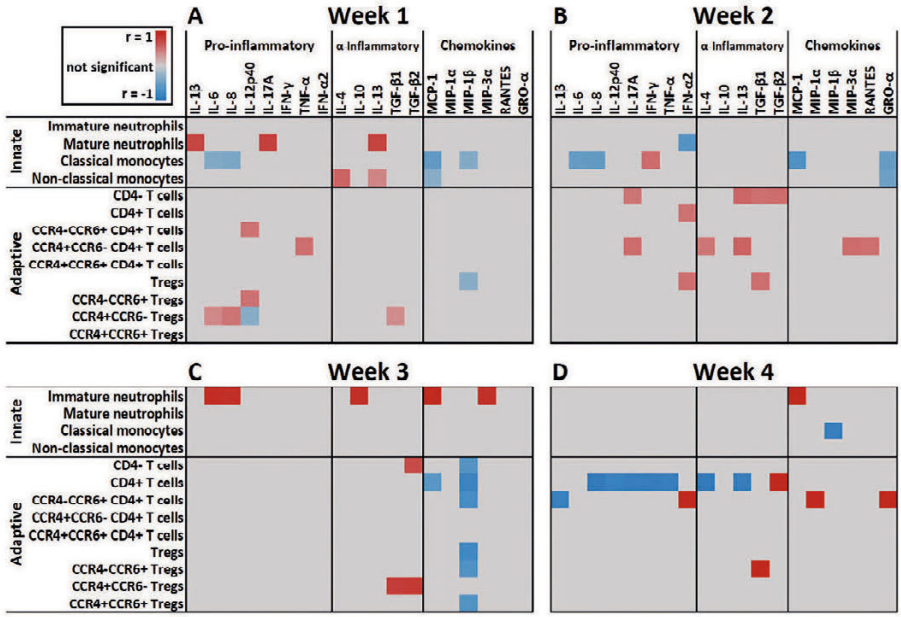

Figure 7. Heatmap of correlation coefficients of immune cells and soluble mediators over time. Significant ($p < 0.05$) correlation coefficients (r) of immune cell counts and fold change of soluble mediators at: (**A**) Week 1 (n = 13 patients); (**B**) Week 2 (n = 15 patients); (**C**) Week 3 (n = 10 patients); (**D**) Week 4 (n = 5 patients) after burn injury. Correlations were measured by Pearson test and results are shown in gray (not significant), red (positive correlation) or blue (negative correlation).

DISCUSSION

Here, we performed a longitudinal study on 20 severely burned patients and investigated the effects of severe burn injury on the systemic immune response. We reveal that upon burn injury there is an immediate surge of innate immune cells, with initially a large contribution of immature neutrophils, but no increase in lymphocyte numbers. These cellular responses could not be correlated to the patient's TBSA, which might indicate maximum response levels. Notably, only patients submitted to the ICU were included in this study. Simultaneously with the cellular influx, increased levels of various pro-inflammatory cytokines were found. This innate immune and cytokine response decreased to some extent over time, but persisted for at least three weeks. From the second week onward, shifts in T cell subpopulations were observed: within the T cell population, there was an increase of CCR4 and CCR6 expressing cells and although Treg numbers increased as well, the overall phenotype of the $CD4^+$ T cells and Tregs appeared to be rather pro-inflammatory than anti-inflammatory.

The increase in granulocytes could mainly be attributed to neutrophils, and within this population both mature and immature neutrophils were increased. In the first week after injury, mature neutrophil counts correlated with IL-17A, which is known to accelerate neutrophil recruitment [27]. Moreover, levels of IL-6 and IL-8 were increased over the complete study period and could be correlated to the number of immature neutrophils in the third week post injury. IL-6 and IL-8 are involved in neutrophil recruitment and chemotaxis [27,28]. In a healthy situation, immature neutrophils are usually absent in the circulation as neutrophils normally mature within the bone marrow before they are released to the bloodstream [29]. The early release of immature neutrophils can be caused by an emergency response of the immune system to acute inflammation, such as trauma, burn or sepsis [25,30,31]. During acute inflammation, neutrophils produce ROS, elastase, myeloperoxidase and release neutrophil extracellular traps (NETs) which can damage tissues by their cytotoxicity and can cause ischemia through thrombocytosis [10,32]. In other studies of patients with large burn injuries (>15% TBSA), expression of CD11b on neutrophils was increased in the first week while expression of CD16 was reduced [33,34]. Other studies also reported a fast decrease of CD16 expression due to the immaturity of neutrophils [35,36]. We also observed reduced CD16 expression on immature neutrophils (data not shown). It has been shown that continuous release of neutrophils into the circulation can lead to bone marrow exhaustion that in turn can lead to compromised innate immunity [37–39]. Although literature on the functions or activities of immature neutrophils is conflicting, some papers state that immature neutrophils are underdeveloped and that high numbers and their active state might induce tissue damage causing secondary progression of the burn injury [13,37,38]. Other

studies have shown that trauma-induced immature neutrophils in blood of patients with systemic inflammation actually show decreased chemotactic activity and increased life-span, and therefore reside longer in the bloodstream then mature neutrophils [40–42]. Also, it was shown that immature neutrophils have a reduced oxidative burst and phagocytic activity and that they are less potent in supporting innate immune defenses [42–44]. Another study showed that the reduced oxidative burst in neutrophils can last for up to 3.5 months [45]. It is however still unclear whether immature neutrophils are overall beneficial or detrimental for wound healing [46]. Nevertheless, our data demonstrate that burns can cause a long-lasting presence of both immature and mature neutrophils that may be harmful for wound healing, distant organs and survival.

During wound healing, the first cells attracted to the site of injury are neutrophils, followed by monocytes, which upon arrival differentiate into macrophages or dendritic cells [47]. In response to acute systemic inflammation, the bone marrow releases its reserve of classical monocytes into the bloodstream to replace the monocytes that migrated into inflamed tissue [48]. CD14, a co-receptor of various Toll-like receptors, is increased on monocytes upon burn injury and helps to detect bacteria in the body [42,49]. We found increased numbers of all three monocyte subtypes in blood, while classical monocytes were the most prevalent. Classical monocytes mainly exert pro-inflammatory functions and can become monocyte-derived macrophages or dendritic cells upon infiltration of inflamed tissue [50]. Negative correlations were found between classical monocytes and MCP-1, a known chemoattractant for monocytes [51]. Binding of MCP-1 to CCR2 on circulating monocytes might have resulted in lower levels of free MCP-1 [52,53]. Alternatively, MCP-1 might have induced migration of classical monocytes toward affected tissues [52]. In this cohort of burn patients, the number of classical monocytes remained elevated for at least 39 days. Other studies on burn patients also found increased levels of classical monocytes during systemic inflammation [54]. Non-classical monocytes, that are described as more anti-inflammatory monocytes are thought to acquire the pro-healing macrophage phenotype (M2) in the injured tissue [52]. Although we found an increase in this monocyte subtype upon burn injury as well, their numbers were much lower than that of classical monocytes. Taken together, this might indicate that the balance of monocyte phenotypes is shifted toward a pro-inflammatory, rather than an anti-inflammatory state, that persists for weeks after burn injury.

Later in the posttraumatic immune response (days to weeks), lymphocytes arrive at the site of injury to regulate the inflammation and support tissue restoration together with pro-healing macrophages [55]. To our knowledge there is no information on the dynamics of T cell activation and differentiation after burn injury in humans. Here, we established that while the number of lymphocytes in blood was largely unaffected upon burn injury,

the T cell subset composition was altered from the second week after injury, indicative of an adaptive immune response [56]. To study the phenotype of circulatory T cells in burn patients, we analyzed CCR4 and CCR6 expression and found increased numbers of $CCR4^+CCR6^+$ $CD4^+$ T cells, which might indicate a shift toward a Th17 T cell phenotype. This notion was supported by increased levels of MIP-3α, a natural ligand of CCR6 [57], from PBD 12 onward, as well as high levels of IL-6, TGF-β1, and TGF-β2, which in combination can induce a Th17 response [18,58]. Additionally, we observed increased numbers of $CCR4^+CCR6^-$ $CD4^+$ T cells, indicative of a Th2 phenotype [59]. This was associated with an increase in IL-13, a Th2 cytokine [60], at PBD 12–21. Animal experiments have also shown that burn injury induces a mixed Th2/Th17 response. Moreover, IL-17 which is released by Th-17 cells is involved in the recruitment and activation of neutrophil [18,61,62]. This might explain the high neutrophil counts that peak during PBD 16–21. Burn injury was also associated with an increase of Tregs, which are likely part of the immune system's attempt to resolve the acute inflammation [63]. Upon in vitro culture, Treg from severely burned patients produced elevated levels of IL-10 in the first 21 days after injury [64]. Here, plasma levels of IL-10 were only increased at PBD 0–3. An early increase of serum IL-10 was also found in severely burned children, which was followed by a small, non-significant elevation of circulatory IL-10 afterward [5,65,66]. This suggests that in vivo, the suppressive response from Tregs might be impaired after PBD 3, possibly due to the high levels of pro-inflammatory cytokines and number of acute phase immune cells. In addition, we found evidence for Treg differentiation, as both $CCR6^+$ and $CCR6^-$ Tregs were present. This suggests that there is a burn-induced mixed phenotype within the Treg population [57,67]. The transformation of Tregs into putative pathophysiologic Tregs has been proposed before [68,69] and, in this case, could be caused by burn-induced DAMPs and pro-inflammatory mediators such as the CCR6 ligand MIP-3α. Although functional assays are needed to verify the phenotype of these Tregs, our data suggest that severe burn injury causes a shift in the T cell subsets toward more pro-inflammatory subtypes, tipping the balance and thereby continuing the inflammation.

The increase in inflammatory mediators is indicative of a persistent systemic inflammatory immune response due to severe burns. However, all included burn patients were at high risk for infection, such as central line-associated bloodstream infection. These infections were not observed in this study but bacterial presence could have influenced the levels of inflammatory mediators. Medication could have affected the immune response, but all patients were treated in a similar manner, involving the administration of antibiotics and analgesics. Although the differences in immune components in the blood between burn wound patients and healthy controls were significant and remained increased over time, the sample size is a limitation of our study. Missing data was caused by less frequent blood withdrawal at the infirmary, delayed start

of study participation, patient discharge and death. In addition, we acknowledge that the difference in age and gender between burn patients and healthy controls represents a limitation of this work, as aging and gender can affect the immune response [70,71]. Unfortunately, the group size was insufficient to analyze these differences in more detail. Supervised gating of flow cytometry data can be challenging due to biological variation and the fact that manual gating relies on the researcher's prior knowledge causing bias in the analysis [72]. To overcome this, we took a combined approach of both supervised and unsupervised analysis of our data, and showed that they largely reached the same outcome. To better understand the behavior of these immune cells, it would be interesting to study functionality of these cells after burn injury. Furthermore, we are curious to see if the observed systemic immune response is reflected by the local immune response to burn injury and will pursue this aim in the near future.

Information on the immunological mechanisms driving burn-induced inflammation and pathophysiology is very limited. Because of the excessive and persistent inflammation, it could be beneficial for burn wound patients to use anti-inflammatory drugs [73]. Directed therapy that either decreases the influx of neutrophils or supports the suppressive arm of the immune system, might lower the risk of complications caused by the systemic inflammation, which in turn should improve wound healing. Because of variation between burn wounds, patients and differences in the intensity of the burn-induced immune response, treatment should be empirical and personalized to improve the outcome.

Taken together, we showed that the burn-induced leukocytosis is mainly due to an increase of neutrophils and monocytes and that burn injury caused a long-lasting influx of immature neutrophils. The persistent elevated levels of pro-inflammatory cytokines and the shifts in neutrophil and lymphocyte composition suggest that the immune system remains in a long-term pro-inflammatory state rather than switching to a resolving state. Because these immune reactions are likely to strengthen one another and keep the inflammation going, we need to search for ways to resolve inflammation in an early stage in order to improve burn treatment, prevent secondary complications, and reduce length of hospital stay.

MATERIALS AND METHODS

Subject Recruitment and Sample Collection

Twenty burn wound patients admitted to the intensive care unit (ICU) of the Burn Center of the Red Cross Hospital in Beverwijk, the Netherlands were included in this study after written consent was obtained from the patient or a legal representative

(for subject details see **Supplementary Table 1A**). The study protocol with number "NL54823.094.15" was approved by the METc of the VU Medical Center (Amsterdam, the Netherlands). Patients were eligible from 18 years of age with a burned total body surface area (TBSA) of ≥15%. Subjects were included between April 2018 and April 2020. Venous blood samples were collected on a daily basis when present on ICU or twice per week when transferred to the infirmary. Blood samples taken from 20 healthy volunteers served as controls (METc approved under protocol number "NL54823.094.15"). Blood levels of C-reactive protein (CRP), albumin and thrombocytes were determined according to standard diagnostic laboratory procedures as part of standard burn care. Blood samples for flow cytometry were collected in ethylenediaminetetraacetic acid (EDTA) tubes and were stored at 4°C until analysis (<3 h). Only blood samples from working days were used for flow cytometric analysis. The frequency of sampling of each individual patient is presented in **Supplementary Table 1B**. The patients were treated according to standard burn care, including fluid resuscitation. All patients received analgesics (including paracetamol, NSAIDs and opiates) and antibiotics One of the included patients died two days after the trauma. Three patients contracted pneumonia, one patient had an infected hematoma and none of the patients had sepsis or full-blown infection due to their burn injuries. Colonization of burn wounds was noted, and the predominant bacterial species were Staphylococcus aureus (10/20), Enterococcus cloaca (10/20), Pseudomonas aeruginosa (7/20) and Escherichia coli (5/20).

Flow Cytometry

Plasma was separated from blood cells by centrifugation for 10 min at 450 × g and stored at -80 °C. Erythrocyte lysis buffer (1.5 mM NH_4Cl, 0.1 mM $NaHCO_3$ and 0.01 mM EDTA (Life Technologies, Paisley, UK) in demineralized water) was used to remove erythrocytes from the blood cells. Blood immune cells were resuspended in Dulbecco's phosphate buffered saline (Gibco, ThermoFisher, Paisley, UK) containing 0.2 mM bovine serum albumin (Fisher Scientific, Pittsburgh, PA) and 0.01 mM EDTA. Cell concentrations were determined by a flow cytometer (MACS Quant Analyzer 10, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Cell suspensions of 2.5 × 105 cells each were stained with different antibody combinations (see panels in **Supplementary Table 2**) and were analyzed by flow cytometry (MACS Quant Analyzer 10). Samples with more than 40% dead cells (based on 7-AAD staining (Miltenyi)) were excluded from the analysis. Singlet events were gated based on FSC. Viable CD45+ cells were gated and subtyped based on expression of the markers in the 3 staining panels: innate panel (CD10, CD11b, CD14, CD15, and CD16), eosinophil panel (CD9, CD15, and CD16), and lymphocyte panel (CD3, CD4, CD25, CD127, CCR4/CD194, and CCR6/CD196). Manual data analysis was performed using the FlowLogic software (Inivai Technologies, Victoria, Australia).

Gating Strategy for Supervised Flow Cytometry

The gating strategy is shown in **Supplementary Figure 1**. Viable CD45+ cellswere gated on FSC and SSC to characterize granulocytes, monocytes and lymphocytes. Subsequently, cells were determined as follows: immature neutrophils ($CD10^{dim}CD15^{+}CD16^{+}$ granulocytes), mature neutrophils ($CD10^{bright}CD15^{+}CD16^{+}$ granulocytes), eosinophils ($CD9^{+}CD15^{+}CD16^{-}$ granulocytes), classical monocytes ($CD14^{bright}CD16^{-}$ monocytes), intermediate monocytes ($CD14^{bright}CD16^{+}$ monocytes), non-classical monocytes ($CD14^{dim}CD16^{+}$ monocytes), T cells ($CD3^{+}$ lymphocytes), and Tregs ($CD3^{+}CD25^{+}CD127^{-}$).

Unsupervised Analysis of Flow Cytometry Data

The innate and lymphocyte panel were used for unsupervised analysis in Cytobank [74]. Viable monocytes, granulocytes or lymphocytes were gated using 7-AAD and CD45 staining and FSC/SSC in MACSQuantify 2.13 software (Miltenyi). The data was uploaded to Cytobank to create Flow Self-Organizing Map (FlowSOM) cluster plots.

Plasma Cytokine Analysis

Plasma samples were thawed, and debris was removed using a filter plate (Multiscreen, Merck KGaA, Darmstadt, Germany). Luminex assay was performed according to the manufacturer's instructions (Merck KGaA). The following assay kits were used: HCYTA-60K, TGFBMAG-64K, HCYTA-60K, HCYP2MAG-62K and HTH17MAG-14K. In short, 25 µL of plasma was used to determine the concentrations of 33 cytokines and chemokines, namely MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), GRO-α (CXCL1), IP-10 (CXCL10), IFN-α2, IFN-γ, TNF-α, TGF-β1, TGF-β2, TGF-β3, CTACK (CCL27), RANTES (CCL5; in a 1:100 dilution), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL- 12p70, IL-13, IL-17A (CTLA-8), IL-17F, IL-18, IL-21, IL-22, IL-23, and IL-33 (NF-HEV). Mean fluorescence intensity of samples was measured with a Flexmap 3D System (Luminex Corp, Austin, USA) and concentrations were calculated using Bio-Plex Manager Software (Bio-Rad Laboratories, Veenendaal, The Netherlands). When cytokine levels were out of range of the standard, either the lowest level of quantification or the highest level of quantification was used. To combine results of multiple assays, we transformed the data to fold changes of healthy controls.

Statistical Analyses

Distribution of the data was checked for normality. For the flow cytometry data, differences between the levels of outcomes of patients on PBD 0–3 and healthy controls were explored using the Mann Whitney U test. Results per time interval (e.g., PBD 0–3) were averaged per patient. Differences in outcomes within patients between time intervals PBD 4–6 through PBD 37–39 vs. PBD 0–3 were analyzed in SPSS version 25 (IBM, Armonk, USA) using linear mixed model to correct for the dependent data structure.

The outcome measurements were used as the dependent variable in the models. Time was entered as a categorical variable in the model as a fixed effect. Level of statistical significance was set at $p < 0.05$. The data was visualized using Graphpad version 5.01 (PRISM, La Jolla, USA).

Data of the soluble immune factors was transformed to fold changes of healthy controls. P values between time intervals and healthy controls were determined using Mann Whitney U test. Because of multiple testing, we considered a p value of <0.01 to be significant. Volcano plots were created using "EnhancedVolcano" version 1.6.0 package in R version 3.6.2.

ACKNOWLEDGMENTS

We are grateful for the work of all involved physicians, surgeons and nurses of the burn center and intensive care department of the Red Cross Hospital. We want to thank Evi Warmerdam and Rosa Rentenaar for their technical assistance and help with data analysis. This research was funded by the Dutch Burns Foundation under grant number WO/17.108 (MMWU).

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the article at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.621222/full#supplementary-material.

Supplementary Table 1. Demographics and sampling of subjects. (**A**) Demographic data is shown per patient and per group. For the burn patients the age range was 18-82 and for the healthy controls this was 23-62. (**B**) Black dots show frequency of blood sampling of individual burn patients. For the analyses, multiple measures of one patient within one time interval were averaged.

A

Subject number	Gender	Age (years)	Total burn surface area (%)			Burn type	Hospital stay (days)	Medication	Complications
			Total	2nd degree	3rd degree				
1	Male	20	34	17	17	Flame	42		
2	Male	35	20	17	4	Flame	22		
3	Male	82	15	15	0	Water	24	Antidiabetics; finasteride	
4	Male	39	17	17	0	Flame	12	Antidiabetics	
5	Female	78	26	6	21	Flame	2 (†)	Antidiabetics; pantoprozole; oxycodone; acetylsalicylic acid; allopurinol; pravastatin; thyrax	Died after 2 days
6	Female	57	23	0	23	Flame	60	Antidiabetics	Thrombosis
7	Male	42	28	28	0	Flame	29		
8	Male	61	47	25	22	Flame	40	Antidiabetics; baclofen; midazolam; laxatives	Pneumonia; glucose dysregulation

Supplementary Table 1. Demographics and sampling of subjects. Continued.

A				Total burn surface area (%)						
Subject	**number**	**Gender**	**Age (years)**	**Total**	**2nd degree**	**3rd degree**	**Burn type**	**Hospital stay (days)**	**Medication**	**Complications**
	9	Female	65	29	1	28	Flame	60		Infected hematoma
	10	Male	18	24	20	5	Flame	23		
	11	Male	33	32	24	8	Flame	19		Thrombophlebitis
	12	Male	39	38	29	9	Electricity	52	Antidiabetics; quetiapine	Pneumonia; cocaine user
	13	Female	43	23	23	0	Flame	37		
	14	Male	60	17	17	0	Oil	26		
	15	Male	57	20	20	0	Flame	24		
	16	Male	49	49	14	35	Electricity	119		Renal insufficiency
	17	Male	56	55	4	51	Flame	68	Antidiabetics; flupentixol	Thrombosis; polyneuropathy
	18	Male	46	34	21	13	Flame	57	Antidiabetics	
	19	Male	68	27	27	0	Flame	71		Pneuomia
	20	Male	73	25	20	5	Flame	75		
Burn patients in mean (±SD)		**16 male; 4 female**	**51.1 (±17.8)**	**29.0 (±11.0)**	**17.1 (±8.6)**	**11.9 (±14.2)**	**NA**	**45.3 (±26.5)**		
Healthy controls in mean (±SD)		**7 male; 13 female**	**43.9 (±10.9)**	**NA**	**NA**	**NA**	**NA**	**NA**		

Post burn day

B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
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11		•		•		•			•	•	•	•	•			•		•		•																				
12				•	•	•	•	•			•	•	•	•	•			•				•					•					•		•					•	
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17					•	•	•	•			•	•	•	•				•	•	•		•			•	•	•					•		•		•				
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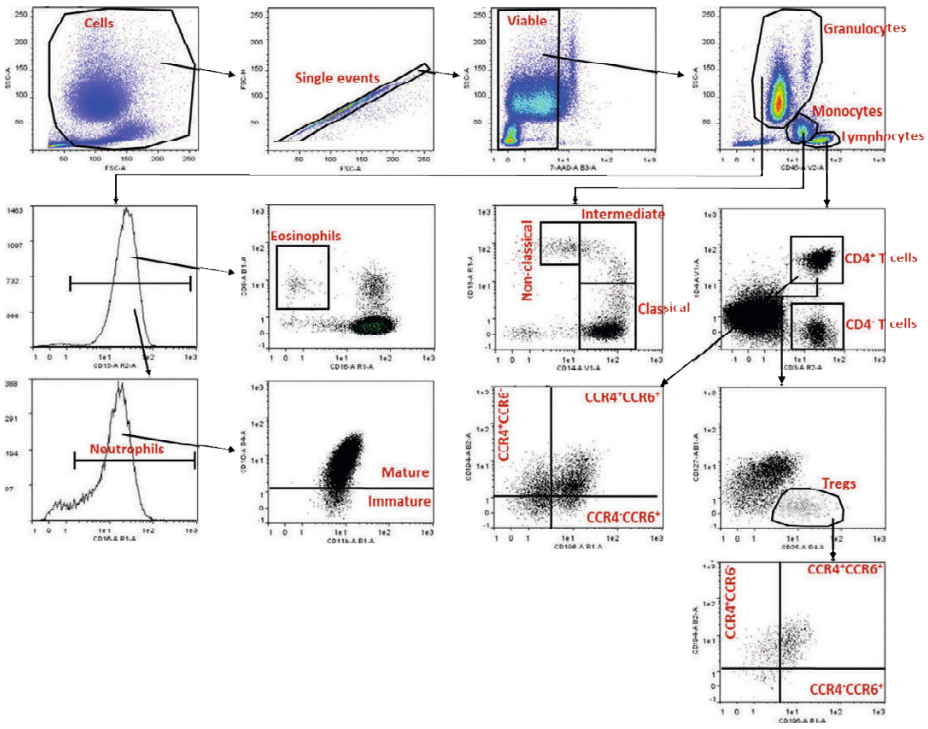
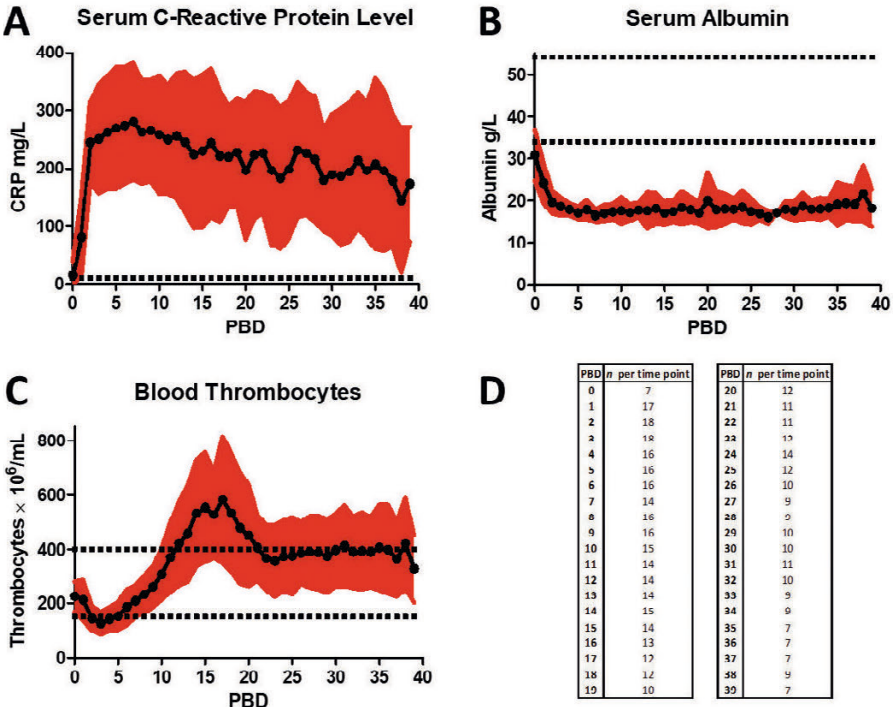
19			•	•			•	•			•			•	•	•	•	•			•		•	•				•		•										
20		•	•	•	•				•		•	•	•		•	•	•		•				•		•		•					•		•			•		•	
# per time point	2	7	7	10	10	10	10	9	7	6	9	10	10	9	7	8	7	6	6	4	6	5	6	6	3	5	6	3	2	4	1	5	1	4	0	3	2	2	4	1
# per time interval	14				15			15			14			13			13			11			10			10			5			6			4			5		

Supplementary Table 2. Antibodies used for flow cytometry. All antibodies were purchased at Miltenyi Biotec GmbH, Bergisch Gladbach, Germany.

Antibody	Panel	Conjugate	Clone
anti-CD3	Lymphocyte	APC-Vio770	REA613
anti-CD4	Lymphocyte	VioBlue	REA623
anti-CD9	Eosinophil	FITC	REA1071
anti-CD10	Innate	PE-Vio770	REA877
anti-CD11b	Innate	FITC	REA713
anti-CD14	Innate	VioBlue	REA599
anti-CD15	Innate, eosinophil	APC-Vio770	REA321
anti-CD16	Innate, eosinophil	APC	REA423
anti-CD25	Lymphocyte	PE-Vio770	REA945
anti-CD45	Innate, eosinophil, lymphocyte	VioGreen	REA747
anti-CD127	Lymphocyte	FITC	REA614
anti-CCR4/CD94	Lymphocyte	PE	REA279
anti-CCR6/CD196	Lymphocyte	APC	REA613



Supplementary Figure 1. **Gating strategy for supervised flow cytometry analysis.** Gating strategy is shown for all three panels.



PBD	*n* per time point
0	7
1	17
2	18
3	18
4	16
5	16
6	16
7	14
8	16
9	16
10	15
11	14
12	14
13	14
14	15
15	14
16	13
17	12
18	12
19	10

PBD	*n* per time point
20	12
21	11
22	11
23	10
24	14
25	12
26	10
27	9
28	9
29	10
30	10
31	11
32	10
33	9
34	9
35	7
36	7
37	7
38	9
39	7

Supplementary Figure 2. Levels of systemic inflammation indicators in blood of burn patients. Laboratory results of: (**A**) Serum CRP levels compared to healthy reference values (dotted lines: 0-10 mg/L). (**B**) Serum albumin levels compared to healthy reference values (dotted lines: 34-54 g/L). (**C**) Blood thrombocyte counts compared to healthy reference values (dotted lines: 150-400 × 10^6/mL). (**D**) Number of patients per time point in A-C. Values of burn wound patients are shown as mean (line and dots) ± standard deviation (colored band).

CHAPTER 5

Burn-injured skin is marked by a prolonged local acute inflammatory response of innate immune cells and pro-inflammatory cytokines

Published in Frontiers in Immunology, **2022***, 13, 1034420*
DOI: 10.3389/fimmu.2022.1034420

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ABSTRACT

The systemic and local immune response in burn patients is often extreme and derailed. As excessive inflammation can damage healthy tissues and slow down the healing process, modulation of inflammatory responses could limit complications and improve recovery. Due to its complexity, more detailed information on the immune effects of thermal injury is needed to improve patient outcomes. We therefore characterized and quantified subsets of immune cells and mediators present in human burn wound tissue (eschar), sampled at various time points. This study shows that after burn injury, the number of immune cells were persistently increased, unlike the normal wound healing process. There was an immediate, strong increase in neutrophils and a moderate increase in monocytes/macrophages and lymphocytes, especially in the second and third week post burn. The percentage of classical ($CD14^{high}CD16^{-}$) monocytes/macrophages demonstrated a steady decrease over time, whereas the proportion of intermediate ($CD14^{high}CD16^{+}$) monocytes/macrophages slowly increased. The absolute numbers of T cells, NK cells and B cells increased up to week 3, while the fraction of γδ T cells was increased only in week 1. Secretome profiling revealed high levels of chemokines and an overall pro-inflammatory cytokine milieu in burn tissue. The local burn immune response shows similarities to the systemic immune reaction, but differs in neutrophil maturity and lymphocyte composition. Altogether, the neutrophil surges, high levels of pro-inflammatory cytokines and limited immunosuppression might be key factors that prolong the inflammation phase and delay the wound healing process in burns.

INTRODUCTION

Burn injury is often accompanied by an extensive, derailed immune response in both burn wound tissue and peripheral blood [1,2]. Regardless of infection, burn patients generally show signs of systemic inflammation caused by high levels of cytokines and danger signals that originate from damaged tissue [3,4]. Necrotized and inflamed tissue stimulates the immune system to recruit acute phase immune cells to the affected site [2,5,6]. Fibroblasts and keratinocytes surrounding the wound site and infiltrating leukocytes release a storm of cytokines, chemokines and growth factors that initiate the inflammation phase [7].

Typically during wound healing, neutrophils and macrophages with a pro-inflammatory (i.e. M1) phenotype will migrate into the wounded skin to remove debris and prevent bacterial colonization [8]. Within days, wound neutrophils will disappear through apoptosis and macrophages will differentiate into a state that supports wound healing (i.e. M2 phenotype) [9]. Generally within one week after injury, lymphocytes will infiltrate the wound site to orchestrate tailored pathogen-eliminating and immune cell regulating responses [10]. The reduction, transition and control of immune cells are crucial for dampening of the inflammatory response and for the establishment of a healthy wound healing process. After burn injury however, the immune system can be overactive and is then likely to cause damage to surrounding tissues, delay wound healing and contribute to the severity of scarring [2,6].

Burn patients who experience persistent inflammation might benefit from immune suppressive treatment, however at the same time they are at risk of contracting infections such as pneumonia or cellulitis, caused by opportunistic bacteria [11]. Therefore, innovative and precise interventions that modulate the immune response could be crucial in the relief of secondary illnesses while improving wound healing and preventing infection. Still, there is only little information on the immune response after burn injury and how exactly it differs from normal wound healing, mainly due to its complexity and variation among cases (e.g. burn size, depth and cause) and burn patients (e.g. age, sex and co-morbidities) [12]. Moreover, present evidence on the processes that underlie burn injury originates mostly from animal research [13], which is only partially translatable to the human situation [14]. We previously showed that in blood from severely burned patients, there was an extreme increase in innate immune cells and pro-inflammatory cytokines [7]. In this longitudinal study, we investigated immune cells and soluble factors present in burn wound tissue (eschar) that was surgically debrided as part of standard treatment [15]. A better understanding of the immune reactions to burn injury will facilitate the design of improved and more targeted treatment approaches for trauma-induced immune dysfunction.

RESULTS

Burn injury is followed by a strong local increase in granulocytes and moderate increase in monocytes and lymphocytes

Local immune effects of burn trauma were investigated in burn tissue that was debrided during routine surgical procedures (subject and sample characteristics shown in **Supplementary Table 1**). We selected viable sections of tissue biopsies and neglected necrotized or blackened segments to ensure the isolation of viable cells. CD45 immunohistochemical (IHC) staining showed an extreme infiltration of leukocytes ($CD45^+$ cells) in burn tissue (**Figure 1A**). The majority of leukocytes were viable after isolation from healthy skin (90.3% ± 6.6) and burn tissue (89.1% ± 10.5) (**Supplementary Figure 2A**). Flow cytometry (FCM)-based quantification revealed that the increase in leukocyte numbers was most abundant at post burn week (PBW) 2-3 (**Figure 1B**). As a result, the percentage of CD45- cells, which include fibroblasts, keratinocytes and endothelial cells, was lower in burn tissue from PBW 2-3 than in healthy skin (**Supplementary Figure 2B**). The leukocytes isolated from healthy skin consisted of approximately 25% granulocytes, 55% monocytic cells (monocytes and macrophages) and 20% lymphocytes (**Figure 1C**). In burn tissue from PBW 1, there were 52% granulocytes, while for the proportion of monocytic cells was 29%. The lymphocytes fraction in burn tissue was similar to healthy skin (19%). In burn tissue from PBW 2-4, the portion of granulocytes was still enlarged (55-62%), while the fraction of monocytic cells decreased further to 13-16% and the lymphocyte fraction increased (24-31%). During PBW 1-3, absolute number of granulocytes, monocytic cells and lymphocytes rose and declined only at PBW 4 (**Figure 1D**). Multiplex spatial phenotyping of healthy skin and burn tissue sections using CD3 and CD15 revealed dense areas populated with granulocytes and T cells in burn tissue (**Figures 1E,F**).

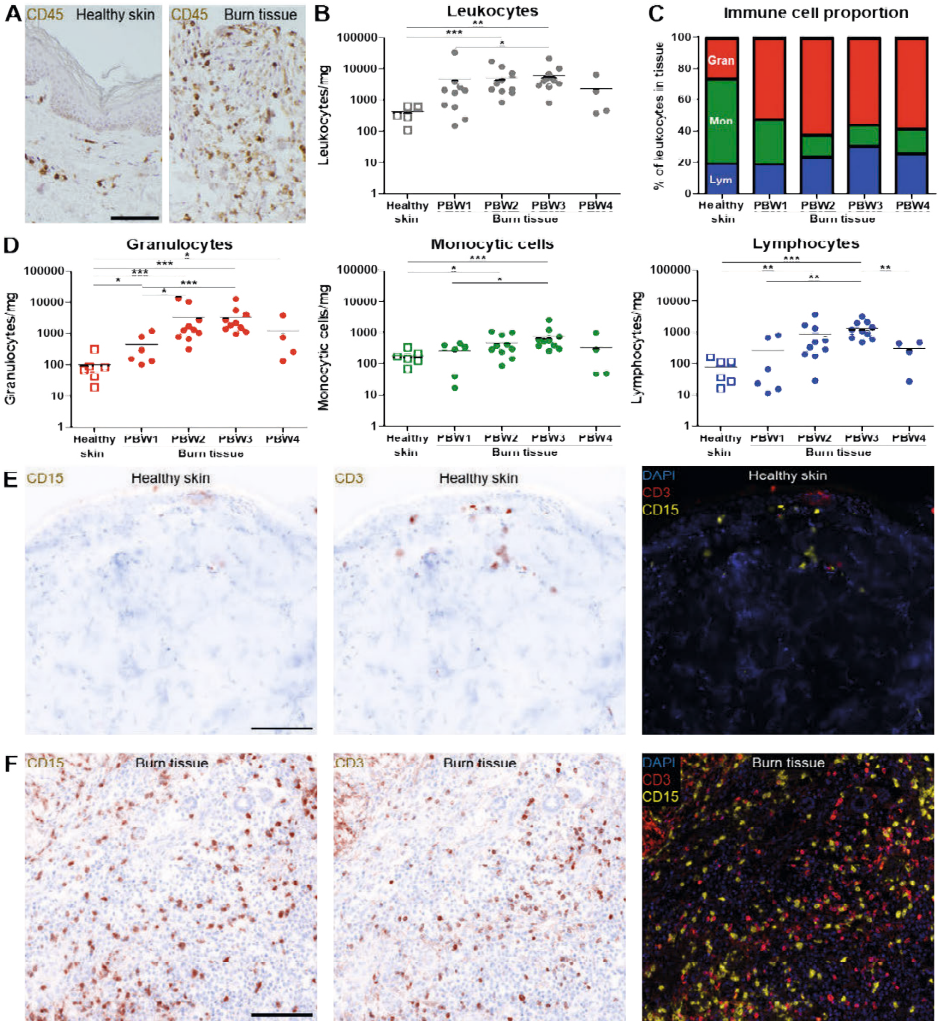

Figure 1. High number of immune cells infiltrate the skin as response to burn injury. (**A**) CD45 immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). Flow cytometry-based quantification of: (**B**) Absolute number of leukocytes per mg tissue (based on side scatter and CD45); (**C**) Proportion of granulocytes (Gran), monocytic cells (Mon) and lymphocytes (Lym) in tissue (based on side scatter and CD45); (**D**) Absolute numbers of granulocytes, monocytic cells and lymphocytes per mg tissue (based on side scatter and CD45). Microscopic image of multiplex DAPI, CD15 (granulocytes) and CD3 (T cells) staining of a representative: (**E**) Healthy skin sample; (**F**) Burn tissue sample (from 25 days post burn), shown separately and as composite (black scale bar = 100 µm). P values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

Granulocytes in burn tissue consist mainly of activated mature neutrophils

IHC analysis of myeloperoxidase (MPO) expression, an enzyme abundantly present in azurophilic granules of neutrophils [16], showed an immediate increase in neutrophil numbers in burn tissue at PBW 1, and an even larger increase from PBW 2 onward (**Figures 2A,B**). This was confirmed by FCM analysis of neutrophils ($CD15^+CD16^+$ granulocytes) (**Figure 2C**). Eosinophils ($CD9^+CD15^+CD16^-$ granulocytes) were increased at PBW 2-3, but to a lesser extent (**Supplementary Figure 2C**). In both healthy skin and burn tissue neutrophils were almost exclusively $CD10^+$, a marker that is associated with neutrophil maturation [17] (**Figure 2D**). Only in burn tissue from PBW 1 there was a slight increase in immature ($CD10^-$) neutrophils. Activation markers CD11b and CD66b were upregulated in neutrophils at PBW 2-3 (**Figures 2E,F**). Self-organizing map clustering of flow data (FlowSOM) using Cytobank displayed cell populations (nodes) and clusters based on marker expression in an unsupervised manner (**Figure 2G**). This analysis highlights some of the burn-specific changes that occur in wound neutrophils. Burn injury caused significant differences in the percentage of neutrophils per cluster (**Figure 2H**). $CD11b^{low}CD14^+CD66b^-$ neutrophils (cluster 1) were decreased early after burn injury, while $CD11b^+CD66b^{low}$ neutrophils (cluster 2) were increased. Although $CD11b^{high}CD66b^+$ neutrophils (cluster 3) seemed more represented in burn tissue than in healthy skin, no significant difference was found. A small population of $CD16^{low}$ neutrophils (cluster 4) was significantly increased at PBW 1 and the percentage of $CD16^{low}CD14^+$ neutrophils (cluster 5) was significantly increased at PBW 4.

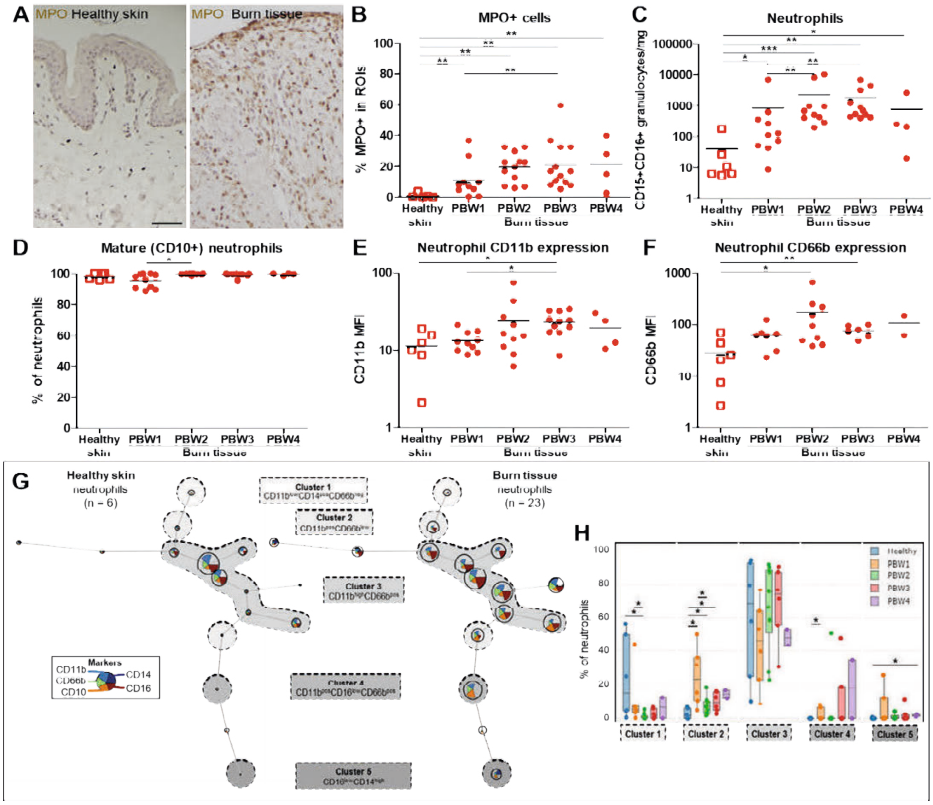

Figure 2. Local neutrophil response to burn injury. (**A**) Myeloperoxidase (MPO) immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). (**B**) MPO$^+$ area of tissue sections. Flow cytometry-based quantification of: (**C**) Absolute number of neutrophils (CD15$^+$CD16$^+$ granulocytes) per mg tissue; (**D**) Percentage of CD10$^+$ (mature) neutrophils (CD15$^+$CD16$^+$ granulocytes); (**E**) MFI of CD11b on neutrophils (CD15$^+$CD16$^+$ granulocytes) in tissue; (**F**) MFI of CD66b on neutrophils (CD15$^+$CD16$^+$ granulocytes) in tissue. (**G**) Unsupervised clustering of neutrophil (CD15$^+$CD16$^+$ granulocytes) flow data from healthy skin and burn tissue, 5 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. (**H**) Percentage of neutrophils within each cluster. Error bars in H show boxplot, p values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

Burn injury increases macrophage numbers and affects differentiation

Macrophage differentiation was assessed by analyzing the CD14 and CD16 expression of the monocytic cell population (among which are monocytes and macrophages) using flow cytometry. In both healthy skin and burn tissue the majority of these cells expressed a classical phenotype (CD14^{high}CD16$^-$) (**Figures 3A,B**). In burn tissue from PBW 3, the proportion of classical monocytic cells decreased while the proportion of intermediate

($CD14^{high}CD16^{+}$) monocytic cells increased. Next, we analyzed the macrophages ($CD68^{+}$ cells) within the monocytic cell population and found a steady increase in macrophages over time after burn injury (**Figure 3C-E**). By both IHC and FCM we could detect a significant increase in macrophages at PBW 3. Macrophage phenotype was further investigated by analyzing CD40 and CD80 expression (indicative for pro-inflammatory phenotype) and CD163 and CD206 expression (hallmarks for pro-healing) (**Supplementary Figure 2D**). The only significant difference we observed was a reduction of CD40+ macrophages at PBW 3 (**Supplementary Figure 2D**). Using FlowSOM analysis of the FCM data, we identified macrophage subtypes with different expression patterns: $CD163^{-}$ macrophages (cluster 1), $CD163^{+}$ macrophages with a low or moderate expression of CD40, CD80 and CD206 (cluster 2) and $CD163^{+}$ macrophages with a moderate to high expression of CD40, CD80 and CD206 (cluster 3) (**Figure 3F**). A significant increase in macrophages in cluster 2 was observed in burn tissue at PBW1 and 3 (**Figure 3G**). Overall, this analysis demonstrated that burn injury increased the number of macrophages and changed their composition.

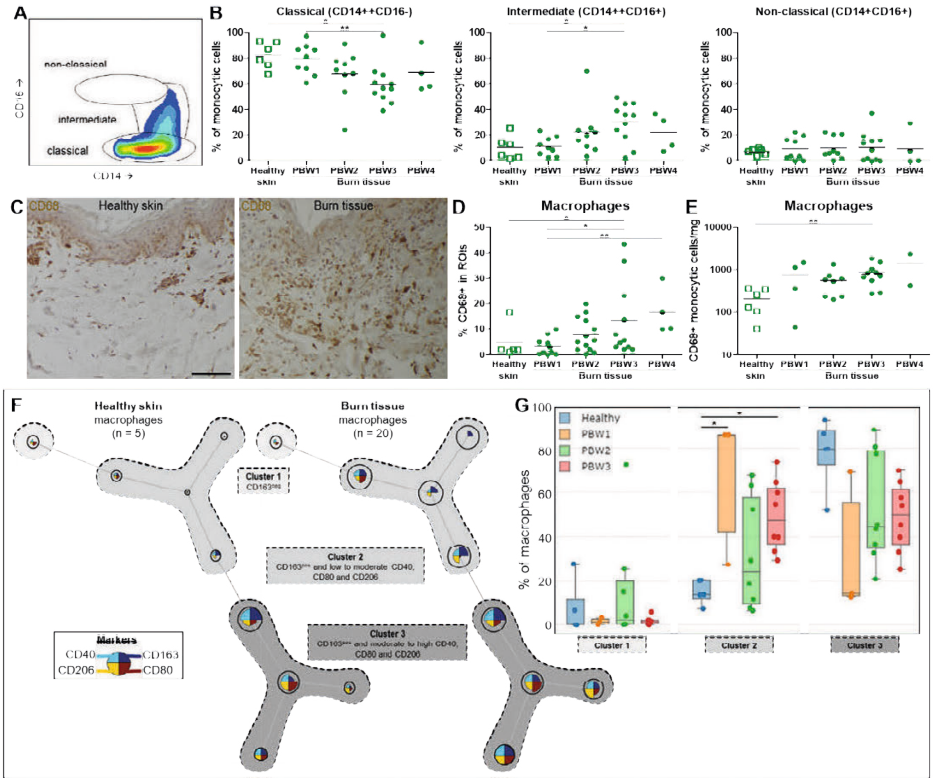

Figure 3. Local macrophage response to burn injury. (**A**) Flow cytometry gating strategy for detection of differentiation stages of monocytic cells (classical, intermediate or non-classical, as based on CD14 and CD16). (**B**) Flow cytometry-based quantification of percentage of monocytic cells within classical, intermediate, non-classical gates. (**C**) CD68 immunohistochemical DAB staining of a representative section of healthy skin and burn tissue (from 15 days post burn) (black scale bar = 100 µm). (**D**) $CD68^+$ area of tissue sections. (**E**) Flow cytometry-based quantification of absolute number of macrophages ($CD68^+$ monocytic cells) per mg tissue. (**F**) Unsupervised clustering of macrophages ($CD68^+$ monocytic cells) in healthy skin and burn tissue, 3 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. (**G**) Percentage of macrophages ($CD68^+$ monocytic cells) within each cluster. Error bars in G show boxplot, p values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *$p < 0.05$; **$p < 0.01$.

Burn injury causes shifts in the lymphocyte composition and increases total T cells at PBW 2-3

T cell ($CD3^+$ lymphocyte) numbers rose significantly at PBW 2-3 (**Figure 4A**), in line with the total lymphocyte increase (**Figure 1D**). A shift towards more $CD4^+$ T cells was detected in burn tissue compared to healthy skin and were highest in burn tissue from PBW 3 as the CD4/CD8 T cell ratio ($CD3^+CD4^+/CD3^+CD4^-$ ratio) was higher in burn tissue than in healthy skin (**Figure 4B** and **Supplementary Figure 2E**). An increase in the proportion of γδ T

cells ($CD3^+CD4^-$ $\gamma\delta TCR^+$ lymphocytes) was found only at PBW 1 (**Figure 4C**), indicating a fast response of γδ T cells after burn injury. The absolute number of γδ T cells steadily increased over time after burn injury (**Supplementary Figure 2F**). The shift towards a higher abundance of γδ T cells at PBW 1 was confirmed by mapping flow cytometry data of T cells using FlowSOM (clusters 3 and 4; **Figure 4D,E**) and shows that the majority of the γδ T cells was $CD25^+$, which is a prominent marker for cellular activation [18]. At PBW 1 there was a relative decrease of T cells with a regulatory phenotype ($CD25^+CD127^-$; cluster 1). We did not observe considerable alterations in the cluster containing $CD3^+CD4^-$ T cells (cluster 5)

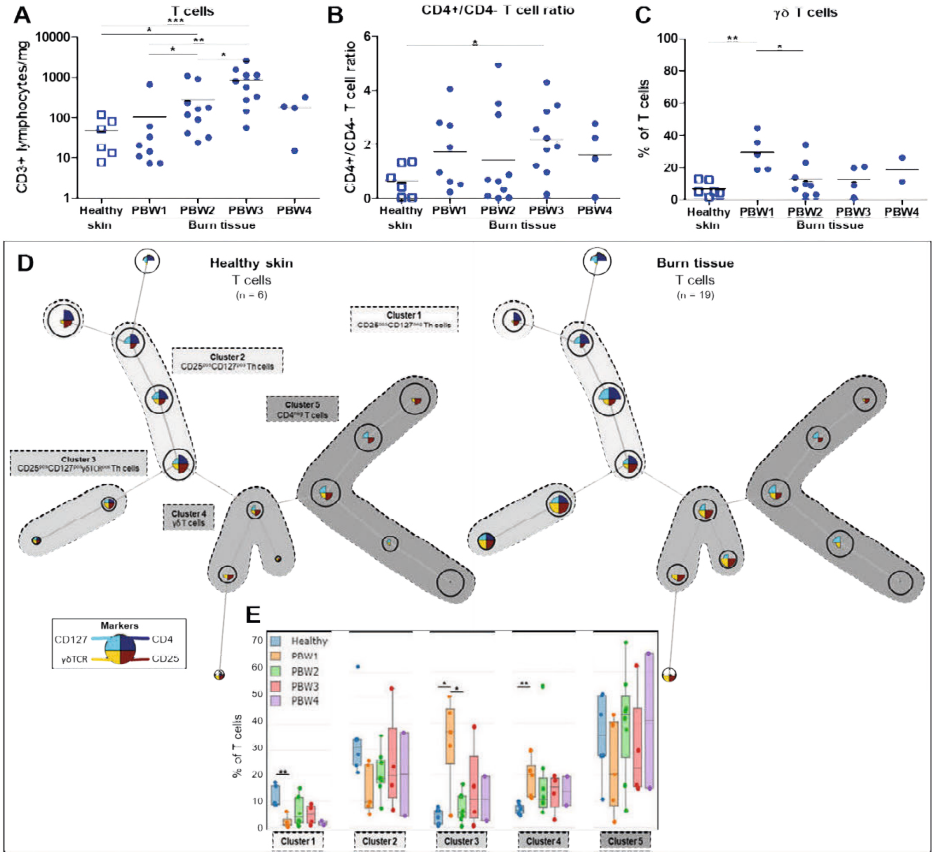

Figure 4. Local T cell response to burn injury. Flow cytometry-based quantification of: (**A**) Absolute number of T cells ($CD3^+$ lymphocytes) per mg tissue; (**B**) $CD4^+/CD4^-$ T cell ($CD3^+$ lymphocytes) ratio in tissue; (**C**) Percentage of T cells ($CD3^+$ lymphocytes) that are γδ T cells ($\gamma\delta TCR^+CD4^-$ T cells). (**D**) Unsupervised clustering of T cells ($CD3^+$ lymphocytes) in healthy skin and burn tissue, 5 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. (**E**) Percentage of T cells within each cluster. Error bars in E show boxplot, p values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Absolute number of NK and B cells increase after burn injury

FCM analysis showed that the absolute number of NK cells ($CD56^+$ lymphocytes) was higher in burn tissue from PBW 2-3 (**Figure 5A**). Relative to total leukocyte numbers, NK cells were significantly reduced in burn tissue from PBW 1 and normalized afterwards (**Supplementary Figure 2F**). In both healthy skin and burn tissue, the majority of the NK cells was $CD16^-$ (**Figure 5B**), which is opposed to the NK cell composition in peripheral blood where approximately 90% of the NK cells are $CD16^+$ [19]. Differences in CD16 expression of the NK cells were not observed between healthy skin and burn tissue or between time points. The absolute number of B cells ($CD19^+$ lymphocytes) were higher in burn tissue from PBW 3 (**Figure 5C**), while the proportion of B cells within the leukocyte population in burn tissue was similar to that of healthy skin (**Supplementary Figure 2G**). We identified 4 clusters using FlowSOM analysis of the FCM data: $CD56^+CD16^+$ NK cells, $CD56^+CD16^-$ NK cells, $CD9^+CD56^+$ B cells and $CD9^{low}CD56^-$ B cells (**Figure 5D**). Clustering analysis showed a clear shift towards more $CD9^{low}CD56^-$ B cells in burn tissue but no significant differences were detected over time (**Figure 5E**).

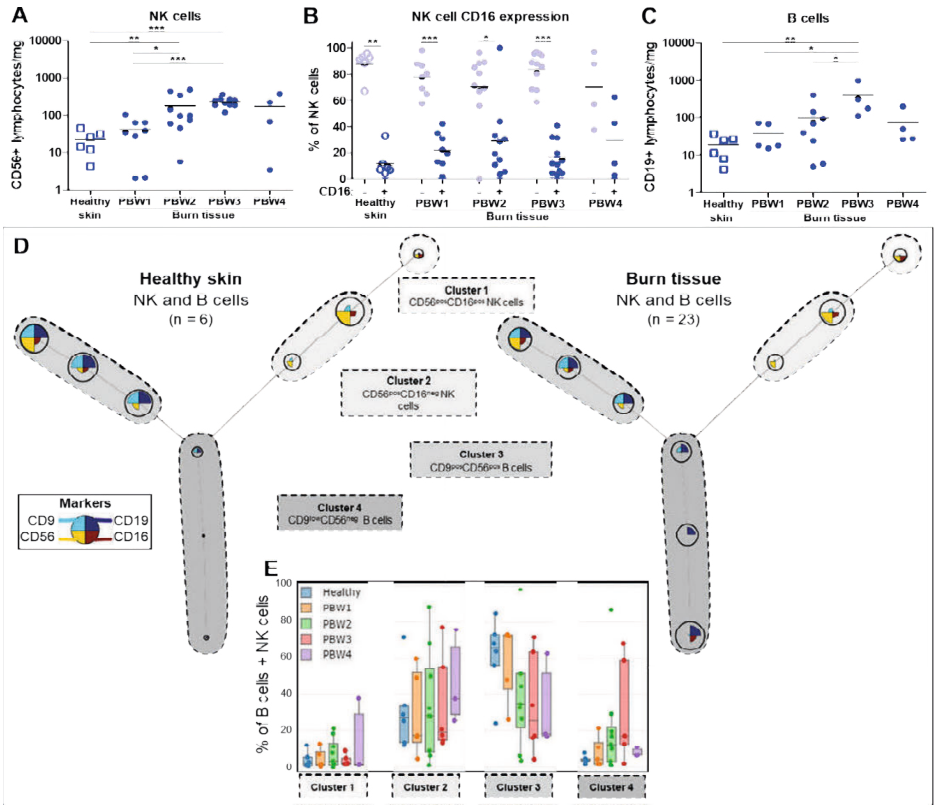

Figure 5. Local NK and B cell response to burn injury. Flow cytometry-based quantification of: (**A**) Absolute number of NK cells ($CD56^+$ lymphocytes) per mg tissue; (**B**) Percentage of NK cells that are $CD16^-$ and $CD16^+$; (**C**) Absolute number of B cells ($CD19^+$ lymphocytes) per mg tissue. (**D**) Unsupervised clustering of NK and B cells in healthy skin and burn tissue, 4 clusters are highlighted. Node size represents relative size of population and node diagram shows expression level of markers. (**E**) Percentage of NK or B cells within each cluster. Error bars in E show boxplot, p values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

Immune cell infiltration coincides with high levels of cytokines, chemokines and growth factors

The concentrations of 37 soluble immune factors were determined in homogenates of burn tissue using Luminex immunoassay (raw data is presented in **Supplementary Figure 3**). **Figure 6** shows an overview of these results using volcano plots and heatmaps at 4 time intervals after burn injury. In burn tissue there was an extremely high expression of IL-6, IL-1β, IFN-γ and TNF-α compared to healthy skin. The levels of these factors were persistently high, but for IL-6 and IFN-γ the levels declined at the later time intervals. Interestingly, increased levels of IL-12p40 and IL-5 were found only late after burn injury

(PBW 3-4). As compared to healthy skin, a decrease was found for IL-1 family members IL-1α, IL-33 and IL-18. This is opposed to the level of IL-1b, which is also an IL-1 cytokine. The levels of IL-1α, IL-33 and IL-18 somewhat normalized at PBW 4 to the levels found in healthy skin. Chemokines MCP-1 (CCL2), IL-8 (CXCL8), GROα (CXCL1), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5) and IP-10 (CXCL10) in burn tissue were increased at all analyzed time intervals, while the levels of T cell attracting chemokines CTACK (CCL27) and MIP-3α (CCL20) were decreased at PBW 1-4 and PBW 4, respectively. Among the growth factors, an increase in VEGF-A and TGF-β1 levels was found at PBW 1-4. From the growth factors, the level of GM-CSF was increased found at PBW 1-3, PDGF-AA at PBW 2-4 and PDGF-AB/BB and TGF-β2 only at PBW 3.

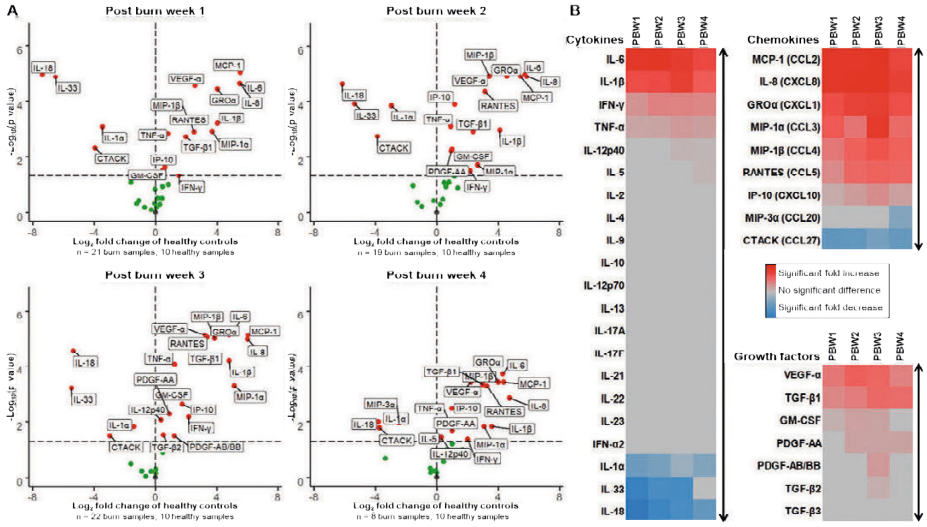

Figure 6. Expression of cytokines, chemokines and growth factors in burn tissue. (**A**) Volcano plot visualization of the expression of soluble factors in burn tissue from 1, 2, 3 and 4 weeks post burn injury. Dots represent soluble factors in burn tissue as Log_2 fold change as found in healthy skin controls. Factors with a statistically significantly different expression ($p < 0.05$) are labeled (values above the black striped line). (**B**) Heatmap visualization of fold increase/decrease of soluble factors in burn tissue compared to healthy skin, categorized by cytokines, chemokines and growth factors. P values were calculated using Mann-Whitney U statistical test.

DISCUSSION

Next to being a protective, physical barrier, the skin carries out immune surveillance to ensure early and effective defense mechanisms against external threats. Besides fibroblasts and keratinocytes, healthy skin is inhabited mainly by lymphocytes and antigen presenting cells that survey the skin and react to foreign structures and danger

signals [20]. Here, we aimed to provide detailed insight in the cellular and soluble immune response in burn injured skin during the first four weeks after injury. In this study, we showed that after burn injury, there is a fast, extensive and long-lasting increase in innate immune cells that is present even in burn tissue debrided 3 to 4 weeks after injury. Lymphocytes also rise in numbers, but mainly at PBW 2-4. In addition, the cytokine composition in these burn tissue samples is highly pro-inflammatory and likely continues the attraction and activation of immune cells. Excessive pro-inflammatory immune responses and a lack or delay of anti-inflammatory responses could complicate wound healing and patient recovery. Limitations of this study that should be addressed are minor differences in treatment between patients such as medication and timing of surgery that could have influenced the inflammatory response. In addition, the broad range in subject age, burn cause and TBSA could have increased variation in the responses.

In tissue samples from PBW 1, the proportion of γδ T cells was increased, indicating that γδ T cells could play a role during the early phase of burn-induced response. γδ T cells possess a unique T cell receptor and can, unlike ab T cells, interact with antigens directly [21]. They execute immune surveilling functions and react to damaged cell structures by producing cytokines and chemokines to recruit immune cells [22]. Mouse studies have shown that γδ T cells regulate the infiltration of innate immune cells shortly after trauma [23,24]. Our data suggests that next to keratinocytes, fibroblasts, mast cells and platelets [25], γδ T cells could be important inducers of the inflammatory response in humans as well. Within the same timeframe (PBW 1), IL-1β, IL-6, IL-8 (CXCL8), MCP-1, and GROα (CXCL1) levels were highly augmented. Others have demonstrated that these cytokines are also elevated in burn wound exudate [26]. These factors are known enhancers of the inflammatory response and attract neutrophils and monocytes/macrophages to wound site [27]. On the contrary, levels of IL-1α, IL-18 and IL-33 in burn tissue were reduced, especially during at PBW 1. These IL-1 cytokine family members are constitutively produced by keratinocytes to maintain the immune surveillance aspect of the skin [28]. Reduction of these factors is presumably caused by extreme loss of keratinocytes due to destruction of the epidermal layer by thermal injury. In burn tissue from PBW 2-4, the levels of IL-1α, IL-18 and IL-33 were returning to the levels in healthy skin, which may be related to the presence of keratinocytes closing the defect. Levels of cytokines, as well as microRNAs [29], could be potential biomarkers to predict disease progression or recovery [30].

The rapid neutrophil response to burn injury is presumably caused by the persisting levels of neutrophil attractants, such as IL-8, MCP-1 and GROα. This can also be observed in the circulation of burn patients, where high levels of neutrophils were accompanied by high

levels of IL-8 and MCP1, especially early after injury [7]. Other studies have also shown that burn tissue contains large numbers of neutrophils in both human [31] and animals [13,32]. The vast majority of neutrophils that infiltrated the wound area were mature, whereas, in peripheral blood from severely burned patients high numbers of immature neutrophils were detected [7]. This release of immature neutrophils may well be a compensatory response by the bone marrow [33]. Nucleus flexibility and chemotactic activity increases with neutrophil age and could explain the presence of mainly mature neutrophils in burn tissue [34]. If only mature neutrophils are able to enter the wound site, immature neutrophils would be trapped in the circulation until they reach maturity. As immature neutrophils are proposedly more active and less predictable in reacting to danger signals [35], they are likely to enhance systemic inflammation, thereby delaying recovery. In burn tissue, we found only a small number of immature neutrophils and only at PBW 1, which could have been released from the blood circulation by capillary leakage caused by the burn injury. Expression of CD11b and CD66b was increased on neutrophils isolated from burn tissue. This highlights the inflammatory state of the infiltrating neutrophils as CD11b and CD66b are important for neutrophil activation, adhesion and migration to inflamed tissue [36,37]. The surges of active neutrophils in the wound could lead to an overproduction of products such as elastase, MPO and ROS which can (further) damage surrounding tissues and organs [38,39].

Blood monocytes are progenitors of both pro-inflammatory macrophages and wound healing macrophages. Although there is little evidence in this respect, it has been suggested that classical monocytes could be predisposed progenitors to pro-inflammatory macrophages [40], while intermediate and non-classical monocytes are biased progenitors to wound healing macrophages [41,42]. The initial monocyte population in burn tissue consisted mainly of classical monocytes. The relative decrease in classical monocytes in PBW 3 could indicate a relevant shift towards more wound healing macrophages, which is assumed to happen during wound healing [8]. In burn tissue, the number of macrophages was increased this population showed a different composition of M1 and M2 markers. $CD163^{+}$ macrophages with low to moderate expression of CD40, CD80 and CD206 were more abundant in burn tissue. M1 macrophage differentiation factor GM-CSF was increased in burn tissue from PBW 1-3 and mediators that are known to be actively produced by M1 macrophages such as TNF-α, IFN-γ, IL-1β, IL-6, IL-8 and MCP-1 [8,43], were all increased in these burn tissues. While typical M2 macrophage factors like IL-4, IL-10, IL-13 were unaffected, the levels of TGF-β1 and VEGF-α, which are also described as M2 mediators [42], were increased in burn tissue. Altogether, the monocyte/macrophage composition and cytokine environment possibly supports the generation of macrophages with a pro-inflammatory phenotype. Timely transition towards more suppressive, regenerative macrophages is however essential for

a healthy healing process, as these cells support fibroblasts in the formation of collagen and enhance re-epithelization [8,42]. Due to the active, continuing inflammation and the presence of danger signals from tissue damage, macrophage transition might be delayed or insufficient, although more research is required to elucidate this.

Immunosuppression from the adaptive arm of the immune system is essential to create an environment in which fibroblasts and keratinocytes can repair the damaged skin [44]. Here, we revealed that lymphocyte numbers, including T cells, NK cells and B cells, were increased at PBW 2-3, which is relatively late after injury [10]. This coincided with a high levels of chemokines MIP-1α, MIP-1β and RANTES (CCL5), which are known to attract lymphocytes to injured skin [10]. Particularly CCL3, CCL4 and CCL5 are involved in the activation of NK cells [45] and could lead to increased cytokine release by NK cells in burn tissue. Information on the response of NK cells and B cells in burn tissue is very limited at this moment. We here showed that after burn injury there is an increased number of NK cells and B cells in burn tissue, however, functional assays are needed in order to speculate about their behavior and involvement in the burn immune response. The levels of CCL3, CCL4, CCL5, IFN-γ, TNF-α and IP-10 (CXCL10) were associated with the number of T cells at PBW 3. IP-10 promotes chemotaxis and inflammation and is likely induced by IFN-γ. Peters et al. previously described an interplay of keratinocytes and T cells and showed that IP-10 is actively produced by keratinocytes in co-culture, even with relatively low numbers of keratinocytes [46]. This interplay is presumably also active during burn wound healing by residual, surrounding or re-epithelializing keratinocytes. Cytokines with anti-inflammatory properties such as IL-4, IL-10 and IL-13 were not detected in these burn tissue samples. Altogether, the soluble factors in burn tissue are likely to support Th1 response, resulting in more attraction of leukocytes to the wound site, while control or suppression of inflammation appears to be limited.

In this study, we showed that after burn injury, the numbers of immune cells were persistently elevated, while during normal wound healing neutrophils disappear within days and lymphocytes counts start to increase in the first week [9,47,48]. Burn injury often leads to a prolonged hyperinflammatory state [2,49] and treatment of burn wounds is therefore a difficult and time-consuming process. Damage to the skin is a trigger for the immune system to recruit immune cells en masse and replenish these immune cells in the blood from the bone marrow. Ancillary damage and chemokine production by immune cells and stressed skin cells will trigger the immune system to react, thereby creating a vicious circle of prolonged inflammation in both the skin and in the blood. Therapy is often empiric due to the large diversity among patients and their injuries, e.g. burn type, size, depth and location. In the present study, there was no indication that burn size or burn cause (water versus flame) affected cellular or soluble inflammatory markers (data

not shown). Excessive and persistent inflammation is also among the causes of long-term complications such as the formation of hypertrophic scars [6]. On top of that, there is a risk of contracting an infection and the activity of the immune system is unpredictable. In clinical practice, patients with burns larger than 15% TBSA are hypermetabolic and often develop SIRS or organ insufficiency. Hence debridement of burn tissue is important to reduce inflammation and promote wound healing while also preventing further tissue necrosis and cellulitis. Possibly, early debridement of burn tissue (noted as post-burn days 2 through 12) or impediment of pro-inflammatory cytokines such as IL-6 might remove inflammatory triggers at an early stage and avoid secondary damage [50,51]. Resolution of excessive inflammation using immune suppressants could increase the patients' recovery rate, but might increase the risk for infection. Moreover, it can be very difficult to discriminate burn-induced SIRS from sepsis. Our analysis of the local immune reactions to burn injury aids in improving our understanding of burn-induced inflammation. This knowledge is needed to design more sophisticated and effective ways to diagnose and treat immune dysfunction and hyperactive inflammation. Immune modulating treatment targeting the disturbed immune processes will improve patients' overall health recovery time and scar quality.

In conclusion, through the characterization of immune cell subsets isolated from human burn tissue we demonstrated that burn injury induced a local persistent surge of pro-inflammatory immune cells and cytokines, while immunosuppression appeared to be limited. These burn-induced immune reactions might be key factors that extent the inflammation phase and thereby obstruct the wound healing process in burn injury.

MATERIALS AND METHODS

Sample collection

Burn wound tissue (eschar) from patients of all ages and thermal burn causes who underwent eschar debridement as part of their treatment at the Burn Center of the Red Cross Hospital in Beverwijk, the Netherlands. Healthy skin samples were obtained from adult patients who underwent cosmetic surgery (abdominoplasty or elective) at the Department of Plastic and Reconstructive Surgery of the Red Cross Hospital. Tissue samples were collected in the period between February 2019 and December 2021. Consent for the use of residual samples was received through the opt-out protocol of the Red Cross Hospital, in accordance with the national guidelines (https://www.coreon.org/). Subjects were informed of this procedure and were able to withdraw at any point. After surgical removal, tissue samples were stored in RPMI 1640 (Gibco, Paisley, UK) containing 1% penicillin and streptomycin (Gibco) as soon as possible to increase

cell survival [52,53]. Samples were stored overnight at 4 °C and processed the following morning. Subject and sample characteristics are shown in **Supplementary Table 1**.

Single cell isolation

This protocol was based on the immune cell isolation procedure from He et al. [54]. Biopsies were taken from viable areas of the burn tissues, i.e. white or red areas with bleeding spots and not blackened or leathery areas. Approximately 600 mg of tissue was used per cell isolation for flow cytometry (FCM). Tissue samples were cut into smaller pieces and subsequently divided over 2 C-tubes (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) containing 5 mL of RPMI 1640 containing 1% penicillin and streptomycin. C-tubes were placed on a tissue dissociator (gentleMACS, Miltenyi Biotec) and program "B" was run. Hundred-fifty µL of 80 mg/mL collagenase I (Merck, St. Louis, MO, USA) in PBS (Gibco) was added and the sample was incubated for 1 h in a shaking water bath at 37 °C. After incubation, the C-tube was placed on the tissue dissociator to run program "B". Samples were passed through a 500 µm and 40 µm cell strainer (pluriSelect, Leipzig, Germany) to obtain a single cell suspension. Suspensions were centrifuged for 10 min at 450 × *g*, and supernatant was discarded. The cell pellet was resuspended in erythrocyte lysis buffer (1.5 mM NH_4Cl, 0.1 mM $NaHCO_3$ and 0.01 mM EDTA in demineralized water) for 10 min at room temperature. Twenty mL of FCM buffer (PBS containing 1% BSA, 0.05% natrium-azide and 1 mM EDTA) was added and the suspension was centrifuged for 10 min at 450 × *g*. The pellet was resuspended in 5 mL of FCM buffer and cells were counted on the flow cytometer (MACS Quant Analyzer 10, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Supervised flow cytometry

From the single cells suspensions approximately 2.5×10^5 cells were used per staining panel. Antibodies used for FCM are displayed in **Supplementary Table 2**. A solution of 7-AAD (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) or propidium iodide (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were used to calculate viability of cells. Stained cell samples were acquired on the MACS Quant Analyzer 10 and analysis was performed using FlowLogic (Inivai Technologies, Victoria, Australia). Gating strategy is shown in **Supplementary Figure 1**. Data was visualized using Graphpad version 5.01 (PRISM, La Jolla, USA) and R (ggplot package).

Unsupervised flow cytometry analysis

Lymphocyte (panel 1), T cell (panel 2), neutrophil (panel 3) or monocytic (panel 4) populations were gated based on FSC/SSC, CD45, CD3, CD15 in MACSQuantify 2.13.3 software (Miltenyi Biotec). Data of these sole populations were uploaded to Cytobank [55] to create Flow Self-Organizing Map (FlowSOM) clusters.

Immunohistochemistry

Kryofix (50% ethanol, 3% PEG300)-fixed paraffin-embedded 5 µm thick sections were used after deparaffinization and rehydration. Endogenous peroxidase was blocked in 1% hydrogen peroxide for 15 min at room temperature. Next, antigen retrieval for different antigens was performed. The blocking step was performed using 5% normal goat serum (Merck) diluted in PBS + 1% bovine serum albumin (BSA). Tissue sections were then incubated with the primary antibodies (**Supplementary Table 3**) for 1 h at RT followed by incubation with a poly-HRP-goat-anti-mouse or rabbit secondary antibody (BrightVision, VWR) for 30 min at RT. Detection of the target protein was established using 3,3′-Diaminobenzidine (BrightDAB, VWR). After immunohistochemical (IHC) DAB staining was successful, sections were counterstained with hematoxylin, dehydrated and mounted with Eukitt Mounting Medium (Merck). Percentage of MPO, CD3 or CD68 positive area was calculated using NIS Elements (Nikon Instruments Europe B.V.) and based on 3 images from representative tissue sections.

Multiplex imaging and analysis

Formalin-fixed and paraffin-embedded 5 µm sections were deparaffinized using xylene and rehydrated with ethanol and distilled water. Antigen retrieval was performed by boiling in TRIS-borate-EDTA buffer for 10 min. A multiplex staining for the detection of neutrophils and lymphocytes was performed performed using the protocol described by Rodriguez et al. [56].

Immunoassay of tissue homogenates

Frozen tissue samples of approximately 60 mg were thawed, minced into smaller pieces and further dissociated in M-tubes (Miltenyi Biotec) by adding lysis buffer (PBS containing 0.01 mM EDTA and protease inhibitor (1 tablet per 10 mL; Pierce, Thermo Scientific)) and running program “Protein_01” on a gentleMACS (Miltenyi Biotec). Debris was removed from the samples using a filter plate (Multiscreen, Merck) and samples were diluted to concentration of 12 mg tissue/mL. Luminex assay was performed according to the manufacturer’s instructions (Merck KGaA). The following assay kits were used: HCYTA-60K, TGFBMAG-64K, HCYP2MAG-62K and HTH17MAG-14K. In short, 25 µL of tissue homogenate was used to determine the concentrations of 37 soluble factors, namely MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), GROα (CXCL1), IP-10 (CXCL10), IFN-α2, IFN-γ, TNF-α, TGF-β1, TGF-β2, TGF-β3, CTACK (CCL27), RANTES (CCL5), IL-1α, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8 (CXCL8), IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A (CTLA-8), IL-17F, IL-18, IL-21, IL-22, IL-23, IL-33 (NF-HEV), GM-CSF, PDGF-AA, PDGF-AB/BB and VEGF-A. For TGF-β1,2,3 samples were acid-treated prior to the assay, according to the manufacturer’s instructions. Mean fluorescence intensity of samples was measured with a Flexmap 3D System (Luminex Corp, Austin, USA) and concentrations were calculated

using Bio-Plex Manager Software (Bio-Rad Laboratories, Veenendaal, The Netherlands). Values below the minimum of the standard were based on extrapolation of the standard curve by the software.

Statistical analyses

Distribution of the data was checked for normality using the Shapiro Wilk test. For the FCM and IHC data, differences between burn tissue and healthy skin, and between burn tissues of different time intervals after injury (PBW 1, 2, 3 and 4) were explored using the Mann Whitney U test in Graphpad version 5.01 (PRISM, La Jolla, USA). Only statistically significant differences are shown and are indicated by black asterisks (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). The data was visualized using Graphpad version 5.01 (PRISM, La Jolla, USA). Levels of the soluble immune factors in burn tissue were transformed to fold change differences compared to the levels in healthy skin. P values of differences between burn tissue and healthy skin were determined using Mann Whitney U test. We considered a p value of < 0.05 to be statistically significant. Volcano plots were created using "EnhancedVolcano" version 1.6.0 package in R version 3.6.2.

ACKNOWLEDGMENTS

We would like to thank Xuehui He for helping with the cell isolation protocol, Bram van Cranenbroek for his assistance on the immunoassay, Mark Gorris for helping with the multiplex imaging and Evi Warmerdam, Rosa Rentenaar and Myrthe Witbaard for their technical assistance during experiments. We are grateful for the work and participation of all involved physicians, surgeons, nurses and patients of the Burn Center and the Department of Plastic and Reconstructive Surgery of the Red Cross Hospital. This research was funded by the Dutch Burns Foundation under grant number WO/17.108 (BKHLB).

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the article at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1034420/full#supplementary-material.

Supplementary Table 1. Sample characteristics. Table shows number of samples, unless indicated otherwise. *Based on TBSA of 35 patients.

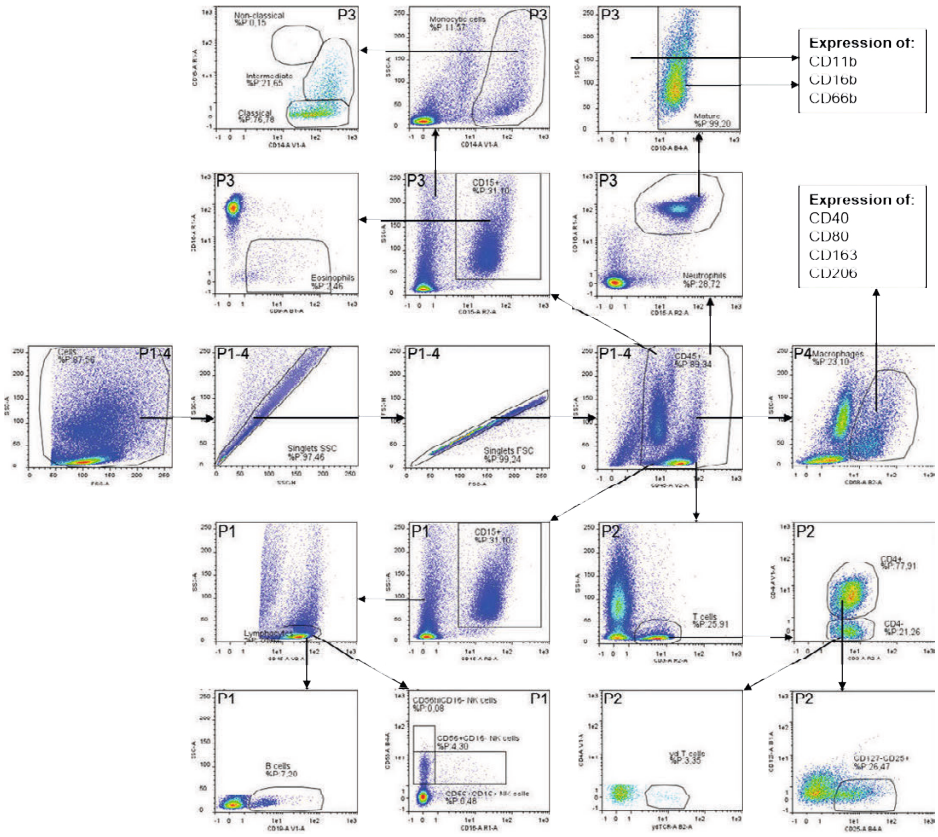
		Burn tissue	Healthy skin
Subject characteristics	Samples	83	20
	Subjects	81	20
	Sex (M/F/unknown)	44/35/2	2/17/1
	Age (mean ± SD)	54 ± 19 y	48 ± 12 y
Anatomical location	Arm	20	1
	Leg	32	1
	Torso	11	12
	Multiple	3	0
	Unknown	17	6
Time after burn injury in weeks	PBW 1	23	
	PBW 2	22	
	PBW 3	28	
	PBW 4	10	
Burn size	TBSA (mean ± SD)*	17 ± 14%	
Burn cause	Flame	50	
	Water	18	
	Oil/wax	6	
	Contact	4	
	Chemical	2	
	Electrical	1	

Supplementary Table 2. Antibodies used for flow cytometric analysis.

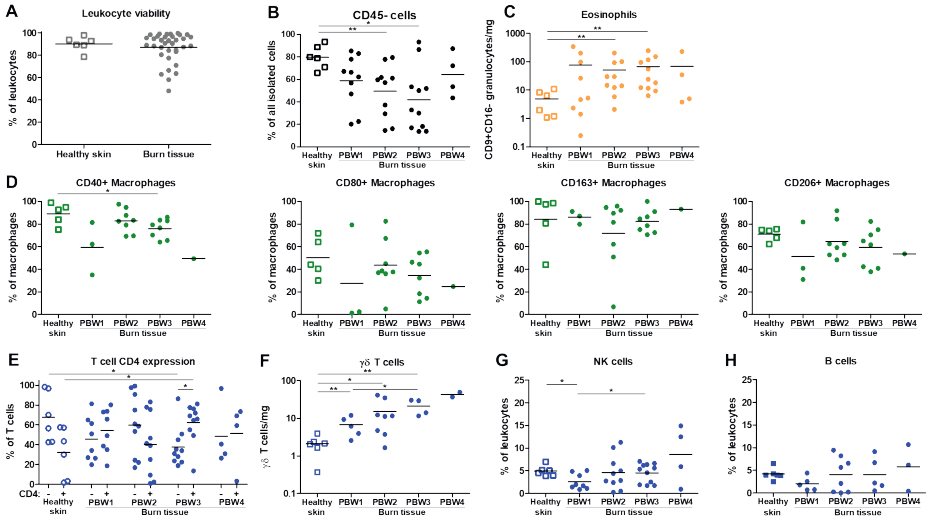
Antibody	Clone	Conjugate	Manufacturer
anti-CD3	REA613	APC	Miltenyi Biotec
anti-CD4	REA623	VioBlue	Miltenyi Biotec
anti-CD10	REA877	PE-Vio770	Miltenyi Biotec
anti-CD11b	REA713	FITC	Miltenyi Biotec
anti-CD14	REA599	VioBlue	Miltenyi Biotec
anti-CD15	W6D3	APC-fire750	BioLegend
anti-CD16	REA423	APC	Miltenyi Biotec
anti-CD19	REA675	VioBlue	Miltenyi Biotec
anti-CD25	REA945	PE-Vio770	Miltenyi Biotec
anti-CD40	REA733	FITC	Miltenyi Biotec
anti-CD45	REA747	VioGreen	Miltenyi Biotec
anti-CD56	REA196	PE-Vio770	Miltenyi Biotec
anti-CD66b	REA306	PE	Miltenyi Biotec
anti-CD68	REA886	APC-Vio770	Miltenyi Biotec
anti-CD80	REA661	APC	Miltenyi Biotec
anti-CD127	REA614	FITC	Miltenyi Biotec
anti-CD163	REA812	PE	Miltenyi Biotec
anti-CD206	DCN228	PE-Vio770	Miltenyi Biotec
anti-γδTCR	REA591	PE	Miltenyi Biotec

Supplementary Table 3. Antibodies used for immunohistochemistry.

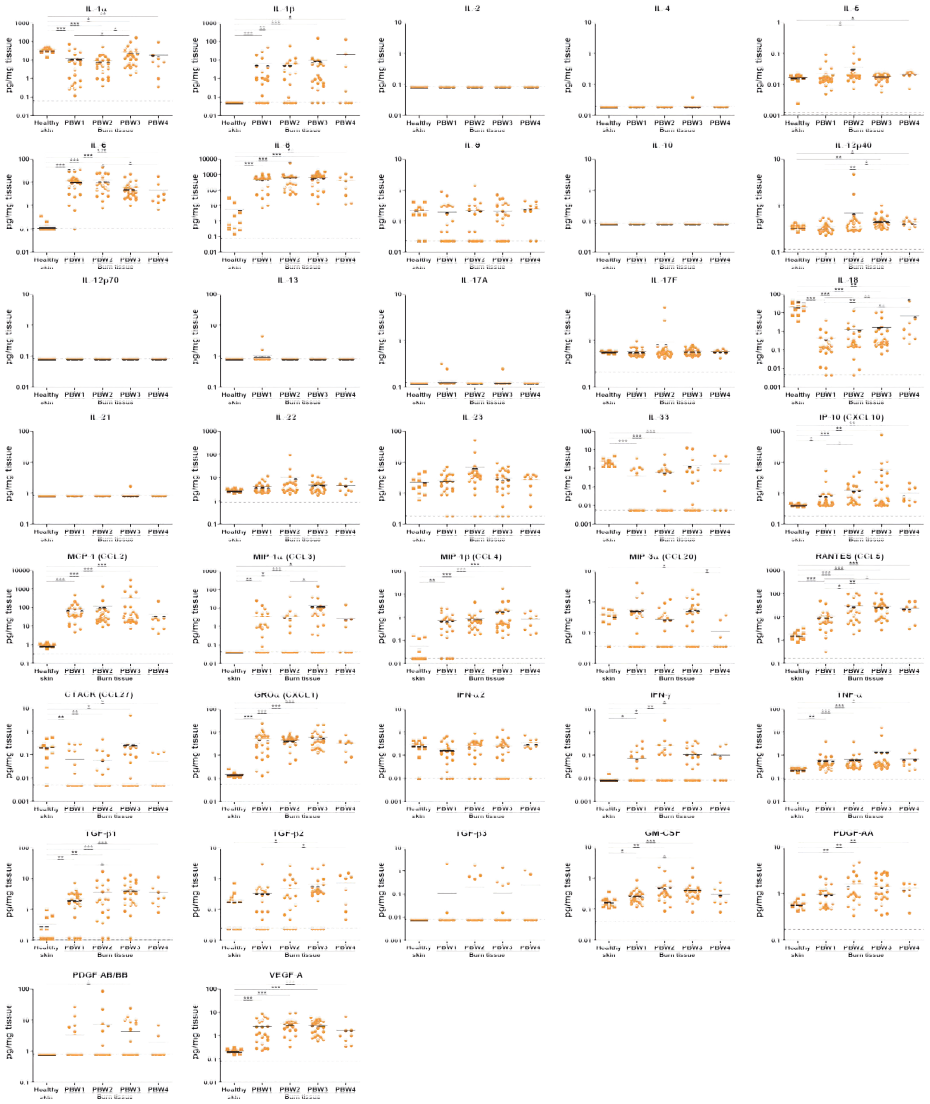
Antibody	Clone	Manufacturer	Dilution	Antigen retrieval	Stain
anti-CD3	Sp7	ThermoFisher	1/200	EDTA	TSA520
anti-CD3	Sp7	Abcam	1/200	EDTA	DAB
anti-CD8	C8/144B	DAKO	1/200	EDTA	TSA690
anti-CD15	MMA	BD Biosciences	1/400	EDTA	TSA520/DAB
anti-CD45	2B11+PD7/26	DAKO	1/100	Citrate	DAB
anti-CD68	KP1	DAKO	1/2000	EDTA	DAB
anti-MPO	Polyclonal	DAKO	1/1200	Citrate	DAB



Supplementary Figure 1. Gating strategy of flow cytometric analysis. At the top of the plots the panel numbers are shown for which the gating was performed (P1, 2, 3 and 4).



Supplementary Figure 2. Cell counts in burn tissue. Flow cytometry-based quantification of: (**A**) Percentage of leukocytes that stained negative for 7-AAD or propidium iodide (viable cells); (**B**) Percentage of isolated cells that is $CD45^-$ (fibroblasts, keratinocytes, endothelial cells, others); (**C**) Number of eosinophils ($CD9^+CD16^-$ granulocytes) per mg tissue; (**D**) Percentage of macrophages that is positive for CD40, CD80, CD163 or CD206; (**E**) Percentage of T cells that are $CD4^-$ and $CD4^+$. (**F**) Number of γδ T cells ($\gamma\delta TCR^+$) per mg tissue; (**G**) Percentage of NK cells ($CD56^+$ lymphocytes) within leukocyte population; (**H**) Percentage of B cells ($CD19^+$ lymphocytes) within leukocyte population. P values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



Supplementary Figure 3. Concentrations of soluble factors in burn tissue. Healthy skin was used as controls. Black lines show mean values and the black striped line represents the lowest limit of detection. P values were calculated using Mann-Whitney U statistical test, significant differences are indicated by black asterisks: *p < 0.05; **p < 0.01; ***p < 0.001.

PART 3

In Vitro Modeling

CHAPTER 6

Full Skin Equivalent Models for Simulation of Burn Wound Healing, Exploring Skin Regeneration and Cytokine Response

Published in Journal of Functional Biomaterials, **2023***, 14, 29*
DOI: 10.3390/jfb14010029

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ABSTRACT

Healing of burn injury is a complex process that often leads to the development of functional and aesthetic complications. To study skin regeneration in more detail, organotypic skin models, such as full skin equivalents (FSEs) generated from dermal matrices, can be used. Here, FSEs were generated using de-epidermalized dermis (DED) and collagen matrices MatriDerm® and Mucomaix®. Our aim was to validate the MatriDerm- and Mucomaix-based FSEs for the use as in vitro models of wound healing. Therefore, we first characterized the FSEs in terms of skin development and cell proliferation. Proper dermal and epidermal morphogenesis was established in all FSEs and was comparable to ex vivo human skin models. Extension of culture time improved the organization of the epidermal layers and the basement membrane in MatriDerm-based FSE but resulted in rapid degradation of the Mucomaix-based FSE. After applying a standardized burn injury to the models, re-epithelization occurred in the DED- and MatriDerm-based FSEs at 2 weeks after injury, similar to ex vivo human skin. High levels of pro-inflammatory cytokines were present in the culture media of all models, but no significant differences were observed between models. We anticipate that these animal-free in vitro models can facilitate research on skin regeneration and can be used to test therapeutic interventions in a preclinical setting to improve wound healing.

INTRODUCTION

Wound healing of deep and large wounds is often problematic and can lead to medical complications such as hyper-inflammation and excessive scarring of the skin. In turn, these complications can lead to delayed recovery and poor aesthetic outcomes [1–4]. To improve the treatment of burn injuries, the processes underlying skin regeneration need to be better understood. Furthermore, there is a need for appropriate in vitro models to facilitate drug discovery and testing [5].

Research on cellular processes in burn wound healing is generally performed on animals [6–8]. Translation to the human situation is, however, difficult due to physiological differences between animals and humans [9,10]. In addition, in our modern society we strive for innovative, animal-free ways of conducting research [11]. Human studies, on the other hand, are limited by the absence of baseline values, heterogeneity among patients, and restrictions in the collection of tissue samples [6,7]. Therefore, there is an important demand for alternative models to study burn injuries.

Organotypic skin models are useful alternatives to animal experimentation and can be used as a research instrument to study defined aspects of skin trauma, based on the behavior of human cells [12–14]. Moreover, these models are easily adjustable to study interactions of specific cell types and can be used to evaluate the effect of therapeutic interventions. Scratch models, in which a scratch is made in a monolayer of a single cell type, can be used to study cell migration and proliferation after wounding. However, such scratch models rely on a single type of cell, usually keratinocytes or fibroblasts [15]. Alternatively, 3D culture models can be used to study skin diseases [12–14,16–19]. 3D culture models resemble a more natural and complete environment for cells; however, they are often produced from hydrogels and seeded with immortalized cell lines or animal cells instead of primary isolated human cells. Furthermore, gel-based models are less suitable for the study of thermal trauma because they might not be strong enough to withstand the injury.

More relevant and robust in vitro culture models for the study of wound healing are full skin equivalents produced from dermal scaffolds seeded with fibroblasts and keratinocytes [20,21]. Such FSEs are uniform, as there is less variation in the matrix, and more representative of the in vivo situation than the aforesaid culture models, because collagen is the predominant component [22]. FSEs produced from dermal collagen-elastin scaffolds provide a durable extracellular matrix architecture that supports cell anchorage [23,24]. In this study, we generated FSEs from the dermal substitutes MatriDerm® and Mucomaix®. These dermal matrices are clinically used in combination with split thickness

autografts to treat full-thickness skin defects and support skin regeneration [25–28]. Therefore, FSEs generated using these matrices are relevant in vitro study models. We validated the different FSE models by studying skin development, cell differentiation, cell viability, and cytokine production. A standardized thermal contact injury was applied to evaluate in vitro wound healing. We compared the performance of these FSEs to cultured ex vivo human skin and de-epidermalized dermis (DED)-based FSEs.

RESULTS

Skin morphogenesis in full skin equivalent models was similar to ex vivo human skin

FSEs were generated from dermal matrices DED, MatriDerm, and Mucomaix. To validate our FSE models, we first studied skin development after 3 weeks of initial culture (indicated as time T0) and compared this to ex vivo normal human skin (**Figure 1A**). At T0, the DED-based models contained a completely developed dermis and a pan-cytokeratin-positive epidermis (**Figure 1B**). At T + 2 weeks, the epidermal and dermal structures developed further and included a thickened stratum corneum that was similar to that of ex vivo human skin. MatriDerm-based FSEs also displayed a well-developed dermis and pan-cytokeratin-positive epidermis that was comparable to the DED-based FSEs and ex vivo human skin. At T + 2 weeks, the dermis remained intact, and the stratum spinosum became thinner, while the stratum corneum grew thicker. Mucomaix-based FSEs developed a complete, pan-cytokeratin-positive epidermis, but its dermis was rather incomplete due to partial degradation and compaction of the matrix. Extension of the culture time improved organization of the epidermal layer and thickening of the stratum corneum but resulted in further degradation of the matrix.

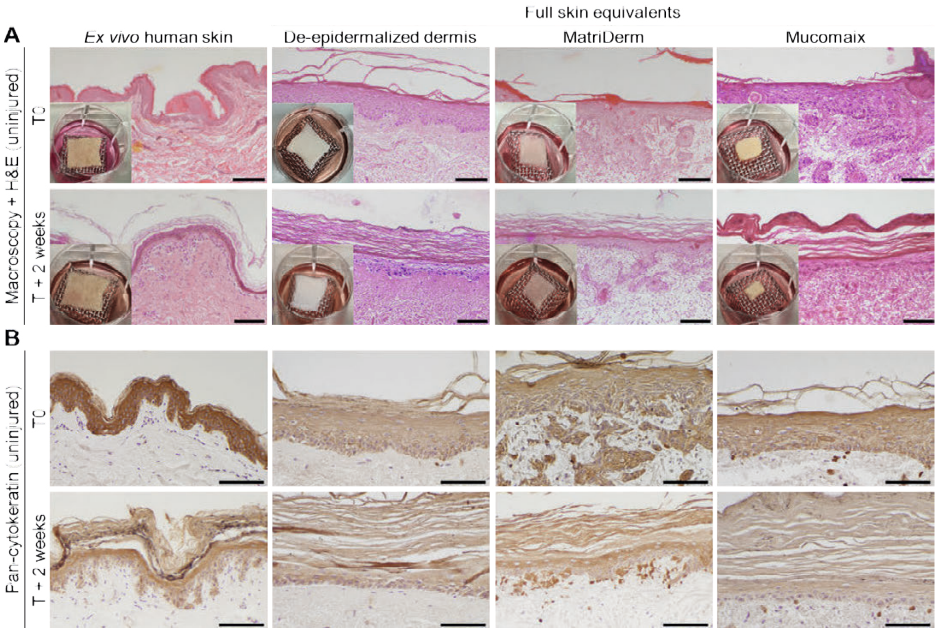

Figure 1. Evaluation of skin development of cultured skin models. Images of ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right) at T0 or T + 2 weeks. (**A**) Macroscopy and H&E staining; (**B**) Immunohistochemical pan-cytokeratin staining. Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, T0 was after the initial 3 weeks of culture. Black scale bar = 100 µm.

Epidermal and dermal structures developed completely in full skin equivalents

Epidermal and dermal development in the FSEs was examined by immunohistochemical analysis. Cytokeratin 15, present in progenitor keratinocytes [29], was consistently expressed in ex vivo human skin and DED-based FSEs from T0 onward (**Figure 2A**). In both MatriDerm- and Mucomaix-based models, cytokeratin-15-positive cells were present but did not display a well-organized basal layer. MatriDerm- and Mucomaix-based FSEs developed a basement membrane, as was shown by collagen IV and laminin α 5 expression at the dermal–epidermal junction (**Figure 2B,C**) [30]. Expression of collagen IV and laminin α 5 gradually increased over time, simultaneously with the improvement of the epidermal architecture. Although organization of the basal layer is not optimal, the FSE models produced an epidermis including a basement membrane, stratum spinosum, and stratum corneum that was similar to ex vivo human skin.

Next, the differentiation status of keratinocytes in the FSEs was assessed by determining the presence of early differentiation marker cytokeratin 10 and late differentiation marker involucrin (**Supplementary Figure 1A,B**) [31,32]. In ex vivo human skin and DED-based

FSEs, cytokeratin 10 was expressed in all suprabasal layers of the epidermis. While in MatriDerm and Mucomaix-based FSEs, the presence of cytokeratin-10-positive cells at T0 was limited, the expression of cytokeratin 10 at T + 2 weeks was consistent in the suprabasal layer. Late differentiation marker involucrin was expressed in the stratum granulosum in ex vivo human skin from T0 onward. The DED-based FSEs showed suprabasal involucrin expression at T0, which shifted to the stratum granulosum at T + 2 weeks. As for the MatriDerm- and Mucomaix-based FSEs, involucrin was present in all suprabasal layers from T0 onward.

Expression of stress marker cytokeratin 17 was not present at T0 in the ex vivo human skin models (**Supplementary Figure 1C**). However, when the ex vivo human skin models were cultured for 1 or 2 weeks, cytokeratin 17 expression was upregulated, similar to the FSEs. In FSEs, cytokeratin 17 was displayed in all epidermal layers. Fibroblast distribution was visualized by analyzing the presence of vimentin in cells in the dermal part of the FSEs (**Figure 2D**). All FSE models showed a fibroblast-populated dermis with a balanced distribution throughout the matrices. Expression of α smooth muscle actin was studied, but it was not detected in any of the FSEs.

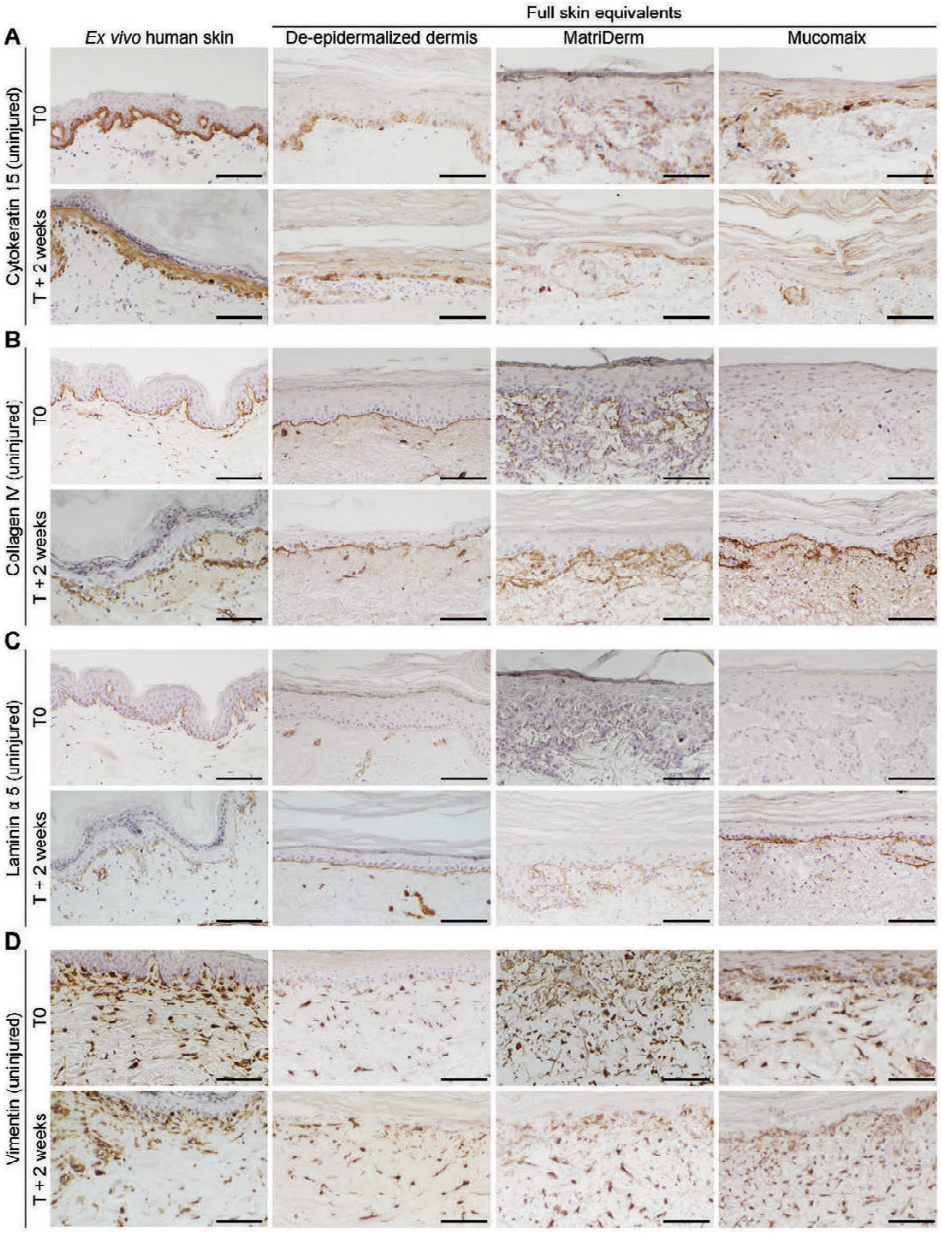

Figure 2. Evaluation of cytokeratin 15, collagen IV, laminin α 5 and vimentin expression in cultured skin models. Images of ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Immunohistochemical (**A**) cytokeratin 15; (**B**) collagen IV (**C**) laminin α 5 and (**D**) vimentin DAB staining. Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, T0 was after the initial 3 weeks of culture. Black scale bar = 100 µm.

Regenerative capacity of MatriDerm-based FSEs was similar to DED-based FSEs and ex vivo human skin at 2 weeks after burn injury

To investigate the ability of the FSEs to function as burn injury models, we subjected the FSEs to a thermal contact injury of 80 °C for 20 s. In preliminary experiments we found that an injury with these settings caused sufficient damage to induce re-epithelization in similar models. Skin morphology and histology of the models were studied at T0, T + 1 week, and T + 2 weeks (**Figure 3** and **Supplementary Figure 2**). At the macroscopic level, burn injuries presented in a rectangular shape which remained visible throughout the duration of the culture. In all models, destruction and release of the epidermis was clearly visible on the H&E-stained sections at T0. At T + 1 week and T + 2 weeks, re-epithelization of the epidermis was apparent in the ex vivo human skin model and the DED- and MatriDerm-based FSEs. In the Mucomaix-based model, re-epithelization did not take place. Although this model was clearly damaged, formation of a neo-epidermis was not detected.

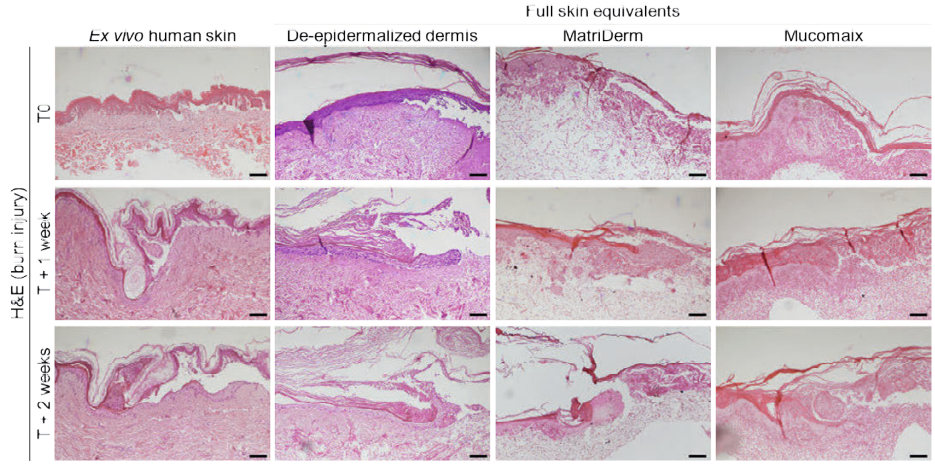

Figure 3. Effect of burn injury on cultured skin models. Images of ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, T0 was after the initial 3 weeks of culture. Black scale bar = 100 µm.

Next, we validated the regenerative capacity of the models. Therefore, we studied the presence of proliferating cells in the neo-epidermis and quantified the length of the re-epithelized epidermis (**Figure 4**). Ki67 staining revealed proliferating cells in and nearby the re-epithelized area in DED- and MatriDerm-based FSEs and the ex vivo human skin model (**Figure 4A**). In Mucomaix-based FSEs, there were hardly any positive cells present. BrdU was used to study 24 h proliferation in the models (**Figure 4B**). BrdU-positive keratinocytes were present in the newly formed basal layer of the ex vivo

human skin model and the DED-based FSE. In the MatriDerm-based FSE, BrdU-positive keratinocytes were only present at the leading edge of the neo-epidermis. Similar to Ki67, only very few BrdU-positive cells were present in Mucomaix-based models. The length of re-epithelization at T + 1 week of the DED- and Matriderm-based models was larger than in the ex vivo human skin model (**Figure 5C**). The length of the re-epithelized area of the wound in DED- and MatriDerm-based models at T + 2 weeks, however, was comparable to that of the ex vivo human skin model. Mucomaix-based models, on the other hand, lacked the capacity to regenerate the burned epidermis. Thus, the DED- and MatriDerm-based models showed regenerative capacity with a neo-epidermis that contained proliferating cells, while regeneration in Mucomaix-based FSEs was not observed.

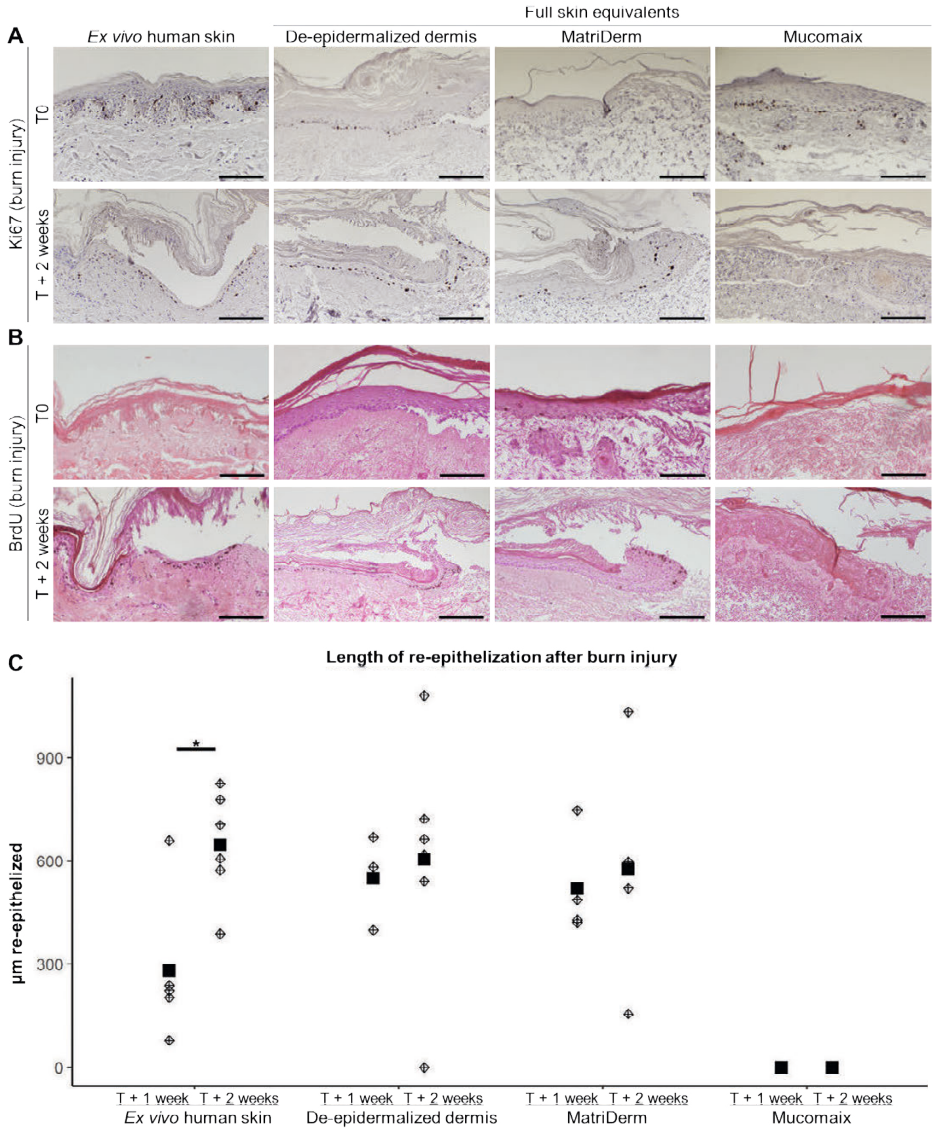

Figure 4. Evaluation of proliferation and re-epithelization in burn-injured skin models. Images of ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right) at T0 and T + 2 weeks after burn. Immunohistochemical (**A**) Ki67; (**B**) BrdU DAB staining. Because the culture of ex vivo human skin models started at T0 and BrdU needed to be added 24 hour prior to termination of the models, no BrdU was present in these models at T0. (**C**) Length of re-epithelization after burn injury at T + 1 week and T + 2 weeks after burn (diamonds represent the mean per model and squares represent the mean of all models). Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, T0 was after the initial 3 weeks of culture. Black scale bar = 100 µm. Statistically significant differences are indicated by asterisks: *: $p < 0.05$.

Cytokine response of burn-injured full skin equivalent models

Cytokine response in the FSEs was explored by determining the cytokine levels in the culture medium at T0, T + 1-4 days, T + 5-7 days, and T + 8-11 days (**Figure 5** and **Supplementary Figure 3**). Of the 13 cytokines that were analyzed, high levels of IL-6, IL-8, and MCP-1 and moderate levels of IL-4 and IP-10 were found in both burn-injured and uninjured models. Only low levels of IL-12p70 and IFN-γ were detected in both burn-injured and uninjured models, while the levels of IL-2, IL-17A, and TNF-α were undetectable. The expression of IL-1β was the highest in ex vivo human skin models, and IL-10 expression appeared to be higher in both ex vivo human skin models and Mucomaix-based FSEs. TGF-β1, on the other hand, was more abundantly expressed in the FSEs than in the ex vivo human skin model.

In this explorative analysis, it seemed that the expression of IL-4, IL-6, IL-8, MCP-1, IFN-γ, and IL-12p70 was more or less consistent over time, as opposed to IL-1β, IL-10, IP-10, and TGF-β1. The levels IL-1β, IL-10, and IP-10 gradually decreased over time in the ex vivo human skin models, but this was not significant. In response to burn injury, the level of IL-4, IL-6, IL-10, and TGF-β1 showed a modest increase only in MatriDerm and Mucomaix-based models at T + 1-4 days, although it did not reach significance. In the Mucomaix-based FSE, burn injury also increased the expression of IL-8, IL-12p70, and IFN-γ. Surprisingly, no differences were found for the ex vivo human skin model or DED-based FSE in reaction to the burn injury. Thus, high levels of pro-inflammatory cytokines were present in the medium of FSE models, similar to ex vivo human skin. The effect of burn injury on pro-inflammatory cytokines was limited and was only evident in the MatriDerm and Mucomaix-based FSEs.

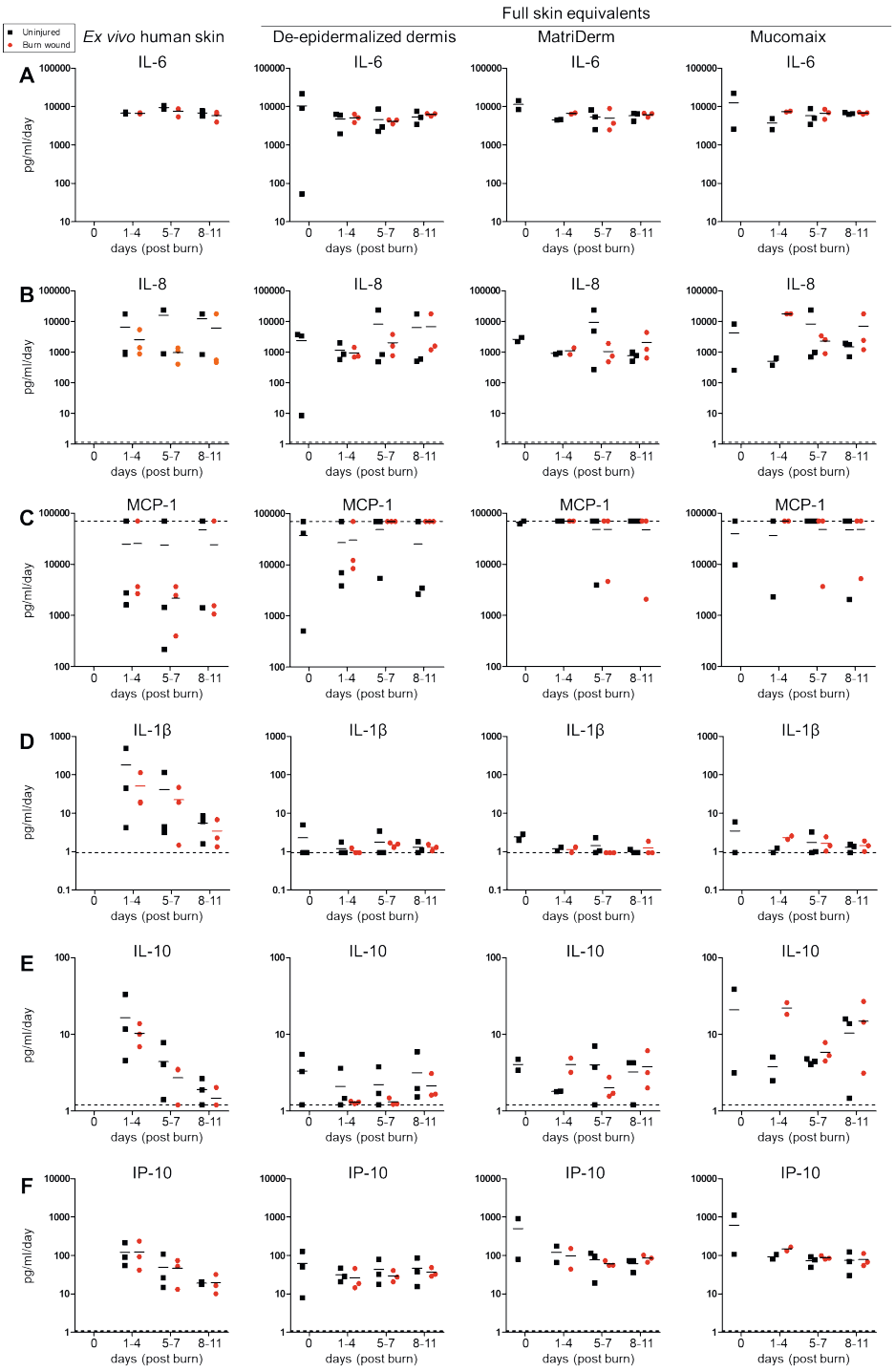

Figure 5. Cytokines detected in medium of burn-injured and uninjured skin models. Ex vivo

human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Level of (**A**) IL-6; (**B**) IL-8; (**C**) MCP-1; (**D**) IL-1β; (**E**) IL-10; (**F**) IP-10 in the culture medium at T0, T + 1-4 days, T + 5-7 days and T + 8-11 days (after burn injury). Samples from biological duplicates were pooled per donor (n = 3 donors) and re-calculated into pg/ml per day of culture to compensate for differences in intervals of medium changes. Striped line indicates the highest or lowest level of quantification. Because ex vivo human skin models were started on the first day, no levels are shown for day 0.

DISCUSSION

Due to issues in the translation of animal data to the human situation, as well as ethical concerns, there is a growing demand for more appropriate, animal-free approaches in preclinical research [11]. Organotypic skin models, such as FSEs, are promising alternatives to animal models because they are more standardized, controllable, and easy to customize with relevant components such as specific cell types [20,21,33]. FSE models are more realistic than models that only use an epidermal layer, as the interplay between keratinocytes and fibroblasts affects skin development and healing [34–36]. Here, we validated FSEs generated from commercially available dermal substitutes MatriDerm and Mucomaix for the use as in vitro skin models to study skin development and burn wound healing. Additionally, we investigated the effect of longer culture times (up to a total of 5 weeks). Studies usually culture organotypic skin models up to 3 weeks [37–40], but in light of preclinical studies, culture times longer than 3 weeks could be required. In the development of 3D models, predominantly the histology, composition of the extracellular matrix, or cell survival is studied [41,42]. Our study not only showed that the FSEs were capable of forming a functional epidermis, but also showed that these models were able to regenerate after thermal injury.

Epidermal morphology of the FSE models after 3 weeks of culture was similar to that of ex vivo human skin. Normal epidermal differentiation was present during this period of culture, as shown by consistent expression of early and later differentiation markers cytokeratin 10 and involucrin. Extending the culture time by 1 or 2 weeks improved the organization of the epidermal structure and led to flattening of the stratum spinosum, as displayed by cytokeratin 10 expression, while the stratum corneum thickened. In FSE models, the expression of several markers of epidermal development was similar to the expression in ex vivo human skin and skin equivalent models from other researchers [20,21,43]. Involucrin was also present in the suprabasal layers of the FSEs, an observation similar to that of Coolen et al. and Thakoersing et al. [20,43]. Premature expression of involucrin is indicative of overactivated cell differentiation and is likely caused by an excess of growth factors in the culture medium or by an imbalance in the ratio of fibroblasts to keratinocytes [35,43,44]. With the extension of culture time,

epidermal organization gradually improved, coinciding with the expression of collagen IV and laminin α 5. Despite a well-formed basement membrane in the MatriDerm- and Mucomaix-based FSEs, the basal layer was not entirely organized and did not improve over time. The disruption of the basal layer could be related to the porosity of the collagen matrices causing keratinocytes to partially descend into the matrix. In all models, a basement membrane was present, which was more mature at T + 2 weeks. All models contained a fibroblast-populated dermis, as was shown by vimentin expression [45].

Both MatriDerm- and Mucomaix-based models appeared suitable for in vitro study of skin development. However, due to the rapid degradation of Mucomaix, this matrix turned out less suitable as a model for extensive culture times. The degradation speed of Mucomaix was also shown in vivo by Udeabor et al. [28]. The MatriDerm matrix also degraded over time but clearly at a slower rate, which might be due to the presence of elastin-hydrolysate, making it less susceptible to enzymatic degradation [46]. Because of its faster degradation rate, Mucomaix could be more suitable for the study of degradation and cell matrix interactions. Clinically, Mucomaix is useful for the repair of intra- and extra-oral defects [28].

Burn injury and regeneration could successfully be studied in MatriDerm- and DED-based FSEs, as they displayed a regenerative and proliferative capacity similar to ex vivo human skin. The faster re-epithelization rate in FSEs at T + 1 week could be related to an increased proliferation in the FSEs due to the culture of cells. In contrast, cells in intact skin, especially keratinocytes, might be programmed more for differentiation rather than proliferation. Several other studies have used in vitro skin models to study the effects of burn injury, but they did not study the rate of re-epithelization [39,47,48].

Cytokines IL-6, IL-8, and MCP-1 were expressed in the FSE models at levels similar to those in ex vivo human skin. Apparently, there is already some degree of stress response in these models that is presumably triggered by the culturing of cells and in vitro skin development. This is supported by the abundant expression of stress marker cytokeratin 17 (**Supplementary Figure 1C**). Cytokeratin 17 was also expressed in ex vivo human skin models, but only after culture. Reports on the cytokine expression of cells in response to culture or skin development are very limited. This cytokine response is, however, important to take into account, because inflammation and skin regeneration can affect each other, thereby potentially delaying wound healing processes. Despite the potential presence of stress and subsequent cytokine responses during in vitro culture, an epidermis and dermis were successfully established in the presented models.

As seen only in ex vivo human skin cultures, the level of IL-1β, IL-10, and IP-10 gradually decreased over time. These cytokines might have been produced by immune cells, such as lymphocytes, that were residing in the ex vivo skin. With increasing culture time, cytokine production would then be reduced due to migration or depletion of these cells. Burn injury had only a limited effect on the level of cytokines and seemed to moderately increase the levels of IL-4, IL-6, IL-8, IL-10, and TGF-β1 in MatriDerm- and Mucomaix-based models early after injury. Possibly, the effect of burn injury was minimal because of the initially high levels in uninjured models. An increase in IL-8 in medium of burn-injured in vitro skin models was shown by Breetveld et al. and was only present early after injury (up to 4 days) [39]. A study from Schneider et al. showed an increase in the levels of IL-6 and IL-8 in similar models, also during the first week after injury [48]. The limited effect of burn injury on these models is likely caused by the absence of blood circulation and immune cells, which are well-known inducers of immune reactions. Because the thermal injury damaged a large portion of cells in these relatively small models, the potential response could only originate from the remaining viable cells. When the population of remaining cells is too small, the response will also be rather limited.

The current FSEs are useful for the study of tissue development and repair and for translational research without the use of animal models [33,49]. When fibroblasts and keratinocytes are kept in frozen stock, these models can be produced on demand, unlike ex vivo skin models, which depend on the availability of donor skin. FSEs are also advantageous because they are more standardized, can minimize donor variation, and are easily adjustable in terms of matrix, cell types, and cell numbers.

The next step in the development of in vitro skin models will be the integration of immune cells, blood vessels, or other relevant skin appendages [50–53] and developing models suitable for drug discovery and testing [54]. Cells from different (disease-related) origins, such as skin cells derived from fetal, burn, or scar tissue, could be used to study their effect on skin regeneration. For example, van den Broek et al. developed a hypertrophic scar model using adipose-derived mesenchymal stem cells [55]. In these scar models, differences in contraction, epidermal thickness, and cytokine response were shown compared to models produced from dermal mesenchymal cells. To study inflammatory responses in a more relevant environment, immune cells can be integrated into FSEs [56]. Finally, such models could be supplemented with skin appendages such as hair follicles, making these models a useful platform to test interventions in the preclinical stage.

Clinically applied matrices MatriDerm and Mucomaix are suitable materials for in vitro skin model development. MatriDerm-based FSEs could be used for extensive culture periods and demonstrated regeneration after thermal wounding. The cytokine response

of FSEs was comparable to that of ex vivo human skin. These models are therefore useful for the study of skin development and wound healing using a uniform dermal component without the need for animal models. Further development of the FSEs could include the addition of various immune cells, which would allow further study of inflammatory processes and testing of novel therapeutics.

MATERIALS AND METHODS

Isolation of keratinocytes and fibroblasts

See **Supplementary Table 1** for the contents of the culture media. Healthy skin samples were obtained from adult patients who underwent elective surgery at the Departments of Surgery or Plastic and Reconstructive Surgery of the Red Cross Hospital. Eleven skin tissue samples were used, originating from abdominal, leg, or arm reconstructions wherein excess skin was removed (donor age: 43.8 ± 11.7 years old; donor sex: 72.7% female). These samples were collected in the period between January 2021 and July 2021. Consent for the use of these anonymized, post-operative residual tissue samples was received through the informed opt-out protocol of the Red Cross Hospital, which was in accordance with the national guidelines (https://www.coreon.org/ accessed on 23 November 2020) and approved by the institutional privacy officers. Subjects were actively informed of this procedure and were able to easily withdraw at any point. Split-thickness samples of 0.3 mm were harvested using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany). The epidermis was separated from the dermis using forceps after incubating the harvested skin samples in 0.25% dispase (Gibco) at 37 °C for 45 min. For fibroblast isolation, the dermal part of the split skin was cut into small pieces and submerged into a 0.25% collagenase (Roche)/0.25% dispase solution at 37 °C for 2 h. After addition of 1 mM EDTA/PBS to inhibit collagenase, the cell suspension was poured through a 500 µm cell strainer and centrifuged for 10 min at 360 × *g*. The cell pellet was resuspended in culture medium (**Supplementary Table 1**) and poured through a 70 µm cell strainer and cultured at 37 °C and 5% CO_2. For keratinocyte isolation, the epidermis was transferred into 0.05% trypsin and incubated for 20 min at 37 °C. The cell suspension was poured through a 70 µm cell strainer and centrifuged for 10 min at 110 × *g*. Next, the cell pellet was washed in culture medium and centrifuged for 10 min at 160 × g. The cell pellet was then resuspended in CellnTec-07S culture medium, and keratinocytes were transferred onto a 1 µg/cm2 collagen-type-IV-coated culturing flask at 37 °C and 5% CO_2.

Full skin equivalent models

De-epidermalized dermis (DED; European Tissue Bank BISLIFE, Beverwijk, The Netherlands), MatriDerm® (thickness 3 mm; MedSkin Solutions Dr. Suwelack AG, Billerbeck, Germany), and Mucomaix® (thickness 3 mm; Matricel GmbH, Hertzogenrath,

Germany) were cut into square pieces of 1.44 cm^2. At day one, 200,000 fibroblasts were seeded onto the matrices (for DED, on the reticular side in a metal ring), and the matrices were submerged in culture medium for 4 days at 37 °C and 5% CO_2. Subsequently, 100,000 keratinocytes (from frozen stock) were seeded on the opposite side (for DED, on the papillary side in a metal ring), and the models were cultured submerged in FSE I medium containing 4 ng/mL KGF and 1 ng/mL EGF for 4 days at 37 °C and 5% CO_2. Next, the FSEs were transferred to a stainless-steel grid and cultured air-exposed in FSE II medium containing 4 ng/mL KGF and 1 ng/mL EGF. From day 11, FSEs were cultured in FSE III medium containing 2 ng/mL KGF and 0.5 ng/mL EGF and from day 15 onward in FSE III medium that was refreshed twice a week. Cell numbers and culture times are based on our preliminary experiments where we optimized these settings.

Ex vivo human skin model

Using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany), 0.5 mm split-thickness skin was harvested from human skin and cut into square pieces of 1.44 cm^2. These models were transferred to a stainless-steel grid and cultured air-exposed at 37 °C with 5% CO_2 in FSE II medium that was refreshed twice a week.

Induction of burn injury

A copper plate (2 mm × 10 mm) attached to a PACE intelliHeat ST50 soldering iron (PACE, Vass, NC, USA) was heated to 80 °C and applied to the epidermal side of the models for 20 s without exerting pressure. The temperature of the copper device was measured by an external digital thermometer (Farnell InOne, Utrecht, The Netherlands). FSEs were put in culture for 21 days before they were burn-injured and then cultured for 2 h (T0), 1 week, and 2 weeks. In parallel, burn injury was induced on the ex vivo human skin models, and these models were then also cultured for 2 h (T0), 1 week, 2 weeks. For both FSEs and ex vivo human skin models, the medium was refreshed twice a week. **Figure 6** shows a scheme of the experiment.

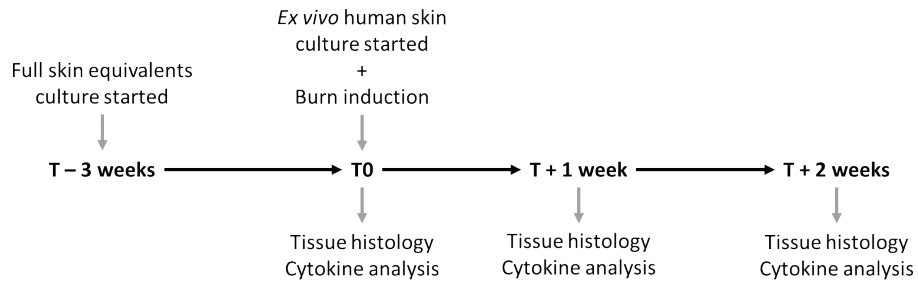

Figure 6. Experiment scheme showing the timing of the performed steps.

Immunohistochemistry

Kryofix (50% ethanol, 3% PEG300)-fixed paraffin-embedded (KFPE) samples were cut into 5 µm thick sections and rehydrated followed by hematoxylin and eosin staining or blocking of endogenous peroxidase using 1% hydrogen peroxide for 15 min at RT. After antigen retrieval was performed (**Supplementary Table 2**), sections were pre-incubated with 5% normal goat serum (Abcam, Cambridge, UK) diluted in PBS + 1% bovine serum albumin. Sections were then incubated with primary antibodies for the detection of pan-cytokeratin, cytokeratin-10, cytokeratin-15, cytokeratin-17, involucrin, collagen IV, laminin α 5, vimentin, aSMA, Ki67, and BrdU (**Supplementary Table 2**) for 1 h at RT followed by incubation with a poly-HRP-goat-anti-mouse or rabbit secondary antibody (Bright Vision, VWR, Amsterdam, The Netherlands) for 30 min at RT. After washing, detection was established using 3,30-Diaminobenzidine (DAB). After DAB staining was completed, sections were counterstained with hematoxylin, dehydrated, and mounted with Eukit Mounting Medium (Sigma-Aldrich, St. Louis, MO, USA). For 5-bromo-20-deoxyuridine (BrdU) staining, culture medium was supplemented with 20 µM BrdU (Sigma-Aldrich, St. Louis, MO, USA) 24 h before termination.

Microscopy

Microscopic visualization was performed with a Zeiss Axioskop40FL microscope (Zeiss, Breda, The Netherlands). Images were acquired using a Nikon Eclipse TS2 camera and the NIS-Elements software version 4.4 (Nikon Instruments, Amsterdam, The Netherlands).

Re-epithelization rate

Re-epithelization length was measured in microscopic images of H&E-stained sections using standardized measurement to calculate µm/pixel in NIS-Elements software. As both sides of each model were measured, the mean was used in the analysis.

Immunoassay of culture medium

Cytokines, chemokines, and growth factors were analyzed in samples of culture medium at T0, T + 1-4 days, T + 5-7 days, and T + 8-11 days (after burn injury). Samples from biological duplicates were pooled per donor (n = 3 donors). Neat samples were measured using the Human Essential Immune Response LegendPlex Multi-analyte Flow Assay kit (cat. 740929; BioLegend), according to the manufacturer's instruction, and were acquired on a flow cytometer (MACS Quant Analyzer 10,Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). This 13-plex immunoassay included: IL-1β, IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IP-10 (CXCL10), MCP-1 (CCL2), IFN-γ, TNF-α, and TGF-β1. Concentrations were determined using FlowLogic software (Inivai Technologies, Victoria, Australia) and recalculated to pg/mL per day of culture to compensate for differences in intervals of

medium changes. When cytokine levels were out of range of the standard, either the lowest level of quantification or the highest level of quantification was used.

Statistical analysis and data visualization

Differences in re-epithelization length and cytokine levels between different time points were explored using the Mann-Whitney U tests in Graphpad version 5.01 (PRISM, La Jolla, CA, USA), and only significant differences were shown in the graphs. A p-value of <0.05 was considered to be statistically significant. The data were visualized using Graphpad version 5.01 and R (ggplot package, open source).

ACKNOWLEDGMENTS

We want to thank Judith Huijgen, Leonore Mastenbroek, and Serena van den Berg of the Association of Dutch Burn Centres for their technical assistance. MatriDerm® was kindly provided as research material by MedSkin Solutions, Dr. Suwelack AG, Billerbeck, Germany. Mucomaix® was kindly provided by Matricel GmbH, Hertzogenrath, Germany. DED was kindly provided by the European Tissue Bank BISLIFE, Beverwijk. This research was funded by the Dutch Burns Foundation under grant numbers WO/16.02 (BKHLB) and WO/17.108 (BKHLB) and by Health-Holland under grant number LSHM16052-HSGF (BKHLB).

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SUPPLEMENTARY MATERIAL

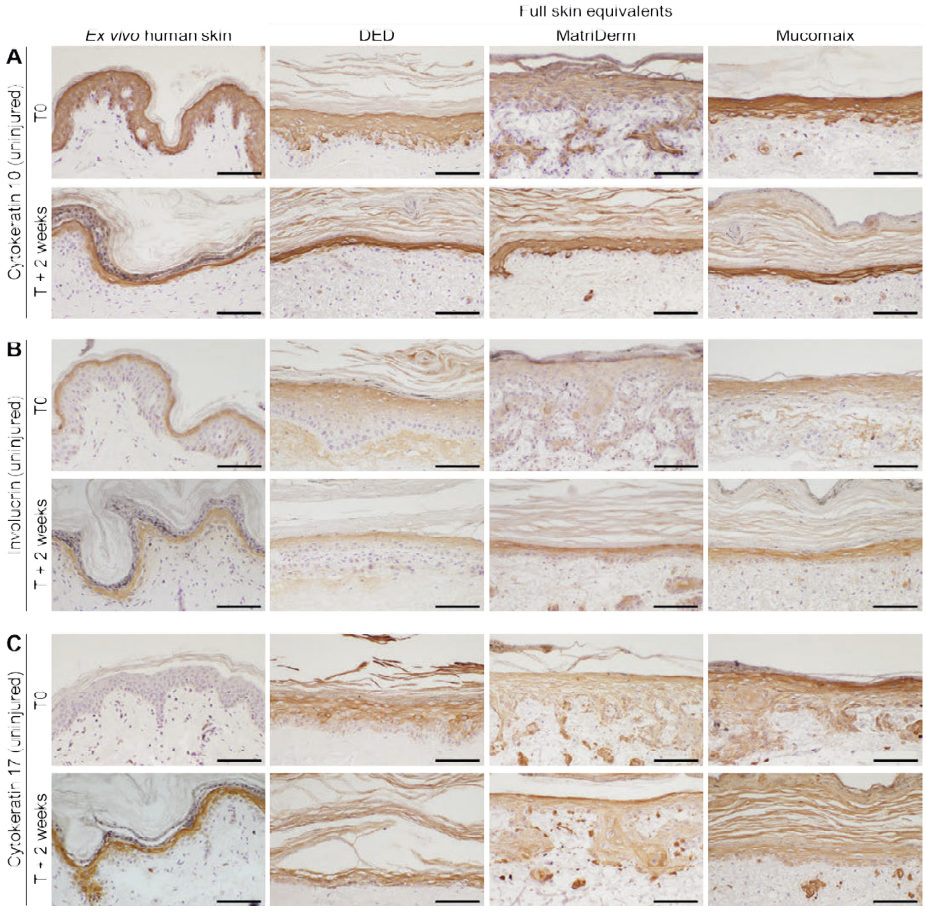
Supplementary material is linked to the online version of the article at: https://www.mdpi.com/2079-4983/14/1/29.

Supplementary Table 1. Culture medium for cells and full skin equivalents.

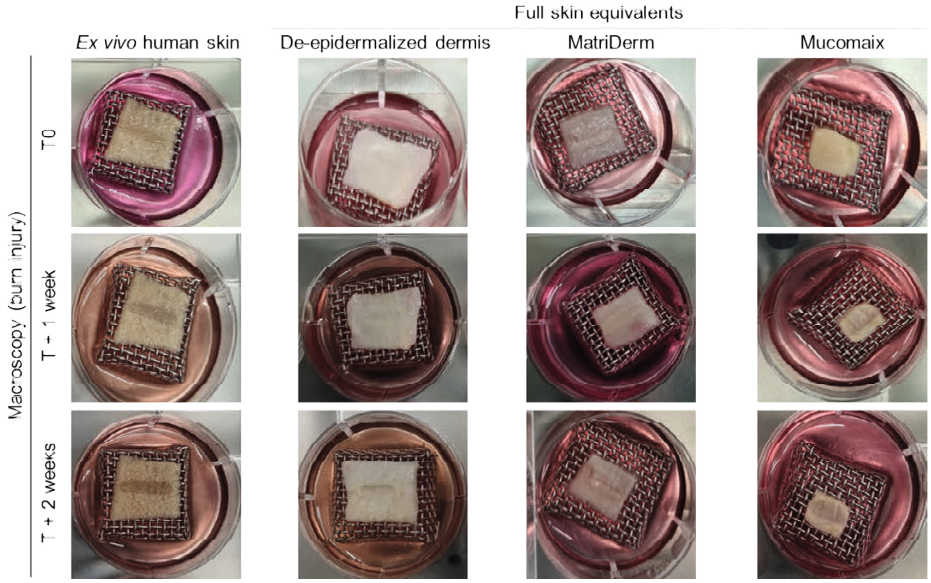
Medium	Contents
Culture medium	Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal calf serum (Fetalclone III, Logan, UT); 1% 200 mM glutamine, antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin; Invitrogen)
FSE I medium	DMEM/Ham's F12 Nutmix (3:1) (Invitrogen), 5% fetal calf serum (Fetalclone III, Logan, UT), 1 mM hydrocortisone, 1 mM isoproterenol, 0.1 mM insulin, a lipid supplement (25 mM palmitic acid, 15 mM linoleic acid, 7 mM arachidonic acid, and 24 mM bovine serum albumin) (all Sigma-Aldrich), antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin; Invitrogen)
FSE II medium	DMEM/Ham's F12 Nutmix (3:1) (Invitrogen), 2% fetal calf serum (Fetalclone III, Logan, UT), 1 mM hydrocortisone, 1 mM isoproterenol, 0.1 mM insulin, 1.9 µM DL-a-tocoferol, a lipid supplement (25 mM palmitic acid, 15 mM linoleic acid, 7 mM arachidonic acid, and 24 mM bovine serum albumin) (all Sigma-Aldrich), antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin; Invitrogen)
FSE III medium	DMEM/Ham's F12 Nutmix (3:1) (Invitrogen), 0.5% fetal calf serum (Fetalclone III, Logan, UT), 1 mM hydrocortisone, 1 mM isoproterenol, 0.1 mM insulin, 1.9 µM DL-a-tocoferol, 130 mg/mL ascorbic acid, a lipid supplement (25 mM palmitic acid, 15 mM linoleic acid, 7 mM arachidonic acid, and 24 mM bovine serum albumin) (all Sigma-Aldrich), antibiotics (100 IU/mL penicillin, 100 mg/mL streptomycin; Invitrogen)

Supplementary Table 2. Antibodies used for immunohistochemistry.

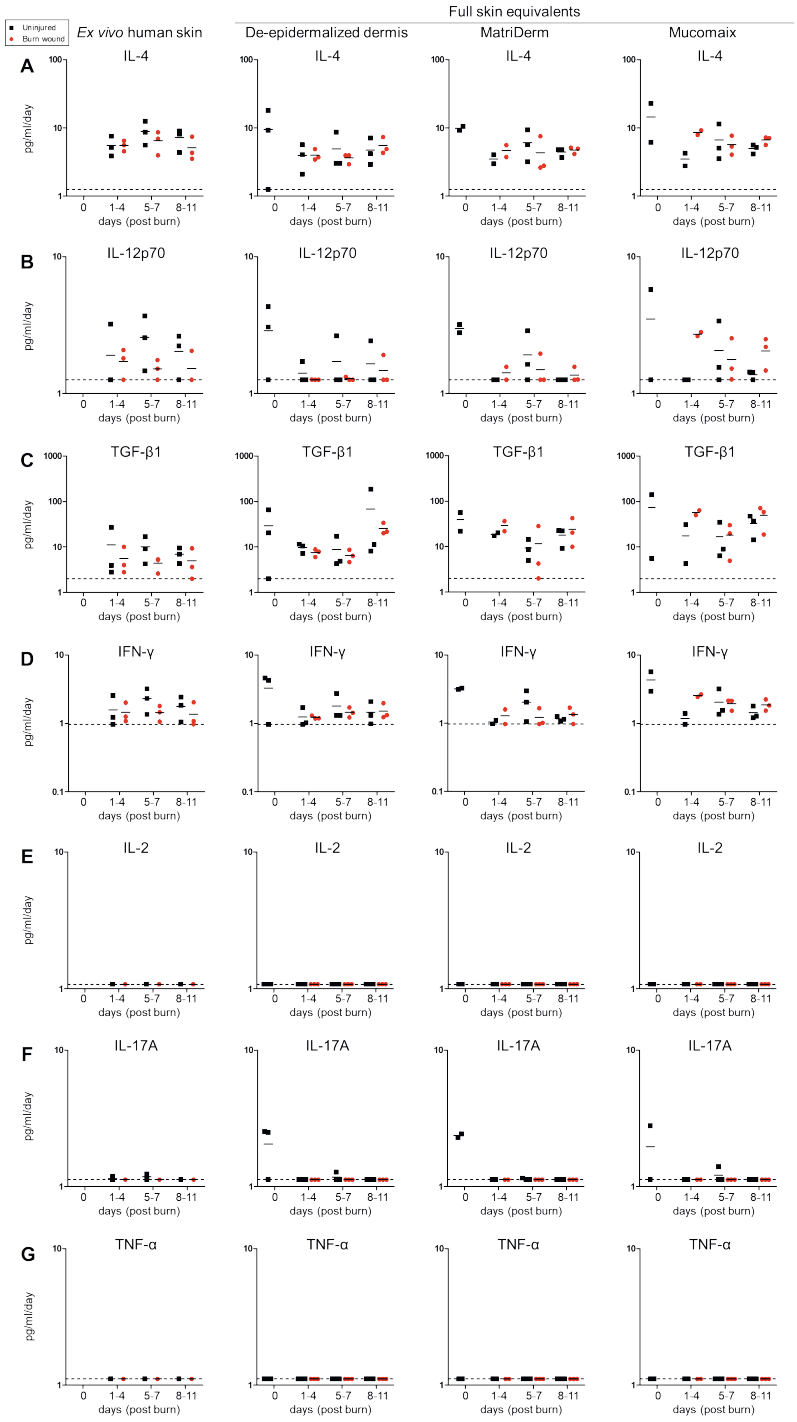
Primary antibody	Clone	Host	Dilution	Manufacturer	Antigen retrieval
Pan-cytokeratin	PCK-26	Mouse	1:200	Sigma	Triton
Cytokeratin 10	Polyclonal	Rabbit	1:5000	Abcam	Citrate
Cytokeratin 15	EPR1614Y	Mouse	1:200	Abcam	Citrate
Cytokeratin 17	E3	Mouse	1:500	DAKO	Citrate
Involucrin	SY5	Mouse	1:250	Novocastra	Triton
Collagen IV	CIV 22	Mouse	1:100	DAKO	Triton
Laminin α 5	4C7	Mouse	1:200	DAKO	Triton
Vimentin	V9	Mouse	1:1000	DAKO	Triton
α-SMA	1A4	Mouse	1:500	DAKO	Tris/EDTA
Ki67	MIB1	Mouse	1:100	DAKO	Tris/EDTA
BrdU	IIB5	Mouse	1:200	MP Biomedicals	HCl/Borax



Supplementary Figure 1. Microscopic images of cytokeratin 10, involucrin and cytokeratin 17 in cultured skin models. Ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Immunohistochemical (**A**) cytokeratin 10; (**B**) involucrin; (**C**) cytokeratin 17 DAB staining. Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, day 0 was after the initial 3 weeks of culture. Black scale bar = 100 µm.



Supplementary Figure 2. Macroscopic images of burn- injured skin models. Ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Models were produced from 3 different skin donors in duplicate. For the full skin equivalent models, day 0 was after the initial 3 weeks of culture.



Supplementary Figure 3. Cytokines detected in culture medium of burn-injured and uninjured

skin models. Ex vivo human skin (left) and full skin equivalents generated from de-epidermalized dermis, MatriDerm and Mucomaix (right). Level of (**A**) IL-4; (**B**) IL-12p70; (**C**) TGF-β1; (**D**) IFN-γ; (**E**) IL-2; (**F**) IL-17A; (**G**) TNF-α in the culture medium at T0, T + 1-4, T + 5-7 and T + 8-11 days (after burn injury). Samples from biological duplicates were pooled per donor (n = 3 donors) and re-calculated into pg/ml per day of culture to compensate for intermittent medium changes. Striped line indicates the highest or lowest level of quantification. Because ex vivo human skin models were started at T0, no levels are shown for day 0. For the full skin equivalent models, day 0 was after the initial 3 weeks of culture.

CHAPTER 7

Monocytes and T cells Incorporated in Full Skin Equivalents to Study Innate or Adaptive Immune Reactions after Burn Injury

Published in adjusted form in Frontiers in Immunology, ***2023****, 14, 1264716*
DOI: 10.3389/fimmu.2023.1264716

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ABSTRACT

Thermal injury often causes excessive and long-lasting inflammation that complicates recovery of patients. There is a lack of appropriate, animal-free models to study the inflammatory processes after burn injury and develop more effective therapies to improve burn care and outcome. Here, we created a human full skin equivalent (FSE) burn wound model in which human peripheral blood derived monocytes and T cells were incorporated. These cells are involved in the innate and adaptive immune response to burn injury. Monocytes in the FSEs differentiated into macrophages. Percentage of HLA-DR$^+$ macrophages and production of cytokines such as IL-1β, IL-6, IL-8 (CXCL8) and IL-12p70 were increased in burn-injured FSEs compared to uninjured FSEs. A portion of T cells actively migrated into the FSE and highly expressed CD25. T cells in the FSE also showed increased expression of markers related to regulatory T cell, Th1 or Th17 activity, coinciding with increased production of cytokines such as IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IP-10 (CXCL10) and TGF-β1. Burn injury did not affect the studied T cell markers, but the levels of IL-10 and IP-10 were decreased. In this pilot study, we set the first steps to develop an immunocompetent skin model for the study of burn-induced innate and adaptive immune reactions, reducing the need for experimental animals.

INTRODUCTION

The immune response that follows burn injury is often excessive and uncontrolled, leading to secondary health complications including systemic inflammation, wound deepening, delayed healing and severe scarring [1–4]. To improve burn wound healing by modulating immune processes, the reactions underlying burn-induced inflammation and skin regeneration need to be better understood. Detailed knowledge on the specific immune cells and cytokines that are involved in the inflammatory response is, however, still limited. There is a need for appropriate human 3D models to study immune dysfunction after burn injury, avoiding the use of animal experimentation.

Shortly after burn injury, pro-inflammatory neutrophils and macrophages accumulate in the wound [5–7]. These phagocytic cells are essential for removal of cell debris and pathogens from the injured site [8]. High numbers of overactive and undirected innate immune cells can, however, lead to damage to healthy tissues and hamper wound healing processes [9–11]. Next to pro-inflammatory macrophages (often called M1), there are anti-inflammatory macrophages (commonly referred to as M2) which dampen inflammatory responses and support wound healing [8,12]. Later in the inflammation phase after burn trauma, T cells migrate to the wounded area to orchestrate specific anti-pathogen responses and control ongoing inflammation to advance wound healing [13,14]. T cell effector subsets Th1 and Th17 cells play a role in the enhancement of inflammation, whereas Th2 and regulatory T cells (Tregs) are involved in the resolution of inflammation [15,16]. Achieving a proper immune balance between pro- and anti-inflammatory responses is essential to establish an uncomplicated and timely transition from inflammation towards wound healing. Yet it is still unknown exactly how the immune reactions after burn injury get distorted and how this can be restored.

Studies in burn patients are limited by the absence of baseline measurements, differences between individuals and injuries, and restrictions in the collection of samples [6,17]. Therefore, most knowledge on burn trauma and the immune response was previously obtained through animal experiments [6,17,18]. However, the use of experimental animals involves certain disadvantages including ethical concerns and problems in translation [19]. Although the collected information is valuable, animals do not accurately resemble the human situation due to differences in skin architecture and wound healing processes [18,20–22], making it challenging to extrapolate relevant findings to burn patients. Therefore, there is a need to find alternative approaches to perform research on burn wound healing [23]. In vitro human skin models are promising alternative experimental instruments to study aspects of skin injury, based on the behavior of keratinocytes and fibroblasts [23–25]**.** Currently, many of the skin models fail to capture the complex

processes of skin inflammation, because they lack essential immune components [26,27]. To make in vitro models more appropriate, relevant immune cells and inflammatory mediators need to be incorporated.

In this study, we generated a human full skin equivalent (FSE) model based on the collagen-elastin matrix MatriDerm® [28,29], as we described previously [30]. MatriDerm is a clinically applied matrix that provides a durable extracellular matrix architecture supporting epithelization in skin defects [31–34]. To study the effect of burn injury on cells from the innate and the adaptive immune system, monocytes and T cells isolated from human buffy coats were cultured in this human FSE. We studied the effect of burn injury on the phenotype and cytokine secretion of monocytes and T cells. By incorporating immune cells into the FSE, we set the first steps in the development of a more relevant skin model for the study of inflammatory reactions that occur after burn injury, while supporting the replacement and reduction of animal experiments.

RESULTS

Human full skin equivalents to study burn injury in vitro

FSEs were produced by seeding human keratinocytes and fibroblasts into MatriDerm matrices containing collagen-elastin (see **Figure 6A** for procedure), as we previously described [30]. After 3 weeks of culture, the FSEs contained a properly developed epidermis and dermis. Burn injury was inflicted on the FSE and subsequently visualized by microscopy (**Figure 1**). Three days after injury, the burn was visible as the epidermis was detached from the injured area of the dermis.

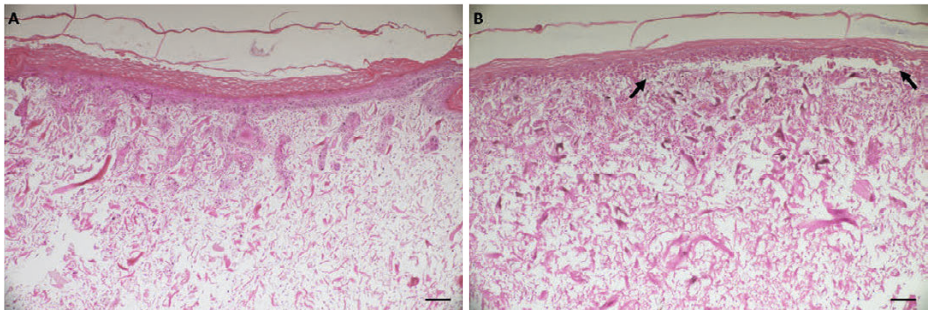

Figure 1. Histology of uninjured and burn-injured FSEs. H&E staining. (**A**) FSE after 3 weeks of culture. (**B**) FSE after 3 weeks of culture and 3 days after burn injury. The detached epidermis caused by the burn is clearly visible. Black scale bar = 100 µm; black arrows indicate burn injured area.

Monocytes differentiated into macrophages in full skin equivalents and upregulated their HLA-DR expression upon burn injury

Unstimulated monocytes were added to (burn-injured) FSEs to simulate an innate immune response to burn injury. About 2.5 × 10^5 monocytes were administered to the dermal side of the FSEs to prevent the cells from adhering to the culture transwell (see **Figure 6B** for procedure). Unstimulated monocytes cultured in suspension or in a matrix without skin cells served as controls. Using microscopy, we confirmed that the monocytes were present in the FSE, irrespective of burn injury. Monocytes seemed to lose or downregulate the monocyte marker CD14 (data not shown) and upregulate the expression of macrophage marker CD68 in the FSEs upon culture regardless of burn injury (**Figure 2A**), suggesting that these cells differentiated into macrophages. To study these monocyte-derived macrophages in more detail, FSEs that were cultured for 7 days were dissociated and the cells were isolated.

Using flow cytometry, we studied the number of macrophages and the expression of several markers: CD68 (macrophage marker), CD14 (monocyte marker), CD11b (activation), HLA-DR (M1 differentiation) and CD163 (M2 differentiation). Uninjured FSEs contained on average 8.0 ×10^4 $CD68^+$ cells (macrophages) (**Figure 2B**). There was high variation in the percentages of $CD68^+$ macrophages that were $CD14^+$ and $CD11b^+$ (**Figure 2C,D**). There were samples with a low percentage of $CD14^+$ and $CD11b^+$ macrophages and samples with a high percentage. This level or rate of macrophage differentiation and activation in the FSE (with or without burn injury) is likely donor-dependent. The percentages of HLA-DR^+ and $CD163^+$ macrophages were lower in the FSEs than in cells cultured in the absence of skin cells (**Figure 2E,F**). Burn injury seemed to increase the average number of macrophages (1.6 × 10^5), although this did not reach significance. The percentage of $CD14^+$ macrophages was significantly decreased after burn injury. Interestingly, burn injury significantly increased the percentage of HLA-DR^+ macrophages and appeared to decrease the percentage of $CD163^+$ macrophages in the FSEs. Thus, we generated a human FSE model with keratinocytes and fibroblasts incorporating unstimulated monocytes that actively differentiated into macrophages upon culture. Burn injury appeared to stimulate differentiation into M1 macrophages.

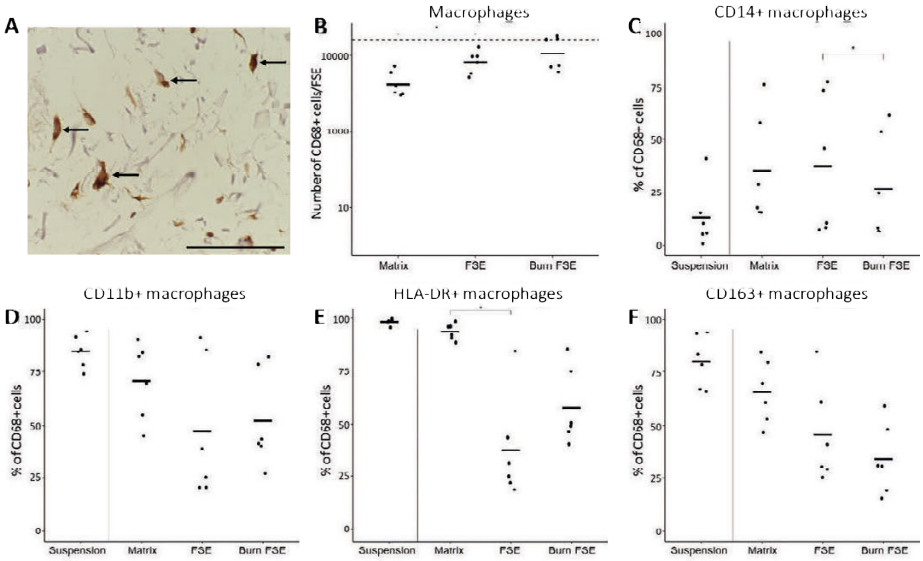

Figure 2. Monocytes after 7 days of culture in (burn-injured) FSEs. (**A**) Immunohistochemical CD68 staining of an injured FSE. (**B**) Number of $CD68^+$ cells (macrophages) per FSE after isolation based on flow cytometry; dashed line indicates the number of monocytes added to the dermal side of the FSE. Percentage of $CD68^+$ cells (macrophages) that were (**C**) $CD14^+$; (**D**) $CD11b^+$; (**E**) HLA-DR^+; (**F**) $CD163^+$. Experiments were performed in duplicate using keratinocytes and fibroblasts from 6 different donors and monocytes from 4 different donors. Only comparisons between monocytes in matrix, in uninjured FSEs and in burn-injured FSEs are shown. Statistically significant differences were calculated using Wilcoxon signed-rank test. Significant differences are indicated by asterisks: *: $p < 0.05$.

Production of inflammatory cytokines seem to be increased upon administration of monocytes into full skin equivalents, regardless of burn injury

Next, cytokine levels in the culture media of the FSEs were determined at day 3 (when medium was changed) and at day 7 (when FSEs were terminated) to study the effect of administering monocytes into the FSE (**Figure 3**). FSEs without monocytes produced high levels of IL-6, IL-8 (CXCL8) and MCP-1 (CCL2) (**Figure 3** and **Figure 5**). Burn injury increased the levels of IL-4, IL-6, IL-8 and IL-12p70 and decreased the level of IP-10 (CXCL10) (**Supplementary Figure 2**) at both day 3 and day 7. The inclusion of monocytes in the FSEs seemed to further increase the levels of IL-4, IL-6, IL-8 and IL-12p70, and burn injury led to a further increase of IL-1β, IL-6, IL-8 and IL-12p70 at both time points. No significant differences between the different conditions were observed for the levels of IL-2, MCP-1 and TNF-α (**Supplementary Figure 2**).

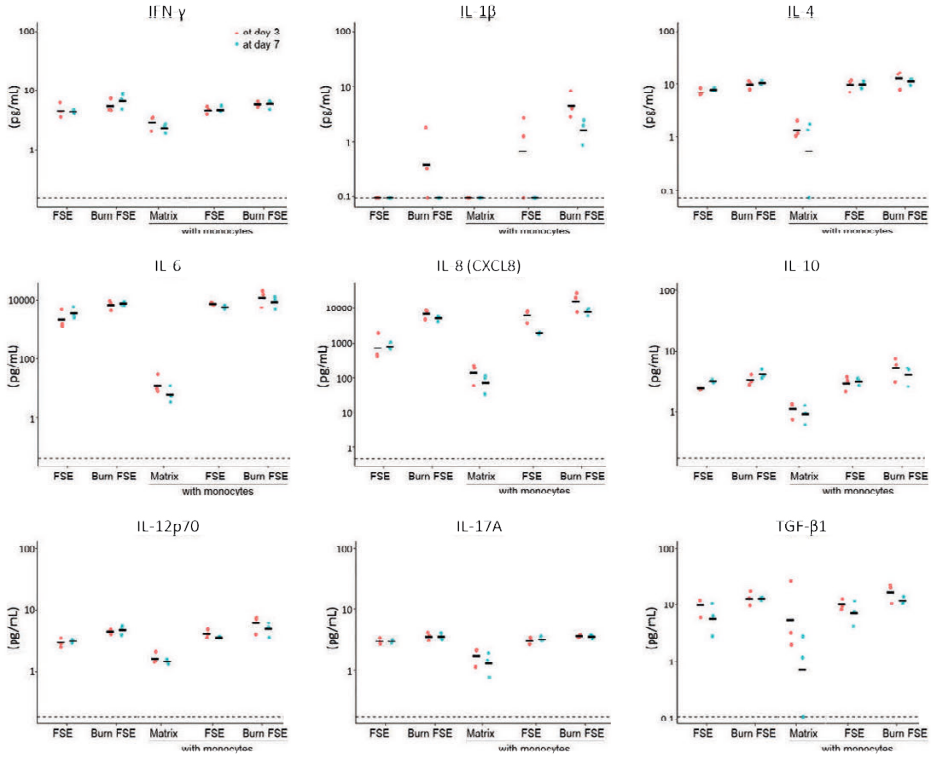

Figure 3. Cytokine levels in medium of (burn-injured) FSEs after 3 or 7 days of culture with monocytes. Samples from biological duplicates were averaged per donor. Concentrations are reported in pg/mL medium. Experiments were performed in duplicate using keratinocytes and fibroblasts from 3 different donors and monocytes from 3 different donors. The dashed line indicates the lowest level of quantification. Statistically significant differences were calculated using Wilcoxon signed-rank test. No comparisons were made with monocytes in matrix only. Significant differences are indicated by asterisks: *: $p < 0.05$.

T cells that migrated into full skin equivalents highly expressed Th1 and Th17 chemokine receptors, irrespective of burn injury

To simulate an adaptive immune response to burn injury, CD3/CD28 bead pre-activated T cells were brought into (burn-injured) FSEs. About 2.5×10^5 T cells were placed between the transwell membrane and the dermal side of the FSEs, and cultured for 3 days (see **Figure 6C** for procedure), based on previous findings [35]. Pre-activated T cells cultured in suspension or in a matrix without skin cells served as controls. Using microscopy, we found that T cells actively migrated into the FSEs (**Figure 4A**). After 3 days of culture, cells were isolated from the FSEs to analyze the T cells by flow cytometry. Based on the flow cytometry analysis, only a small portion (2.8×10^3) of T cells migrated into the FSEs (**Figure 4B**). About 86.7 % of these T cells were $CD4^+$ (**Figure 4C**). The majority of T cells in

the FSEs were $CD25^+$, confirming that the migrated T cells were indeed activated (**Figure 4D**). The percentage of $CD25^+CD127^-$ T cells, which might be an indication for Tregs, was higher in the FSEs than for the T cells cultured in suspension (**Figure 4E**). In FSEs, a clear increase in the percentage of $CXCR3^+$ T cells was observed, which is indicative of Th1 activity (**Figure 4F**). Similarly, we observed an increase in the percentage of $CCR4^+CCR6^+$ T cells in the FSEs, suggesting enhanced Th17 activity (**Figure 4G**). On average the number of T cells in burn-injured FSEs was similar to that found in uninjured FSEs. Burn injury did not affect the analyzed T cell markers. Together, it was shown that particularly activated T cells migrated into FSEs and that Treg and Th1/Th17 activation might be enhanced, irrespective of burn injury.

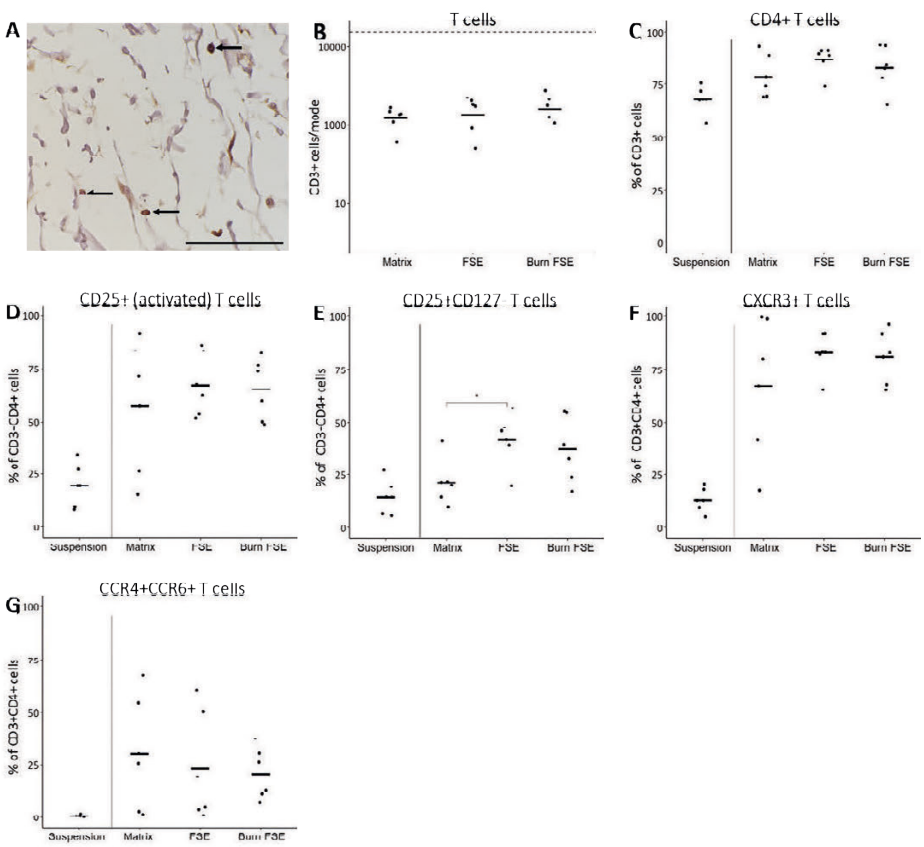

Figure 4. Pre-activated T cells after 3 days of culture in (burn-injured) FSEs. (**A**) Immunohistochemical CD3 staining of an injured FSE. (**B**) Number of T cells ($CD3^+$ cells) per FSE after isolation using flow cytometry; dashed line indicates the number of T cells added to the transwell. (**C**) Percentage of $CD3^+$ (T cells) that are $CD4^+$. Percentage of $CD4^+$ T cells that were (**D**) $CD25^+$; (**E**) $CD25^+CD127^-$; (**F**) $CXCR3^+$; (**G**) $CCR4^+CCR6^+$. Experiments were performed in duplicate using keratinocytes and fibroblasts from 6 different donors and T cells from 5 different donors. Only comparisons between T cells in matrix, in uninjured FSEs and in burn-injured FSEs are shown. Statistically significant differences were calculated using Wilcoxon signed-rank test. Significant differences are indicated by asterisks: *: $p < 0.05$.

T cells increase cytokines levels in (burn-injured) full skin equivalents

To study the effect of incorporating T cells in (burn-injured) FSEs, cytokine levels were analyzed in the culture medium at day 3 (**Figure 5**). The levels in FSEs without T cells and the effect of burn injury on these FSEs were reported in the previous section (**Figure 3**). The inclusion of T cells in the FSEs significantly increased the levels of IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IP-10 and TGF-β1, irrespective of burn injury. However, the levels of IL-10 and IP-10 were reduced after burn injury. Significant

differences between the different conditions were not observed for the levels of IL-1β, IL-2, MCP-1 and TNF-α (**Supplementary Figure 3**). So, in general, the inclusion of T cells in the FSEs appeared to further increase both pro- and anti-inflammatory cytokines, while burn injury specifically reduced the levels of IL-10 and IP-10.

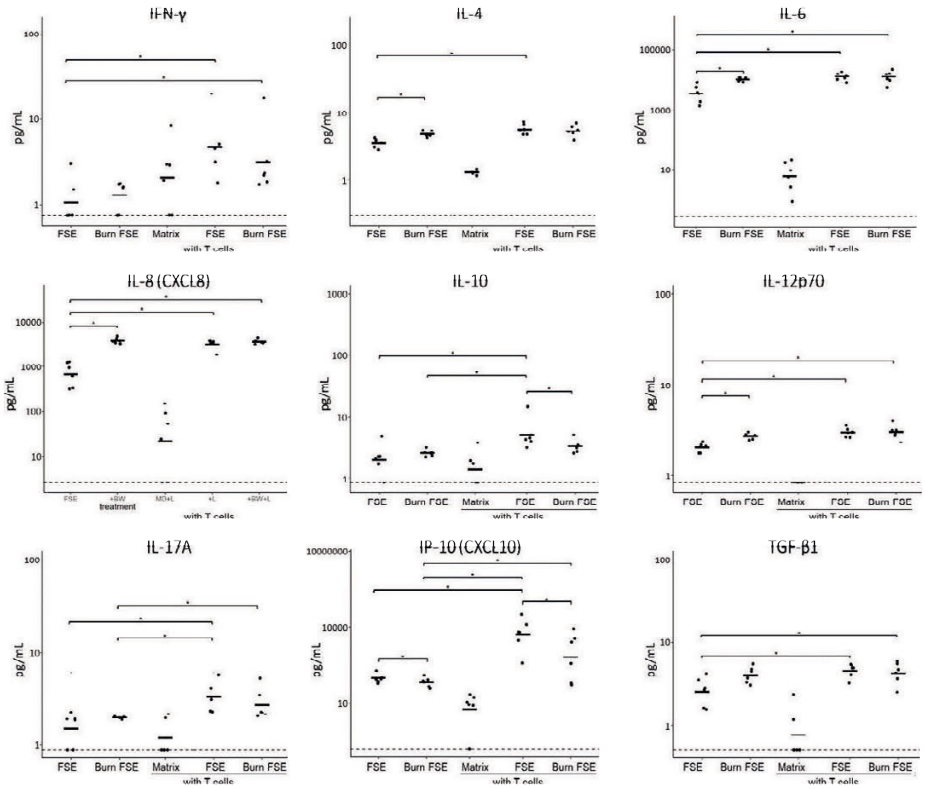

Figure 5. Cytokine levels in medium of (burn-injured) FSEs after 3 days of culture with pre-activated T cells. Samples from biological duplicates were averaged per donor. Concentrations are reported in pg/mL medium. Experiments were performed in duplicate using keratinocytes and fibroblasts from 6 different donors and T cells from 5 different donors. The dashed line indicates the lowest level of quantification. Statistically significant differences were calculated using Wilcoxon signed-rank test. No comparisons were made with the monocytes in matrix. Significant differences are indicated by asterisks: *: $p < 0.05$; **: $p < 0.01$.

DISCUSSION

There is a strong need for appropriate, animal-free models to study immune reactions that occur after burn injury. As standard FSEs are unable to catch these complex immune reactions [26,27,30,36–38], we developed an FSE with monocytes or T cells to simulate

an innate and an adaptive immune reaction to burn injury. Cells of the innate immune system such as neutrophils and monocytes/macrophages are actively involved in the acute inflammatory phase after burn injury, while cells of the adaptive immune system such as T cells are crucial for regulation of ongoing inflammation [5,39]. In this study, we analyzed our immune cell model using microscopy and flow cytometry and focused on changes in cell phenotype and cytokine production. Unlike other models, we used human primary monocytes and were able to analyze them by flow cytometry 1 week after culturing them in FSEs and investigate the effect of burn injury. This model brings us another step closer to a more realistic skin model that is useful for the study of burn-induced inflammation and therapeutic interventions to improve burn wound healing.

Here, we showed that monocytes differentiated into macrophages within 7 days of culture in FSEs. Based on immunohistochemistry, we observed that monocytes in FSEs upregulated their CD68 expression over time while CD14 expression decreased, which is in line with findings of Smith et al. [40]. The percentage of HLA-DR$^+$ macrophages was higher in burn-injured FSEs than in uninjured FSEs, which could be a reaction of the macrophages to the burn injury. Notably, in monocytes/macrophages cultured in suspension there was an even higher percentage of HLA-DR$^+$ cells, which might have been induced by culturing on cell repellent surface. HLA-DR is an MHC class II receptor that is upregulated upon inflammatory stimuli and typifies M1 activity [41]. Furthermore, IL-1β production was only increased in burn-injured FSEs with macrophages, which is a characteristic M1 cytokine [41,42]. Also, CD163 expression, indicative of M2 activation, appeared to be slightly decreased in burn-injured FSEs, but this was not statistically significant. We observed much donor variation for markers CD14 and CD11b, which could be related to distinct activation or differentiation rates of different PBMC donors. Overall, monocytes differentiate into macrophages upon culture in FSEs and there was an indication that burn injury enhanced this polarization further, but more research is needed to clarify this finding.

Other researchers have developed comparable skin models with macrophages to study skin diseases such as inflammatory skin disorders or carcinoma. Chung et al. co-cultured FSEs with RAW264.7 cells to simulate skin inflammatory responses [43]. In this model, the FSE was placed on a transwell membrane while RAW264.7 cells were cultured underneath the transwell. Using this co-culture system, the researchers demonstrated interactions between the skin cells and macrophages that affect cytokine production and the degree of inflammation. Griffoni et al. [44] made co-cultures of polarized PBMC-derived macrophages and FSEs and checked cell viability and expression of M1 and M2 specific genes. These researchers stressed the need for appropriate culture media to create more standardized and accurate immunocompetent skin models. Linde et al. created a

human skin squamous cell carcinoma model with PBMC-derived macrophages that was cultured up to 3 weeks. The researchers analyzed macrophage polarization and found M2 activation of macrophages in the tumor model [45]. In another proof-of-principle study, Bechetoille et al. produced an FSE with anti-inflammatory dermal-type macrophages and studied the effect of co-culture on cytokine production and phagocytic potential of the macrophages [46]. As opposed to these models, we studied the effect of burn injury on PBMC-derived monocytes by means of flow cytometry and cytokine production.

When pre-activated T cells were added to the FSE, a portion of T cells actively migrated into the FSEs. In this population of migrated T cells, the percentage of Th1 receptor CXCR3 expressing cells [47] and Th17 receptors CCR4/CCR6 [48] expressing cells was increased, regardless of burn injury. This coincided with increased levels of pro-inflammatory cytokines IFN-γ, IL-6, IL-8, IL-12p70, IL-17A and IP-10. It has been shown before that the production of chemokines such as IP-10 can be induced by IFN-γ, especially in inflamed tissue [49,50]. IP-10 is a chemoattractant for T cells and binds to only one receptor, namely CXCR3 [51]. The decrease in IP-10 production in the burn-injured FSEs could be related to a loss of keratinocytes caused by the burn injury. The percentage of $CD25^{+}CD127^{-}$ T cells, possibly Tregs, was also increased. Simultaneously, levels of IL-4, IL-10 and TGF-β1 were elevated. The production of IL-10 was, however, reduced in the burn-injured FSEs. This could also be related to the destruction of keratinocytes as about 18% of the model was burn injured or be the result of impaired regulatory activity caused by burn injury. Nevertheless, more research is needed to elucidate this.

Our FSE with T cells was based on previous work, where T cells were cultured in a skin model to study cross-talk between keratinocytes and T cells [35]. Other researchers used similar skin models to focus on skin diseases such as psoriasis or atopic dermatitis [35,52,53]. In studies by Shin et al. and Lorthois et al. T cells were stimulated towards Th1/Th17 to study their role in psoriatic skin models [52,53]. Our model was unique for the study of burn injury. Of the pre-activated T cell pool, only a small portion of T cells migrated into the FSEs. This could be related to incomplete activation of T cells, T cell death, insufficient migratory activity, or suboptimal isolation from the FSEs. The isolation of T cells from FSEs was performed in the absence of collagenase, because collagenase treatment is known to affect the presence of chemokine receptors. This might have limited the yield of T cells compared to monocytes/macrophages from FSEs. Migratory activity of T cells can be enhanced by adding an additional chemotactic stimulus such as T cell chemokines MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5) [54]. Finally, our T cell preparation technique could be improved by magnetic or fluorescence cell sorting to establish an enriched population of T cells before placing the cells in the model.

An important advantage of our FSE model over other models is the use of primary cells instead of cell lines, making these models more representative for the in vivo situation. In other studies, macrophages or T cells were studied by microscopy, but cells were not quantified or analyzed by flow cytometry after culture. A limitation of this study was the absence of keratinocyte and fibroblast markers in the flow cytometry panel. As we showed that an interplay occurs between immune cells and skin cells, it would be interesting to study the effect on keratinocytes and fibroblasts as well. This could give more information on the effect of monocytes or T cells on the healing process in a skin environment. In the current set-up, the effect of burn injury on studied monocyte and T cell markers appeared to be limited. It would be interesting to observe the effect of burn injury on monocytes and T cells for a longer period of time. Moreover, the effect on other monocyte/macrophage and T cell markers or cytokines could be studied. This model can also be used to study the effect of specific immune cell subsets on wound healing. Monocytes can be activated towards M1 or M2 macrophages and T cells can be skewed towards Tregs, Th1, Th2 or Th17 cells. Similarly, the addition of neutrophil subsets, so far still a technical and logistical challenge, would be of great interest to advance our knowledge on the effect of immune cells on burn wound healing [5,55]. However, culturing neutrophils in FSEs will be difficult due to the short lifespan neutrophils and inability to cryopreserve them [56]. Nevertheless, we anticipate that these immunocompetent models will be useful to study therapeutics that modulate inflammatory reactions in the burned skin.

In this pilot study, we developed an FSE model that incorporated monocytes and T cells for the study of burn injury. With this model, we come another step closer to the development of more realistic skin models that will allow the study of both innate and adaptive immune reactions related to burn injury while avoiding the need for animal experimentation.

MATERIALS AND METHODS

Human skin samples

Skin samples were obtained from adult patients who underwent abdominoplasty at the Red Cross Hospital in Beverwijk, Medical Clinic in Velsen or Spaarne Gasthuis in Haarlem. Samples from 17 different donors were used (donor age: 48±13 years; sex: 93% female). Consent for the use of these anonymized, post-operative residual tissue samples was received through an informed opt-out protocol, in accordance with the national guidelines (https://www.coreon.org/) and approved by the institutional privacy officers. Subjects were actively informed of this procedure and were able to easily withdraw at any

point. Split-thickness samples of 0.3 mm were harvested using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany).

Isolation of human keratinocytes and fibroblasts

See **Supplementary Table 1** for the contents of culture media. Harvested skin was incubated in 0.25% dispase (Gibco, ThermoFisher Scientific, Paisley, UK) at 37 °C for 45 min. The epidermis was separated from the dermis using forceps. For fibroblast isolation, the dermal part of the split skin was cut into small pieces and submerged into a 0.25% collagenase A (Roche, Basel, Switzerland) solution at 37 °C for 2 h. After addition of 1 mM EDTA (Life Technologies, Paisley, UK) + PBS (Gibco) to inhibit enzyme activity, the cell suspension was poured through a 500 µm cell strainer (PluriSelect, Leipzich, Germany) and centrifuged for 10 min at 360 × *g*. The cell pellet was resuspended in culture medium and poured through a 70 µm cell strainer (PluriSelect) and cultured at 37 °C with 5% CO_2. For keratinocyte isolation, the epidermis was transferred into 0.05% trypsin (Gibco) and incubated for 20 min at 37 °C. The cell suspension was poured through a 70 µm cell strainer and centrifuged for 10 min at 110 × *g*. Next, the cell pellet was washed in culture medium and centrifuged for 10 min at 160 × *g*. The cell pellet was then resuspended in CnT-07 medium (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) and keratinocytes were transferred onto a 1 µg/cm^2 collagen type IV (Sigma-Aldrich, Saint Louis, MO, USA)-coated culturing flasks (Starstedt AG & Co. KG, Nümbrecht, Germany) at 37 °C with 5% CO_2.

Human Full skin equivalents

MatriDerm® (MedSkin Solutions Dr. Suwelack AG, Billerbeck, Germany) with a thickness 3 mm was cut into circular pieces of 1.13 cm^2. At day one, 2 × 10^5 fibroblasts were seeded onto the matrix and the matrix were submerged in culture medium containing 65 µg/mL ascorbic acid for 4 days at 37 °C with 5% CO_2 (**Figure 6A**). Subsequently, 1 × 10^5 keratinocytes were seeded on the opposite side and the models were cultured submerged in FSE I medium containing 2 ng/ml KGF (ImmunoTools GmbH, Friesoythe, Germany) and 0.5 ng/ml EGF (R&D Systems, Inc., Minneapolis, MN, USA) for 4 days at 37 °C with 5% CO_2. Next, the FSEs were transferred to transwells (Starstedt) and cultured air-exposed in deep well plates (Greiner Bio-One BV, Alphen aan den Rijn, the Netherlands) with FSE II medium containing 4 ng/ml KGF and 1 ng/ml EGF. From day 11 onward FSEs were cultured in FSE III medium containing 4 ng/ml KGF and 1 ng/ml EGF and from day 15 onward in FSE III medium that was refreshed twice a week. Cell numbers and culture conditions are based on preceding experiments [30]. At day 22, immune cells were added to the FSEs.

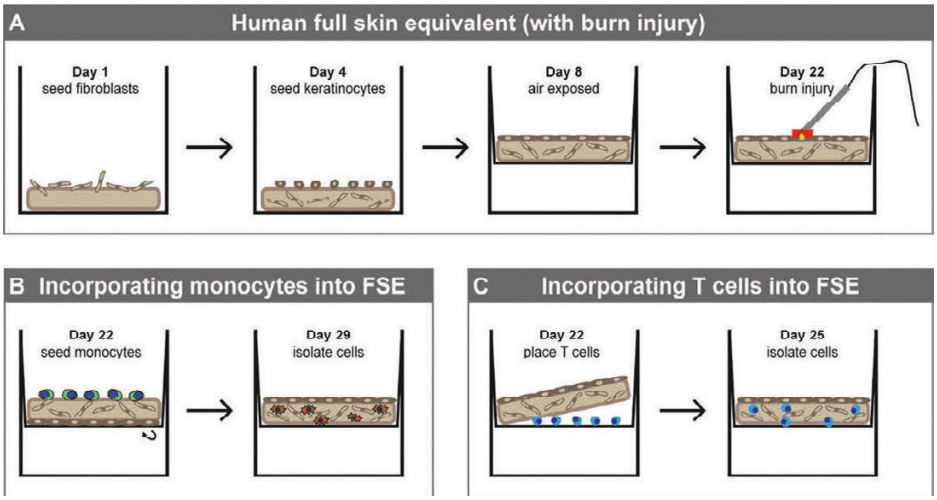

Figure 6 Development of human full skin equivalent (burn wound) model with monocytes or T cells. (**A**) Development of FSE. (**B**) Incorporating monocytes into FSE. (**C**) Incorporating T cells into FSE.

Induction of burn injury

A copper plate (2 × 10 mm) attached to a PACE intelliHeat ST50 soldering iron (Vass, USA) was heated to 80-90 °C and applied to the epidermal side of the models for 20 sec without exerting pressure (**Figure 6A**). The temperature of the copper device was measured by an external digital thermometer (Farnell InOne, Utrecht, the Netherlands).

PBMC isolation from human buffy coat

PBMCs were isolated from buffy coats obtained from healthy donors (Sanquin, Amsterdam, the Netherlands) by density gradient centrifugation using Lymphoprep (Stemcell Technologies, Vancouver, Canada). The buffy coat was diluted in PBS and layered over the density gradient medium. After centrifugation at 1000 × *g* for 15 min (without brakes), the PBMCs were collected in FSE I medium. Cells were resuspended in 50% fetal bovine serum (Gibco) + 40% FSE I medium + 10% dimethyl sulfoxide. After 24 h storage in Mr. Frosty (ThermoFisher scientific) with isopropanol at -80 °C, cells were stored in liquid nitrogen until use.

Monocyte FSE

PBMCs were incubated with anti-CD14 beads (Invitrogen, Waltham, MA, USA) at a bead/cell ratio of 2.5:1 at 2-8 °C for 20 min on a tube roller. Monocytes were isolated from the PBMCs using a magnet (Invitrogen Dynal AS, Oslo, Norway). Monocytes were resuspended in FSE I medium and 2.5 × 10^5 cells were added to the dermal side of FSEs. Inverted FSEs with monocytes were incubated at 37 °C for 2 h and subsequently placed back onto

the transwells (**Figure 6B**). FSEs with monocytes were cultured for 7 more days with a medium change at day 3.

T cell FSE

Lymphocytes were isolated by culturing PBMCs in a culture flask. After 24 h, adherent cells were removed. T cells were activated by adding anti-CD3/CD28 Dynabeads (Gibco) at a bead/cell ratio of 5:1 at 37 °C for 4 h. After the activation, cells were resuspended in FSE I medium and 2.5×10^5 cells were placed between the transwell membrane and the dermal side of the FSE (**Figure 6C**), based on previous findings [35]. FSEs with T cells were cultured for 3 more days.

Cell isolation from immune cell model

The immune cell isolation procedure was based on a protocol from He et al. [57]. Macrophage FSEs were incubated with 0.25 U/ml collagenase A (Roche) at 37 °C in a shaking water bath for 20 min. Because enzymes affect the expression chemokine receptor [58], T cell models were not dissociated using collagenase A. FSEs were then put in C-tubes (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with 5 mL of PBS containing 1 mM EDTA and further dissociated by running program "B" twice on a tissue dissociator (gentleMACS, Miltenyi Biotec GmbH). Samples were passed through a 500 µm and 40 µm cell strainer (PluriSelect) to obtain a single cell suspension.

Flow cytometry

Single cell suspensions were stained using the macrophage or T cell panel (**Supplementary Table 2**). Zombie Aqua (BioLegend, San Diego, CA, USA) was used in the macrophage panel and propidium iodide (Miltenyi Biotec GmbH) was used in the T cell panel to determine viability of cells. Stained cell samples were acquired on the flow cytometer (MACS Quant Analyzer 10, Miltenyi Biotec GmbH) and gating (**Supplementary Figure 1**) was performed in FlowLogic (Inivai Technologies, Victoria, Australia).

Immunohistochemistry

See **Supplementary Table 3** for antigen retrieval and primary antibodies. Kryofix (50% ethanol + 3% PEG300 in demineralized water)-fixed paraffin-embedded samples were cut into sections with a thickness of 5 µm and rehydrated followed by hematoxylin and eosin staining or blocking of endogenous peroxidase using 1% hydrogen peroxide at room temperature for 15 min. After antigen retrieval was performed, sections were pre-incubated with 5% normal goat serum (Sigma-Aldrich) diluted in PBS + 1% bovine serum albumin (ThermoFisher). Sections were then incubated with primary antibodies at room temperature for 1 h followed by incubation with a poly-HRP-goat-anti-mouse or rabbit secondary antibody (BrightVision, VWR, Amsterdam, the Netherlands) for

at room temperature for 30 min. After washing, detection was established using 3,3′-diaminobenzidine (DAB). After DAB staining was completed, sections were counterstained with hematoxylin, dehydrated and mounted with Eukit Mounting Medium (Sigma-Aldrich).

Microscopy

Microscopic visualization was performed with a Zeiss Axioskop40FL microscope (Zeiss, Breda, The Netherlands). Images were acquired using a Nikon Eclipse TS2 camera and the NIS-Elements software version 4.4 (Nikon Instruments, Amsterdam, The Netherlands).

Immunoassay

Cytokines, chemokines and growth factors were analyzed in samples of medium. Neat samples were measured using the Human Essential Immune Response LegendPlex Multi-analyte Flow Assay kit (cat. 740929, BioLegend), according to the manufacturer's instructions and were acquired on the flow cytometer. This 13-plex immunoassay included: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A, IP-10 (CXCL10), MCP-1 (CCL2), TNF-α and TGF-β1. Concentrations were determined using FlowLogic software. When cytokine levels were lower than the standard range, the lowest level of quantification was used. When cytokine levels were higher than the standard range, the levels were estimated based on the fluorescent signal in the assay.

Statistical analysis and data visualization

Differences in cell number/percentages and cytokines levels between different modeling conditions were explored using Wilcoxon signed-rank test in R (ggpubr and ggplot2 packages, open source). Data was visualized using R (ggplot2 package, open source) and significant (p value of < 0.05) differences were indicated by asterisks.

ACKNOWLEDGMENTS

MatriDerm® was kindly provided as research material by MedSkin Solutions, Dr. Suwelack AG, Billerbeck, Germany. This research was funded by the Dutch Burns Foundation under grant numbers WO/17.108 (BKHLB) and WO/22.106 (PPGM).

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Culture media used for cell and FSE culture.

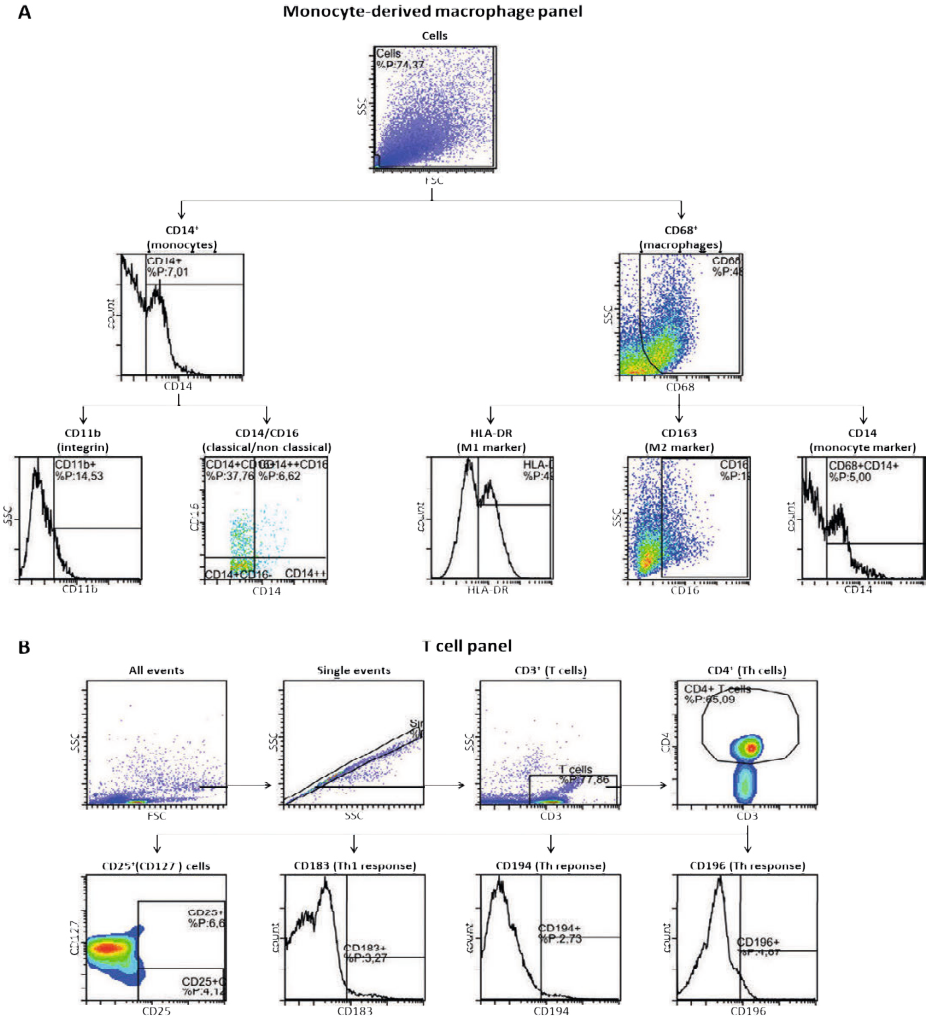
Medium	Contents
Culture medium	Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal calf serum (Fetalclone III, Logan, UT); 1% 200 mM glutamine, antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin (all Invitrogen))
FSE I medium	DMEM + Ham's F12 Nutmix (3:1) (Invitrogen, Paisley, UK), 5% fetal calf serum (Fetalclone III), 1.1 µM hydrocortisone, 1 µM isoproterenol, 0.09 µM insulin, a lipid supplement (25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid, and 24 µM bovine serum albumin (all Sigma-Aldrich)), antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin)
FSE II medium	DMEM + Ham's F12 Nutmix (3:1) (Invitrogen), 2% fetal calf serum (Fetalclone III), 1.1 µM hydrocortisone, 1 µM isoproterenol, 0.09 µM insulin, 1.9 µM DL-α-tocoferol, 5.01 µM β-cyclodextrin, 10.1 µM L-carnitine, 9.99 µM serine, a lipid supplement (25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid, and 24 µM bovine serum albumin), antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin)
FSE III medium	DMEM + Ham's F12 Nutmix (3:1) (Invitrogen), 0.5% fetal calf serum (Fetalclone III), 1.1 µM hydrocortisone, 1 µM isoproterenol, 0.09 µM insulin, 1.9 µM DL-α-tocoferol, 5.01 µM β-cyclodextrin, 130 µg/mL ascorbic acid, 10.1 µM L-carnitine, 9.99 µM serine, a lipid supplement (25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid, and 24 µM bovine serum albumin (all Sigma-Aldrich)), antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin)

Supplementary Table 2. Antibodies used for flow cytometry.

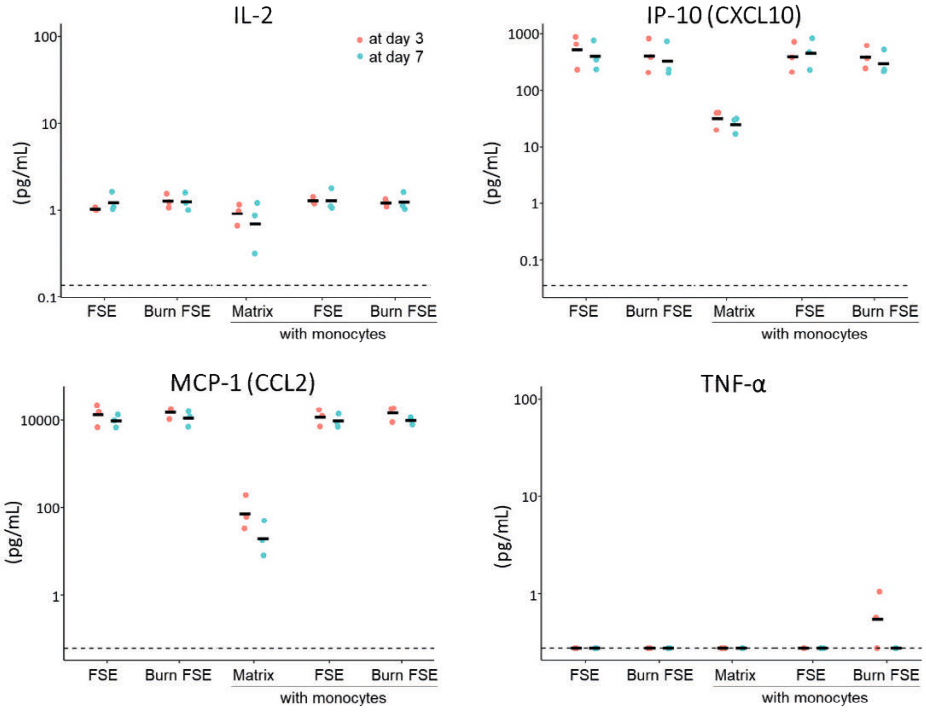
Panel	Primary antibody	Clone	Conjugate	
Macrophage	anti-CD11b	REA713	FITC	Miltenyi Biotec GmbH
	anti-CD14	REA599	VioBlue	
	anti-CD16	REA423	APC	
	anti-CD68	REA886	APC-Vio 770	
	anti-CD163	REA812	PE	
	anti-HLA-DR	L243	PerCP/Cyanine5.5	BioLegend
T cell	anti-CD3	REA613	APC-Vio770	Miltenyi Biotec GmbH
	anti-CD4	REA623	VioBlue	
	anti-CD25	REA945	PE-Vio770	
	anti-CD127	REA614	VioBright FITC	
	anti-CD183 (CXCR3)	G025H7	Briljant Violet 510	BioLegend
	anti-CD194 (CCR4)	REA279	PE	Miltenyi Biotec GmbH
	anti-CD196 (CCR6)	REA190	APC	

Supplementary Table 3. Antibodies used for immunohistochemistry.

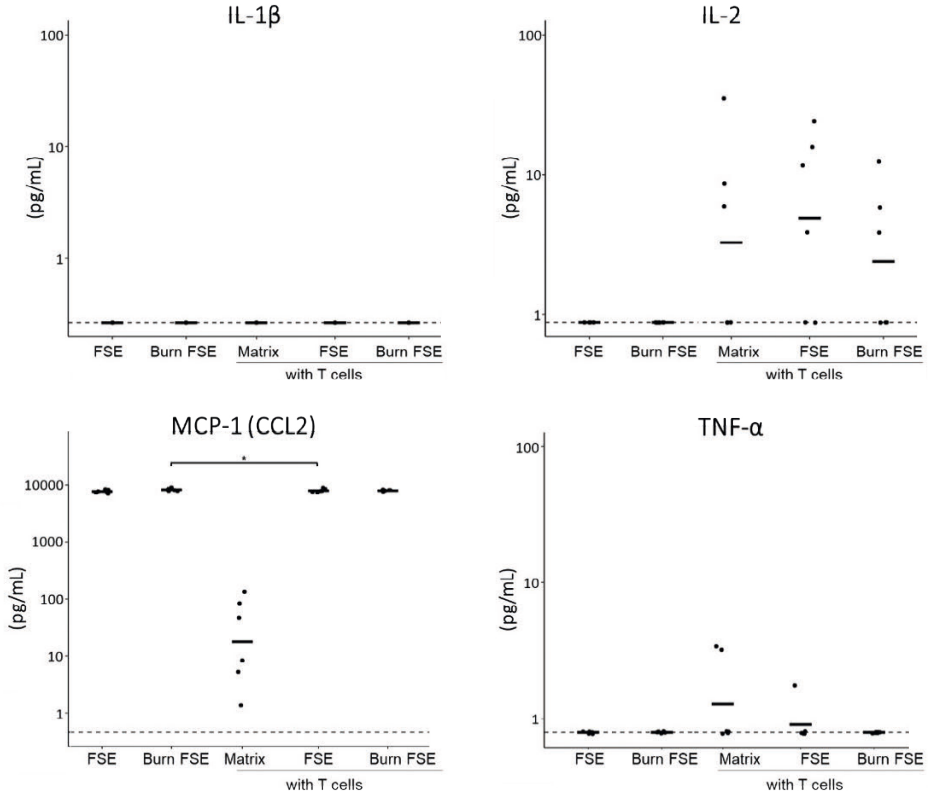
Primary antibody	Clone	Host	Dilution	Manufacturer	Antigen retrieval
anti-CD3	Sp7	Rabbit	1/200	Abcam	EDTA
anti-CD14	Sp192		1/150	Sigma-Aldrich	Citrate
anti-CD68	KP1	Mouse	1/2000	DAKO	EDTA
BrdU	IIB5		1/200	MP Biomedicals	HCl/Borax



Supplementary Figure 1. Gating strategy. Gating strategy is shown for (**A**) monocyte-derived macrophage panel and (**B**) T cell panel.



Supplementary Figure 2. Cytokine levels in medium of FSE models with or without burn injury after 7 days of culture with monocytes. Samples from biological duplicates were pooled per donor (n = 3 donors). Concentrations are reported in pg/mL. The striped line indicates the lowest level of quantification. Statistically significant differences were calculated using Wilcoxon signed-rank test. No comparisons were made with the monocytes in matrix. Significant differences are indicated by asterisks: *: $p < 0.05$.



Supplementary Figure 3. Cytokine levels in medium of FSE models with or without burn injury after 3 days of culture with pre-activated T cells. Samples from biological duplicates were pooled per donor (n = 6 donors). Concentrations are reported in pg/mL. The striped line indicates the lowest level of quantification. Statistically significant differences were calculated using Wilcoxon signed-rank test. No comparisons were made with the monocytes in matrix. Significant differences are indicated by asterisks: *: $p < 0.05$.

DISCUSSION AND SUMMARY

CHAPTER 8

General Discussion

Burn injury induces a multitude of reactions in the body that can become harmful to healthy tissues and even life-threatening [1]. The immune system is actively involved in wound healing processes, essentially to eliminate invading bacteria, remove damaged cells and ensure a timely recovery [2,3]. After burn injury, the immune system can be overstimulated by inflammatory triggers, leading to immune dysfunction and out-of-control inflammation, that in turn slows down re-epithelization and remodeling processes of wound healing [4–6]. Information on response levels and the role of specific immune cells and associated inflammatory mediators during burn wound healing is still largely inadequate. With an improved understanding, treatment strategies can be developed to limit health complications that are a result of excessive inflammation in burn patients.

Studies in burn patients are challenging due to the sudden onset, large variation between injuries, absence of baseline measurements and restrictions in sampling. Therefore, the majority of evidence originates from animal studies. This knowledge was, however, scattered over individual studies and could hardly be compared to the human situation [7,8]. The aim of this thesis was to improve our understanding of the immune response after burn injury. To reach this goal, we reviewed existing animal experimental data, and investigated immune cells and inflammatory mediators in patient samples using flow cytometry and multicolor microscopy. This knowledge was then used to develop skin models wherein the observed processes of burn wound healing and immune reactions can be studied without a need for animal experimentation. In this chapter, we discuss different facets of the burn-induced immune response and opportunities for improvement of burn research and treatment.

BURN-INDUCED INFLAMMATORY MEDIATORS ATTRACT IMMUNE CELLS AND KEEP INFLAMMATION GOING

As thermal injury destroys layers of the skin, it releases danger-associated molecular patterns (DAMPs) [9–11]. The immune system will be activated by DAMPs such as HMGB1 and IL-1α, and will send immune cells towards the site of injury [12,13]. Fibroblasts and keratinocytes that are stressed by the injury, as well as responding immune cells, will produce cytokines thereby influencing the intensity and duration of inflammation [14–17]. We investigated a large range of relevant inflammatory mediators in blood and wound tissue of both experimental animals (**Chapter 2** and **Chapter 3**) and burn patients (**Chapter 4** and **Chapter 5**), at a scale that has not been done before. This extensive, longitudinal analysis of the inflammatory mediators released upon burn injury provides a unique insight into inflammatory pathways activated by burn injury. For instance, we showed that neutrophil attractants and activators (HMGB1, IL-1β, IL-6,

TNF-α, G-CSF, GRO-α (CXCL1) and IL-8 (CXCL8)) were highly increased in wound tissue from animals and patients [18–23]. Furthermore burns increased the level of MCP-1 (CCL2), a chemoattractant for monocytes and supporter of differentiation of monocytes towards macrophages [24,25]. Based on this cytokine milieu, the excessive response of both neutrophils and monocytes is expected to be severe. On the other hand, in wound tissue from burn patients we found that lymphocyte attractants (MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5)) peaked at the second or third week after burn injury [26]. This could mean that while the innate immune system is highly activated immediately after burn, lymphocyte activation could be delayed. This is in line with a studies from Finnerty et al. and Jeschke et al. who showed elevated levels of cytokines that activate innate immune cells in burned children, adults and elderly [27–30]. Other studies have shown that the cytokine profile after burn is associated with an immediate, severe and prolonged innate immune response and the levels of IL-1, IL-6 and MCP-1 can even be linked to increased morbidity and mortality [31,32]. The accumulation of activated immune cells can cause damage to surrounding tissues. This then leads to the release of more DAMPs and cytokines, establishing a vicious circle of inflammation that delays wound healing [10,33].

In **Chapter 6**, we used full skin equivalents (FSEs), consisting of fibroblasts and keratinocytes, to study skin development and skin regeneration after burn injury. In **Chapter 7**, either monocytes or T cells. were incorporated into these FSEs. An important advantage of this models is combinations of incorporated cells lead to the release of specific cytokines. For example, we discovered that IL-6, IL-8 and MCP-1 were highly expressed in FSE models that included only fibroblasts and keratinocytes. This high expression was probably related to a stress response induced by the in vitro culture of these cells [34]. When burn injury was inflicted on these FSE models without immune cells the production of IL-4, IL-6, IL-8 and IL-12p70 increased even further. The release of IL-1β, which was highly expressed after burns in animals and humans, was only detected when monocytes were included in FSE models. Likewise, the expression of IFN-γ and IP-10 was significantly higher when T cells were present in the models, regardless of burn injury. The increased percentage of T cells expressing IP-10 receptor CXCR3 (CD183) might be related to this. These findings demonstrate that interactions between skin cells and immune cells can drive cytokine production and will impact the immune response. Other researchers also showed that co-culture of T cells with keratinocytes increased the levels of chemokines, particularly MCP-1, MIP-3α (CCL20), MIG (CXCL9) and IP-10 (CXCL10) [35–37]. Presumably, T cells produce IFN-γ which stimulates keratinocytes to produce chemokines, especially in inflamed tissue [35,38]. Crosstalk might also happen between T cells and fibroblasts during inflammation [39], which could play a role in (preventing) fibrosis.

NEUTROPHIL RESPONSE TO BURN INJURY IS SEVERE AND MIGHT HAMPER WOUND HEALING

One of the first immune cells to respond to trauma are neutrophils [40–42]. The meta-analysis in **Chapter 2** shows the immediate accumulation of neutrophils both in blood and wound tissue from experimental animal models. The neutrophil response in burn patients is very similar and surges of neutrophils can remain even 4 weeks after injury, as we showed in **Chapter 3** and **Chapter 4**. While textbook schemes dictate that the neutrophil response during wound healing is attenuated within a couple of days [2,8,43], we clearly showed that this is not the case after burn injury. In-depth analysis of blood from burn patients revealed an enormous and long-lasting influx of immature ($CD10^-$) neutrophils (**Chapter 4**). The release of immature neutrophils is part of an emergency compensatory response of the bone marrow (i.e. left-shift) and is associated with inflammatory disorders, bone marrow dysfunction and cancer [44–47]. Neutrophils were highly active in the circulation, evidenced by increased levels of elastase, myeloperoxidase, citrullinated histone H3 and complement factor C3a, likely worsening the condition of burn patients [48].

In burn tissue, we only found mature neutrophils (**Chapter 5**), suggesting that immature neutrophils are trapped in the circulation and are only able to migrate into the skin after they reach maturity. Likely, chemotactic activity increases with age because the flexibility of nucleus increases when neutrophils mature [49]. Literature on behavior of immature neutrophils in blood is indecisive as to whether immature neutrophils are beneficial or detrimental for wound healing [40,50]. Several studies suggest that immature neutrophils are highly active, undirected and show enhanced production of factors such as reactive oxygen species, elastase, myeloperoxidase and neutrophil extracellular traps, causing damage to surrounding tissues [51–56]. Additional damage to tissues can convert the zone of stasis (i.e. salvageable area of decreased tissue perfusion) into an area of complete tissue loss [57,58]. This will expand the wound area and worsen disease complications. Other studies indicate that circulating immature neutrophils exhibit reduced oxidative burst and phagocytic activity and less potency to support innate immune defenses [59–61]. We also showed reduced antibacterial activity of neutrophils in animal burn models, in **Chapter 2**. Thus, from the results in this thesis, we can conclude that neutrophils accumulate in blood and wound tissue for weeks after burn injury and their activity might be increased, yet they are less efficient at killing bacteria, thereby increasing susceptibility to infection and possibly hampering recovery of burn patients.

To study the precise role of neutrophils during burn injury, neutrophils can be isolated from patient blood or wound tissue and studied in functional assays. This could provide more detail about their inflammatory state and anti-bacterial potential at the different stages of wound healing. The in vitro skin models we described in **Chapter 7** might be useful to study the effect of burn injury on cell phenotype and cytokine expression and the effect of neutrophils on re-epithelization. However, because neutrophils are short-lived cells [62,63] and difficult to culture in vitro, multiple administrations of different batches of neutrophils to the skin models will be required to study effects over a longer period of time. Nevertheless, in vitro culturing of neutrophils has been demonstrated before in chemotaxis assays [64]. Several studies have even shown that the lifespan of neutrophils can be increased by specific culture conditions [65–67]. Another opportunity to supplement skin models with neutrophils is the use of the HL-60 promyeoloblast cell line that can easily be differentiated to neutrophil-like cells [68]. Experimental skin models in which neutrophils from burn patients at different phases of wound healing are incorporated could be used to determine the exact role of neutrophils in burn-induced inflammation. It would be interesting to see how immature neutrophils behave compared to mature neutrophils. Moreover, such models could be used to test the effect of manipulation of neutrophil behavior on wound healing in a pre-clinical setting.

BURN INJURY CAUSES HIGH LEVELS OF CLASSICAL MONOCYTES IN BLOOD AND ACCUMULATION OF MACROPHAGES WITH AFFECTED DIFFERENTIATION IN WOUND TISSUE

Macrophages are, similar to neutrophils, early responders to burn injury to ensure the removal of invading pathogens and damaged tissue [69]. When circulating monocytes migrate into tissues they differentiate into dendritic cells or macrophages [70]. After trauma, the bone marrow will release its reservoir of monocytes into the bloodstream to compensate for monocytes that enter tissues [71]. In this thesis, we showed that the number of blood monocytes is highly increased for several weeks after burn injury in both animals (**Chapter 2**) and patients (**Chapter 4**). Monocytes are progenitors to both macrophages with a pro-inflammatory phenotype (i.e. M1) and macrophages that are supporters of wound healing processes (i.e. M2). Studies have suggested that classical monocytes are more likely to differentiate into M1-like macrophages, while intermediate and non-classical monocytes are progenitors to M2-like macrophages [72–74]. Analysis of CD14 and CD16 expression on monocytes in patient blood (**Chapter 4**) and burn tissue (**Chapter 5**) revealed that the classical monocyte was the most prevalent subtype and that their numbers were increased compared to healthy subjects. High numbers of $CD14^{high}CD16^{-}$ (classical) monocytic cells were also detected in burn tissue. This could be an indication of enhanced M1 macrophage activity in the burn wound. The increased

proportion of $CD14^{high}CD16^{+}$ (intermediate) monocytic cells in burn tissue at post burn week 3 could indicate a relevant shift towards more M2-like macrophages. This shift that is needed to support wound healing [75] might be delayed after burn injury, slowing down the healing process.

Inflammatory mediators that typify M1 macrophage activity (e.g. IL-1β, IL-6, IFN-γ, TNF-α, MCP-1 and IL-8) were increased in burn tissue, while the increase of M2 mediators (e.g. IL-4, IL-10, IL-13, TGF-β1 and VEGF-A) was limited [74–77]. On the other hand, investigation of the expression of M1 (CD40 and CD80) and M2 markers (CD163 and CD206) on macrophages revealed that most macrophages in burn tissue express markers of both subtypes. It was therefore impossible to classify macrophages based on these cell markers. Williams et al. found that increased number of M1 monocytes and decreased M2 monocytes was associated with slow wound healing and hypertrophic scar formation [78]. Because the classification of macrophages based on their marker expression is difficult, it is suggested to include analysis of gene expression and functionality as well [79,80]. Altogether, the composition of monocytes/macrophages and cytokine profile after burns might support M1 activity, while limiting or delaying M2 activity. This in turn will negatively affect wound repair functions.

To study how burn injury exactly affects macrophage differentiation and how the different macrophage subtypes influence wound healing, macrophage functionality should be investigated. This research might include more determinative markers for macrophage polarization and analysis of protein and gene expression signatures. Other researchers described the use of markers such as CD11c, CD40, CD80, CD86, HLA-DR, iNOS, to study M1-like macrophages and CD163, CD204 (macrophage scavenger receptor 1), CD206 (mannose receptor), CD192 (CCR2), CD181 (CXCR1), CD182 (CXCR2), CD209 (DC-SIGN) for M2-like macrophages [77,81–84]. Nevertheless, some researchers have suggested that the binary M1/M2 classification might be too simplistic to catch the complexity of wound healing. Therefore, functional assays are required to better understand the behavior of monocytes/macrophages in blood and wound of burn patients during the different stages of wound healing. For example, the effect of burn-induced DAMPs on macrophages polarization and secretion profile could be investigated. In **Chapter 7**, we utilized our FSE models to study the effect of burn injury on the phenotype and cytokine production of PBMNC-derived monocytes. We showed monocytes differentiated into macrophages and that burn injury increased the amount of $HLA\text{-}DR^{+}$ macrophages and inflammatory cytokines such as IL-1β, IL-6, IL-8 and IL-12p70. In future experiments, these macrophages could be polarized before they are added to the models to study the effect of different subtypes or compositions on wound healing. Ultimately, manipulation of the monocyte/

macrophage composition towards more pro-healing activity might be a useful therapy to speed up wound healing in patients.

BURN INJURY LEADS TO A SHIFT IN LYMPHOCYTE SUBSETS, YET REGULATION OF INFLAMMATION APPEARS LIMITED

In general, by the end of the first week after tissue injury, lymphocytes start to respond [26,85], allegedly to ensure specific anti-pathogen reactions and regulation of inflammation [86]. However, evidence on the role of specific lymphocyte subsets including T cells, B cells and NK cells during wound healing is limited [85]. To complement this knowledge, we investigated the levels of B cells, NK cells, T cells and related cytokines in blood and burn wound tissue. Furthermore, we assessed markers that are typical for T cell differentiation towards regulatory (Treg) or inflammatory T helper cells (Th1/Th17). In **Chapter 5**, we showed that chemokines MIP-1α, MIP-1β, and RANTES were highly increased in burn tissue from patients, especially at post burn week 3. This coincided with increased levels of B cells, NK cells and T cells that were likely attracted by these lymphocyte chemokines [87].

Unexpectedly, blood lymphocyte numbers did not increase in patients (**Chapter 4**) and even decreased in experimental animals (**Chapter 2**). Moreover, the neutrophil/lymphocyte ratio, a marker for systemic inflammatory response syndrome [88], was highly increased during at least the first 9 days after burns in animals. Extreme replenishing of innate immune cells by the bone marrow can lead to a shortage of lymphocytes in the circulation [45], delaying the regulatory response of lymphocytes that occurs during normal wound healing [26,89]. Studies in burn patients reported that persistent leukocytosis in combination with lymphopenia is associated with persistent (systemic) inflammation, arrested wound healing, increased susceptibility to opportunistic infections, and increased mortality [4,90,91].

While the total T cell response appears to be delayed, there was a shift towards more γδ T cells in burn tissue during the first week after injury, supporting the proposal that γδ T cells are involved in the early response to injury [92,93]. Unlike αβ T cells, γδ T cells can interact with antigens directly and are presumably involved in immune surveillance and might produce cytokines and chemokines to recruit immune cells upon sensing damaged cell structures [94]. We showed that from the second week after burn injury onward, a portion of T cells acquired a pro-inflammatory phenotype (Th1 or Th17) (**Chapter 4**). We also found evidence for Treg differentiation in blood, but this T cell population also showed increased expression of chemokine receptors (CCR4 and CCR6), suggesting that these T cells might be putative pathophysiologic Tregs [95–98]. Tregs represent a versatile

and adaptive cell type that is able to convert its phenotype and change its functionality over time [99,100]. It has also been suggested that Tregs are able to parallel their effector counterparts (Th1/Th17) by mimicking their phenotype [101]. The exact mechanisms and the precise role of Tregs in wound healing are yet to be determined. Although we could not study the expression of chemokine receptors on T cells in burn tissue, because collagenase used for cell isolation cleaves off these receptors, the cytokine profile is likely to support a Th1 response, while Treg activity appeared limited. Overall, long-lasting high levels of pro-inflammatory cytokines and immune cells after burn injury and the lack of immunosuppression suggest that the immune system remains in a long-term inflammatory state instead of switching to a resolving state to support wound healing processes.

To better understand the role of T cells during wound healing, research should include functional assays. Organotypic skin models with T cells have been produced to study pathogenesis and therapeutic interventions for skin diseases such as psoriasis and atopic dermatitis [102–104]. In this light, we developed burn skin models where pre-activated T cells were added to FSEs (**Chapter 7**). T cells actively migrated into these models and increased the production of inflammatory cytokines IFN-γ, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IP-10 and TGF-β1, demonstrating an interplay between T cells and skin cells. Both IP-10 and IL-10 were decreased when burn injury was applied to the models. In future experiments with these models, the effect of specific Th subsets on wound healing (re-epithelization, proliferation) can be studied.

FUTURE OUTLOOK ON BURN CARE

Research on the burn-induced immune response

This thesis provides insights that are essential for the future of burn research and aids the development of improved treatment strategies. An important limitation of this thesis is the lack of information on functionality of the various immune cells. Also, we did not include markers to detect the presence of Th2 cells in patient blood. In the future, we would like to study T cell responses in more detail. Another limitation is that in several cases the sample size was too small to perform relevant subgroup analyses. Moreover, high variation between donors sometimes made it difficult to draw harsh conclusions. Small patient groups and variation amongst injuries and conditions are common in burn research and complicate studies in human.

To advance burn research, we need to focus on hiatuses in our current understanding of the physiological responses to burn injury. Translation of animal data to the human situation and understanding how certain factors (e.g. aging, burn type and severity)

influence clinical courses (discussed in **Chapter 2** and **Chapter 3**) are critical challenges in biomedicine that need to be overcome. Generalizing observations from preclinical models to patients is complicated due to differences in physiological processes between animals and humans such as signaling pathways, wound contraction and scar formation [8,105,106]. Translatability of experimental findings can for instance be improved by using humanized animal skin models, multi-omics data analysis or by making use of systems biology, machine learning and computational networks [107,108].

A point of interest in burn research is how exactly burn wound healing differs from normal wound healing (e.g. blunt trauma, excisional wounds, scratches or punctures). Burn wounds are more complex and differences might be related to more severe systemic (metabolic) responses and the release of more danger-associated molecular patterns in burns [13,109,110]. A quick return towards a normal wound healing process could speed up recovery of burn patients. Another relevant topic is the precise role of the adaptive immune system in wound healing. While the role of the innate immune system has become clearer during recent years, the contribution of cells from the adaptive immune system during wound healing is still poorly understood. This knowledge gap became especially clear when we screened for relevant articles to include in the systematic reviews (**Chapter 2** and **Chapter 3**). Therefore, we analyzed the levels of lymphocyte subsets and associated mediators in blood and wound tissue from burn patients (**Chapter 4** and **Chapter 5**). However, functionality and effect of adaptive immune cells on wound healing remain to be investigated. Furthermore, it is still unclear how certain burn patients develop systemic inflammatory response syndrome, while others suffer from progressive immunosuppression. The adaptive immune system proposedly plays an important role in these clinical conditions. These questions stress the strong need for more sophisticated research models, novel treatment options to restore the balance in the immune response and markers to predict clinical disease courses and complications.

Modeling of burn injury and immune response

To support the reduction, refinement and replacement of animal experimentation, future research should focus on alternative approaches to study the effects of burn injury [111]. Animal-free research methods include the re-analysis of existing data, patient studies and studies using simulation models. We demonstrated the use of these animal-free approaches in this thesis. The use of in vitro models for research and therapeutic testing is an important step towards animal-free drug development [112]. Here, we proposed a burn wound model with monocyte-derived macrophages or pre-activated T cells. Other immune cells such as neutrophils or specific cell subtypes (Th subsets, M1/M2 macrophages) or combinations of immune cells could also be studied in these or similar skin and wound models [113]. Moreover, another important step forward in skin tissue

engineering will be the integration of relevant accessory structures such as blood vessels, sebaceous glands or hair follicles to make in vitro models even more similar to the situation in patients [112,114–117].

Another appealing animal-free research strategy is the use of in silico models. In such models specific wound healing processes can be simulated and predicted using computational and mathematical models [118,119]. Collaborations have been initiated to use the animal and patient datasets generated in this thesis to support the development of in silico models for the simulation of burn wound healing and related inflammatory processes. These models are advanced tools that can incorporate the complex mechanisms of burn injury and might be used for the prediction of complications and for therapeutic decision-making. Nevertheless, even with these animal-free approaches, animal experiments cannot be abandoned completely and remain necessary. For example, animals will still be needed for safety and dose-finding studies in drug development. In such situations, we recommend that experiments should be set up with caution for factors that can influence immune-related outcomes in order to correctly interpret the results, as we discussed in **Chapter 2** and **Chapter 3**.

Many studies failed to adhere to the ARRIVE guidelines and contacting authors for data requests often remained unanswered, making it difficult or impossible to reuse data in advanced analyses and complicates study quality assessment [120–122]. Future research will most certainly benefit from more standardized designs, complete reporting and effortless access to raw datasets. Furthermore, burn care in general could be improved by participation from health care workers who bring approaches from different disciplines, including clinical (e.g. patients, physicians, surgeons, nurses, therapists), biomedical (e.g. biologists, engineers) and computational sciences (e.g. mathematicians, biostatisticians) to better understand and predict burn-induced pathologies. The combination of different viewpoints, including that of burn patients, can possibly lead to new insights, diagnostic tools and interventions, advancing both burn research and care.

Diagnostics and prediction of clinical course

Some burn patients develop systemic inflammatory response syndrome (SIRS), while others develop progressive immunosuppression. These opposing conditions can even occur at the same time and are sometimes described as sepsis [123]. These conditions are likely linked to the persistent acute phase response and impaired function of the adaptive immune system. There is a strong need for better tools to diagnose and predict the clinical course that patients will follow so that appropriate therapy can be applied as soon as possible. Burn patients are routinely monitored by checking clinical parameters such as body temperature, white blood cell count, c-reactive protein and procalcitonin

levels, erythrocyte sedimentation rate and plasma viscosity [124]. It is, however, often difficult to diagnose sepsis, SIRS or compensatory anti-inflammatory response (CARS) based on these parameters, because it can be hard to discriminate bacterial from sterile sepsis. It might be worthwhile to include other inflammatory parameters such as the level of inflammatory mediators (HMGB1, IL-1α, IL-1β, IL-6, TNF-α) or specific immune cell types (neutrophil/lymphocyte ratio, presence of immature neutrophils, M1/M2 activity) in routine diagnostics of burn patients [88,125–127]. Moreover, fluid phase pattern recognition molecules, cell-free DNA, non-coding RNAs, miRNAs, proteins, metabolites or soluble receptors might also have important implications for diagnostics [127,128]. Suitable biomarkers are needed that can help in diagnosis and therapy decision-making.

Modulation of the immune response

Management of burn injuries is primarily focused on wound closure, preventing deepening of the wound (loss of the zone of stasis) and infection, relieving pain and limiting fibrosis [129]. Hyper-inflammatory reactions in patients with severe burns often cause a complicated clinical course, increase mortality and contribute to excessive scar development [6,123,130,131]. To further improve treatment, we should explore how manipulation of the immune reactions might benefit burn patients' recovery [132]. Dampening excessive inflammatory responses might prevent secondary burn wound progression, saving areas in the zone of stasis and reducing disease complications [57,58]. There are multiple ways to modulate the immune response, some of which are summarized in **Table 1**. This list is far from complete and successful restoration of the immune balance could require a combination of strategies. Modulation could be realized by the use of immunosuppressive drugs such as glucocorticoids [133,134]. Another option is the removal of DAMPs such as HMGB1 or cytokines, to eliminate inflammatory triggers at an early stage [135]. This could be performed as a general therapeutic approach by early debridement of eschar (burn tissue) which contains high levels of pro-inflammatory cytokines (as shown in **Chapter 5**). More specifically, inflammatory mediators can be targeted via a blocking intervention such as Tocilizumab, Infliximab or other inhibitors [136,137].

Next to targeting inflammatory mediators, therapy could be directed at immune cells. As shown in this thesis, neutrophil and macrophage numbers rise to extreme levels in blood and wound tissue, possibly hampering wound healing via mechanisms such as respiratory burst, extracellular traps and the release of proteases [51–55]. Manipulation of the neutrophil response might be achieved by removing neutrophil chemoattractants (such as HMGB1, IL-1β, IL-6, TNF-α, G-CSF, GRO-α or IL-8) or by inhibition of released inflammatory products (among which are elastase, myeloperoxidase, citrullinated histone H3 and complement factor C3a). For macrophage activity, M1 differentiation can

be suppressed and M2 differentiation should be enhanced [69,74]. Lymphocyte activity could be regulated by suppressing Th1/Th17 T cell activity or enhancing Th2/Treg activity.

Distortion in the immune response after trauma not only leads to long-lasting, excessive inflammation, but can also lead to CARS induced immune paralysis. CARS causes defects in the adaptive immunity and will increase the patient's overall susceptibility to infection [6,138–140]. It is therefore of utmost importance to accurately monitor the immune status of patients before applying immunosuppressive therapies. Treatments aimed at reversing the immunosuppression such as inhibition of the programmed cell death protein 1/programmed death ligand (PD-1/PD-L) pathway on T cells are used and tested in diseases like cancer and sepsis [141]. Such interventions might also be of use for burn patients to restore immune paralysis. As there is much variation between patients, burn injuries and clinical progression, therapy should be tailored to individual needs. Moreover, we showed in **Chapter 2** and **Chapter 3** that factors such as age, sex, burn severity and burn agent can influence the immune response. Overall, therapy should be personalized and aimed at a timely restoration of the immune balance by modulating the intensity and duration of inflammatory responses.

Table 1. List of potential strategies to re-establish the balance after immune dysfunction instigated by burn injury. This list is not complete, but proposes several options that might be useful for the improvement of therapy.

Aspect of burn injury	Potential target	Potential strategies
Inflammatory triggers	DAMPs	Blockade of DAMPs: [132]
		Early debridement of burn tissue: [136,142,143]
	Inflammatory mediators	Haemofiltration to remove cytokines: [130]
		Blockade of specific cytokines: [132,137,144]
		Blockade of chemokines: [137]
		Use of glucocorticoids: [133,134]
Neutrophil response	Neutrophil recruitment	Blockade of chemokine signaling: [145]
	Neutrophil activity	Suppress protease activity: [55]
	Neutrophil apoptosis	Cyclin-dependent kinase inhibitors: [146]
Macrophage response	M1 macrophage activity	Suppress M1 differentiation: [69]
	M2 macrophage activity	Enhance M2 differentiation: [69,74,147]
Lymphocyte response	Pro-inflammatory activity	Suppress Th1/Th17 response: [148]
	Anti-inflammatory activity	Enhance Th2/Treg response: [26,149]
Immune paralysis	Restore immune function	Checkpoint inhibition: [141]

CONCLUSIONS

Altogether, this thesis provides valuable insights into immune dysfunction after burn injury, while supporting the reduction of animal experimentation. We showed evidence that the response of innate immune cells is excessive and that adaptive immunity might be delayed and impaired, leading to long-lasting inflammation. Burn patients are likely to benefit from a timely restoration of their immune balance. Multiple approaches to achieve this have been proposed and discussed, paving the way for novel and more personalized treatment strategies. Future burn care will need more sophisticated and refined research models, collaborations between different disciplines, and validated biomarkers to predict clinical courses for safe application of therapies that timely restore the immune balance and support healing.

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CHAPTER 9

English Summary

Burn injury is a prevalent cause of disability and mortality throughout the world and its consequences affect patients both physically and mentally. Over the years, it has become increasingly evident that the immune system plays an indispensable role in the (patho)physiological processes after burn injury. An improved understanding of the burn-induced immune response is necessary to limit secondary pathologies in patients with burns. The aim of this thesis was to improve our understanding of the immune response after burn injury by reviewing existing experimental data from literature and by investigating patient samples through advanced analysis of cells and inflammatory mediators. This knowledge was then used to develop skin models wherein aspects of burn wound healing and immune response can be studied in vitro without a need for animal experimentation.

PART 1: IMMUNE RESPONSE IN ANIMAL BURN MODELS

All available empirical evidence on immune reactions in animal burn models was comprised into two comprehensive systematic overviews, combining more than 500 individual studies. The compilation of these data improves accessibility to important findings, without a need to perform additional animal experiments. These reviews put in perspective the uncontrolled, hyperactive response of immune cells (**Chapter 2**) and inflammatory mediators (**Chapter 3**) that persists for weeks after burn trauma (**Figure 1**). Meta-analyses revealed that shortly after burn injury the numbers of immune cells (such as neutrophils, monocytes and thrombocytes) and many inflammatory mediators (e.g IL-1β, IL-6, TNF-α, CCL2, CXCL1, G-CSF and CRP) were increased in blood. In contrast, the number of lymphocytes and the level of IL-12 were reduced compared to healthy control animals.

Burn wound tissue contained increased levels of several cytokines, chemokines and growth factors and surges of neutrophils and macrophages, whereas mast cell numbers first decreased and then increased after 2 weeks. Cell function was also affected as migration of leukocytes and inflammatory mediator production by neutrophils and macrophages were enhanced, while antibacterial activity of neutrophils was reduced. Subgroup analysis were performed to investigate differences between burn techniques and animal models. Furthermore, study quality, risk of bias and adherence to ARRIVE guidelines was discussed and the importance of complete reporting, correct use of nomenclature and effortless access to raw datasets emphasized. These reviews will help to improve future burn research into the post-burn immune response and can be used to design targeted interventions such as removal of inflammatory triggers, cytokine blockade or regulation of immune cells to improve burn treatment and outcome for patients.

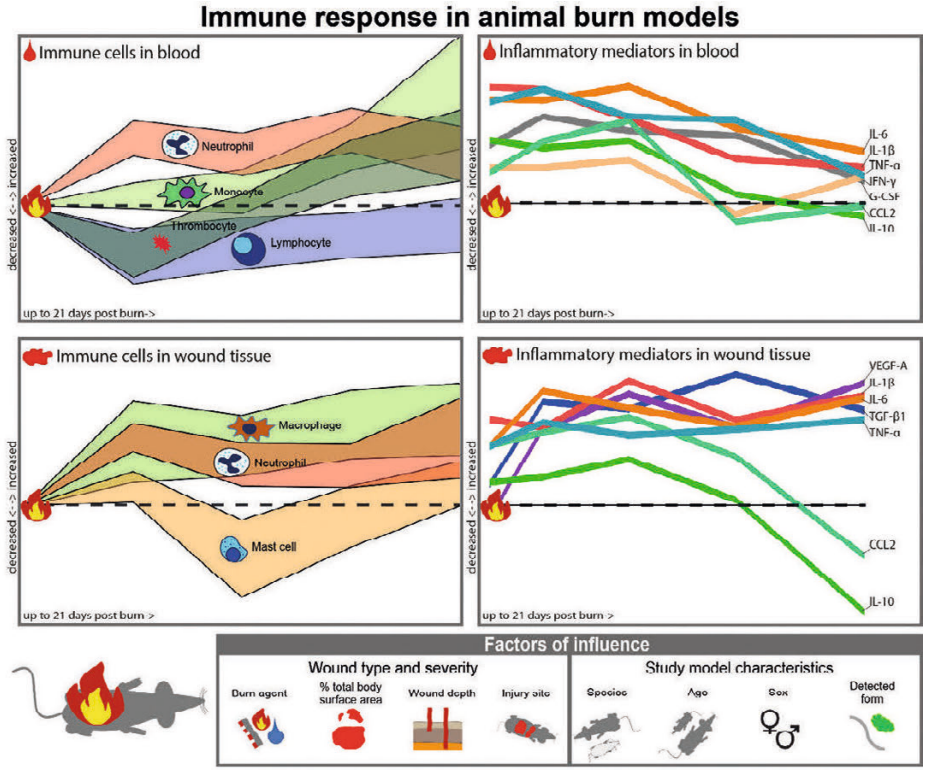


Figure 1. Graphical summary of Part 1: Immune response in animal burn models.

PART 2: IMMUNE RESPONSE IN BURN PATIENTS

Blood and burn tissue samples were collected to investigate the immune response in burn patients. Using both unsupervised and supervised flow cytometry as well as multiplex microscopy and immunoassays, the levels of immune cell subsets and inflammatory mediators present in the circulation (**Chapter 4**) and wound tissue (**Chapter 5**) were delineated. Longitudinal analyses using blood samples from 20 severely burned patients revealed an immediate surge of innate immune cells with initially a large contribution of immature neutrophils, but no increase in lymphocyte numbers. From the second week onward, shifts in T cell subpopulations were observed: there was an increase of CCR4 and CCR6 expressing cells and although regulatory T cell (Treg) numbers increased as well, the overall phenotype of the $CD4^+$ T cells and Tregs appeared to be rather pro-inflammatory than anti-inflammatory. Simultaneously with leukocytosis, increased levels of various pro-inflammatory cytokines were found.

In burn wound tissue, there was a fast and extensive increase in innate immune cells that was present even in tissue debrided 3 to 4 weeks after injury. Lymphocytes also rose in numbers, but considerably late (2-4 weeks after burn injury). Cytokine composition in burn tissue was highly pro-inflammatory and likely continued the attraction and activation of immune cells. The long-lasting increase in pro-inflammatory cytokines and shifts in neutrophil and lymphocyte composition indicate that after burn injury the immune system remains in a long-term pro-inflammatory state rather than switching to a resolving state. This will in turn delay the phases of proliferation and maturation of healing wounds (**Figure 2**).

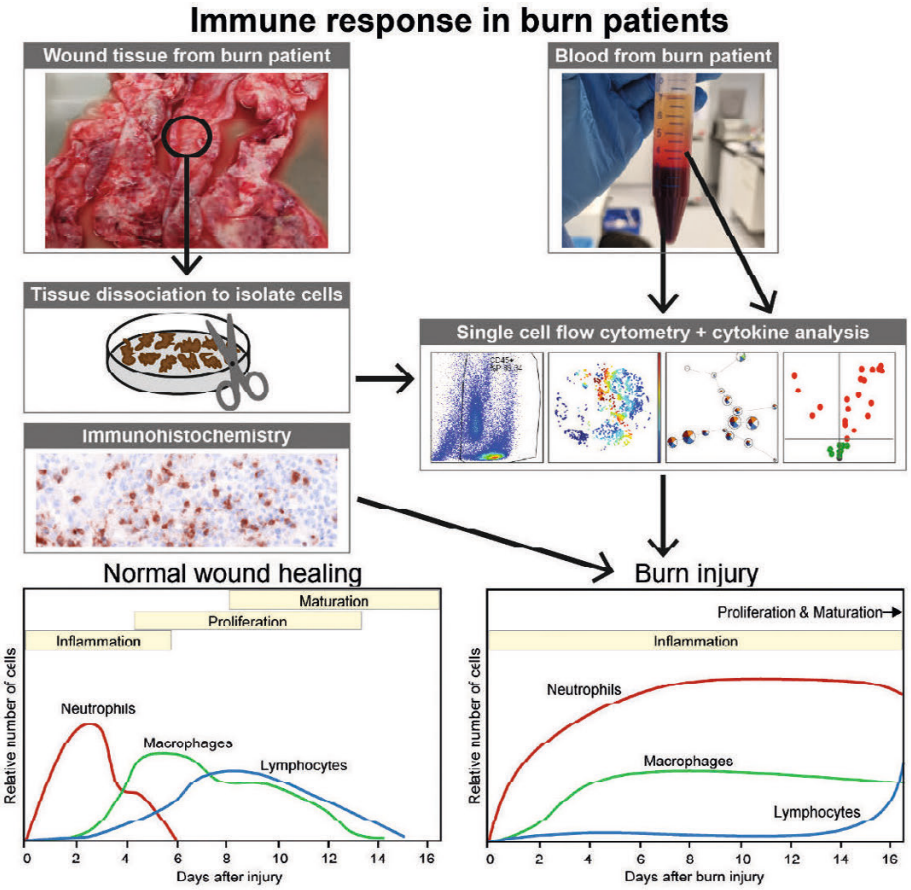


Figure 2. Graphical summary of Part 2: Immune response in burn patients.

PART 3: IN VITRO MODELING

There is a growing demand for more appropriate, animal-free approaches in preclinical research due to both ethical and scientific concerns around animal experimentation. Organotypic skin models are promising alternatives for animals and are standardized, controllable, and easy to customize. To develop a model wherein burn injury and specific immune reactions could be studied, full skin equivalent models (FSEs) were generated using various clinically applied dermal matrices (**Chapter 6**). Using immunohistochemistry techniques and cytokine assays, the epidermal and dermal development and cell proliferation and inflammatory response were assessed. FSEs based on MatriDerm displayed many similarities to ex vivo human skin and showed re-epithelization after a burn injury was applied.

In **Chapter 7**, we simulated innate and adaptive immune reactions by incorporating monocytes or T cells into the MatriDerm-based FSE. In the FSE, monocytes differentiated into macrophages and burn injury seemed to increase the percentage of HLA-DR$^+$ (M1-like) macrophages. The inclusion of monocytes further increased inflammatory cytokines such as IL-1β, IL-6 and IL-8. T cells that actively migrated into the FSE showed enhanced expression of Treg and Th1/Th17 markers, irrespective of burn injury. The inclusion of T cells in the model upregulated the production of inflammatory cytokines such as IFN-γ, IL-10, IL-17A and IP-10, demonstrating the interplay between T cells and skin cells. These immunocompetent models enable the study of skin development, wound healing and specific immune reactions using a uniform dermal component. They will facilitate the testing of novel therapeutic approaches that may treat burn injuries more effectively.

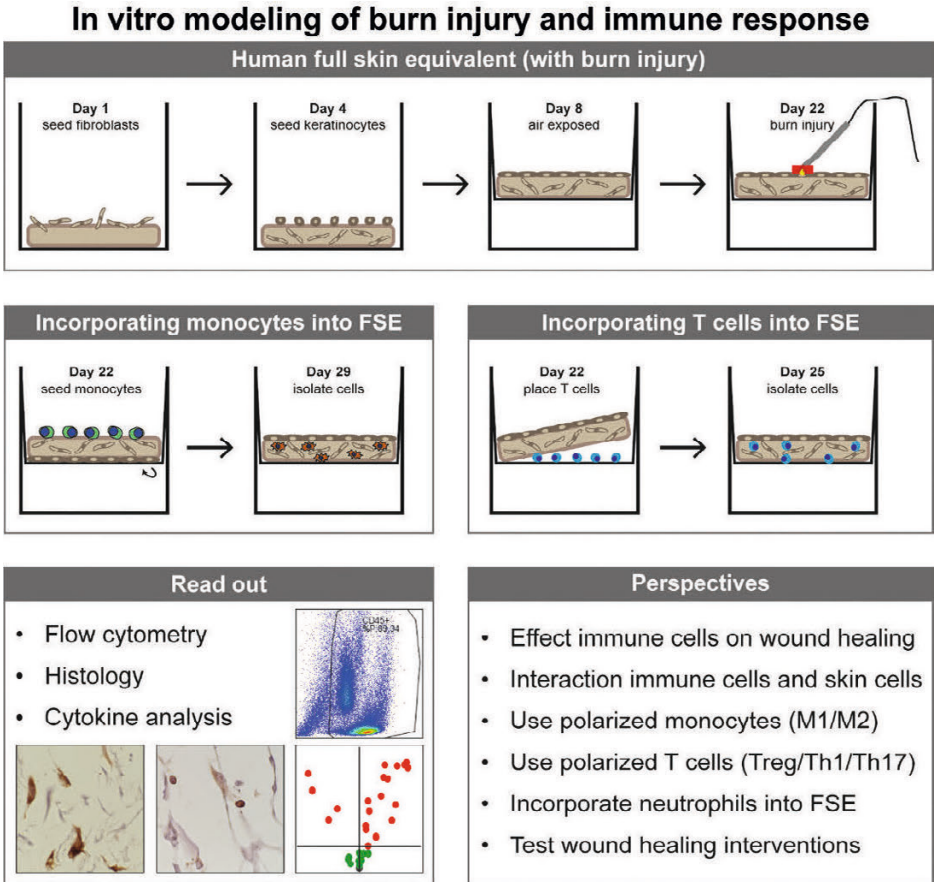
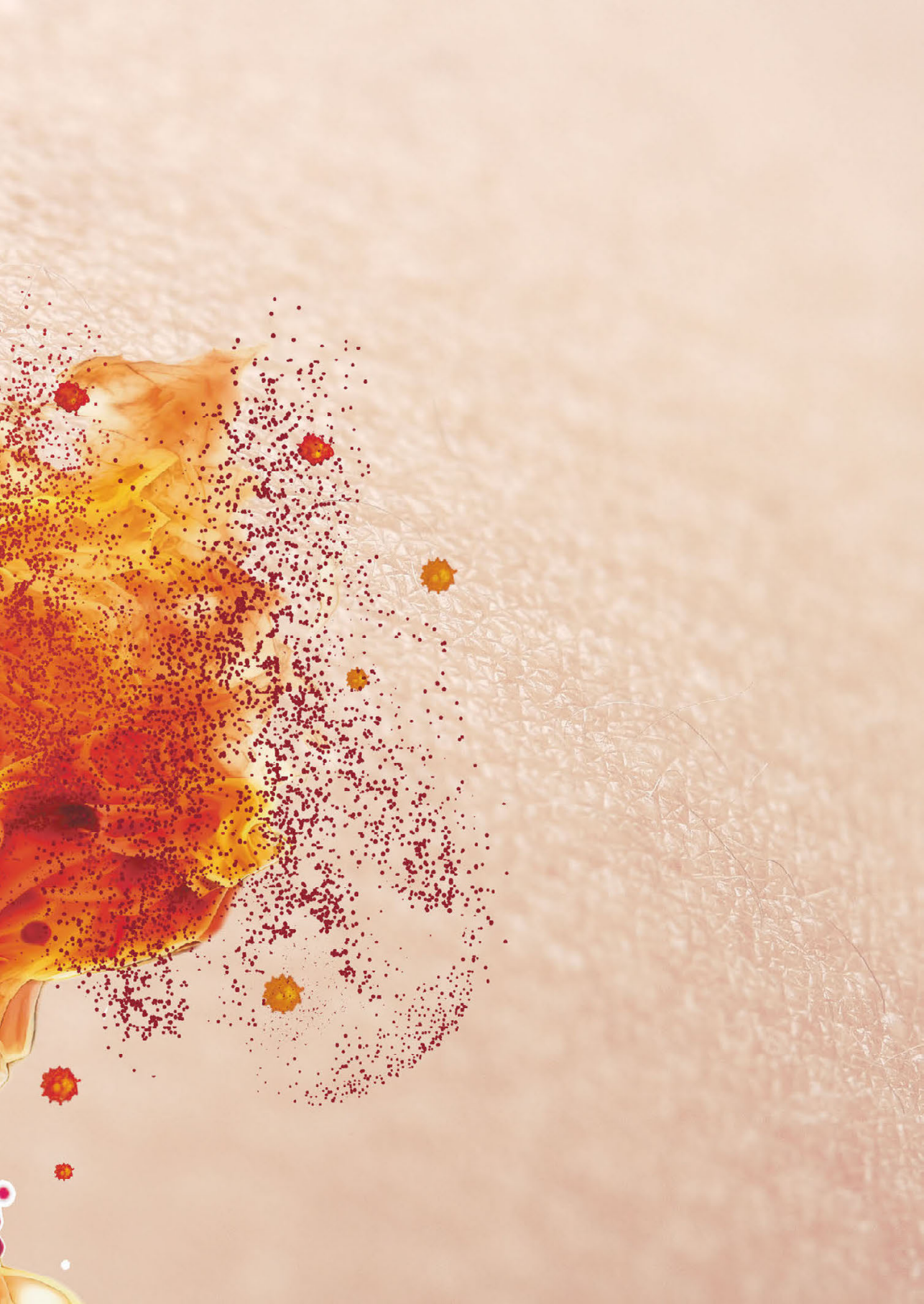


Figure 3. Graphical summary of Part 3: In vitro modeling.



CHAPTER 10

Dutch Summary - Nederlandse Samenvatting

Brandwonden zijn een veel voorkomende oorzaak van ziekte en sterfte en de gevolgen ervan treffen patiënten zowel lichamelijk als geestelijk. Door de jaren heen is steeds duidelijker geworden dat het afweersysteem een belangrijke en soms nadelige rol speelt bij de reactie van het lichaam op brandwonden. Om extra ziekteverschijnselen zoveel mogelijk te beperken, is het noodzakelijk te weten hoe het afweersysteem reageert op brandwonden. Het doel van dit proefschrift was om deze afweerreactie na brandwonden beter te begrijpen. Om dit doel te bereiken hebben we een groot literatuuronderzoek gedaan. Daarnaast hebben we verschillende onderdelen van het afweersysteem in brandwondpatiënten nauwkeurig onderzocht. Daarvoor hebben we gekeken naar celtypen en hun signaalstoffen in het bloed en in de wonden. Deze kennis werd vervolgens gebruikt om huidmodellen te ontwikkelen. Daarin kunnen brandwonden en de ontstekingsreactie bestudeerd worden zonder dat daar dierproeven voor nodig zijn.

DEEL 1: ONTSTEKINGSREACTIE IN EXPERIMENTEEL DIERONDERZOEK

Alle beschikbare literatuur over de ontstekingsreactie bij brandwonden in dieren werd samengevoegd om een totaal overzicht van de ontstekingsreactie te krijgen zonder daarvoor nieuwe dierproeven uit te voeren. Dit overzicht laat zien dat er kort na verbranding een ongecontroleerde, hyperactieve reactie van ontstekingscellen (**Hoofdstuk 2**) en verwante signaalstoffen (**Hoofdstuk 3**) plaatsvindt, die weken aan kan blijven houden. Zo is er kort na verbranding een snelle toename van het aantal ontstekingscellen (zoals neutrofielen en monocyten) en signaalstoffen (waaronder cytokines, chemokines en groeifactoren) in het bloed. Lymfocyten kunnen een ontsteking afremmen, maar hun aantal was juist verlaagd.

Brandwonden bevatten verhoogde niveaus van verschillende cytokines, chemokines en groeifactoren. Daarnaast was er een overvloed aan neutrofielen en macrofagen. Door brandwonden werd ook de functie van de ontstekingscellen beïnvloed: de migratie van leukocyten en de productie van signaalstoffen door neutrofielen en macrofagen namen toe, terwijl de antibacteriële werking van neutrofielen werd verminderd. Daarnaast werd de mate van de ontstekingsreactie bepaald door het soort brandwond en het type diermodel. De kwaliteit van dit soort studies kan verbeterd worden door volledig te rapporteren, de juiste naamgeving van cellen en signaalstoffen te gebruiken en toegang tot datasets te vereenvoudigen. Deze overzichten zullen helpen om toekomstig onderzoek naar de ontstekingsreactie na brandwonden te verbeteren. Ze kunnen worden gebruikt om gerichte therapieën te ontwerpen die de ontstekingsreactie in goede banen kan leiden en zo de behandeling van patiënten met brandwonden te verbeteren.

DEEL 2: ONTSTEKINGSREACTIE IN PATIËNTEN MET BRANDWONDEN

Om de ontstekingsreactie bij patiënten met brandwonden te onderzoeken werden bloed en biopten uit brandwonden verzameld. Ontstekingscellen en signaalstoffen werden bestudeerd met behulp van een cel-analyse apparaat en een speciale microscoop onderzocht. De verschillende ontstekingscellen en signaalstoffen in het bloed (**Hoofdstuk 4**) en in brandwonden (**Hoofdstuk 5**) werden getypeerd en gekwantificeerd. Bloed en brandwonden werden op verschillende momenten na ongeval (dagen tot weken) onderzocht. In het bloed werd een directe en langdurige toename van neutrofielen en monocyten waargenomen, terwijl het aantal lymfocyten min of meer gelijk bleef. Vanaf de tweede week na verbranding vond er een verschuiving plaats in de samenstelling van de lymfocyten. Deze verandering leek de ontsteking voornamelijk te verergeren in plaats van af te remmen. Gelijktijdig werden verhoogde niveaus van verschillende signaalstoffen gevonden die kenmerkend zijn voor hevige ontsteking.

In brandwonden was er een snelle en uitgebreide toename van neutrofielen en macrofagen die zelfs in brandwonden van 3 en 4 weken na ongeval nog aanwezig was. Lymfocyten stegen ook in aantal, maar aanzienlijk laat (namelijk pas 2 tot 4 weken na verbranding). De signaalstoffen in brandwonden trekken ontstekingscellen aan waardoor de ontsteking in stand blijft. Door de langdurige ontsteking na brandwonden kan de wondgenezing van patiënten worden vertraagd waardoor goed herstel uit blijft. Op zijn beurt kan dit bijdragen aan het ontstaan van nieuwe ziekteverschijnselen zoals septische shock en ernstige littekens.

DEEL 3: NABOOTSTEN VAN BRANDWONDEN EN ONTSTEKING

Door zowel ethische als wetenschappelijke bezwaren rond dierproeven, is er een groeiende vraag naar proefdiervrije manieren om onderzoek naar brandwonden te doen. Het gebruik van huidmodellen is een veelbelovend alternatief voor dierproeven en kunnen op een gestandaardiseerde manier worden toegepast voor verschillende onderzoeksvragen. Echter is er op dit moment nog geen huidmodel in staat om de complexe ontstekingsreactie na verbranding na te bootsen. In **Hoofdstuk 6** werd een model ontwikkeld waarin brandwonden bestudeerd kunnen worden. Deze huidmodellen zijn gemaakt door menselijke cellen uit huidresten (afkomstig van operaties) te kweken in collageenmatjes. Deze collageenmatjes worden doorgaans gebruikt om wonden patiënten met brandwonden te bedekken. De groei van de huidcellen in het huidmodel werd onderzocht van behulp van microscopie. Vervolgens werd het effect van een brandwond op het huidmodel bestudeerd. Deze huidmodellen vertoonden veel

overeenkomsten met de menselijke huid en na het aanbrengen van een brandwond vond herstel plaats.

In **Hoofdstuk 7** bootsten we de ontstekingsreactie na door monocyten of lymfocyten op te nemen in het huidmodel. Monocyten veranderden in het huidmodel tot macrofagen, zoals ook in het menselijk lichaam gebeurt als monocyten vanuit het bloed de huid ingaan. Wanneer een brandwond werd aangebracht op het huidmodel leken de macrofagen de ontsteking te bevorderen en nam het niveau van signaalstoffen toe. Lymfocyten die het huidmodel introkken, vertoonden verhoogde activiteit. Interactie tussen lymfocyten en huidcellen was duidelijk zichtbaar door verhoging van bepaalde signaalstoffen. Deze brandwond-ontstekingsmodellen maken het mogelijk om huidontwikkeling, wondgenezing en specifieke cel interacties te bestuderen. Deze modellen zullen het testen van nieuwe geneesmiddelen vergemakkelijken om uiteindelijk brandwonden effectiever te kunnen behandelen.

APPENDIX

SCIENTIFIC OUTPUT

PUBLICATIONS

Scientific publications related to this thesis

- **Mulder PPG**, Vlig M, Elgersma A, Rozemeijer L, Mastenbroek LS, Middelkoop E, Joosten I, Koenen HJPM and Boekema BKHL. Monocytes and T cells Incorporated in Full Skin Equivalents to Study Innate or Adaptive Immune Reactions after Burn Injury. *Frontiers in Immunology*. **2023**, 14, 1264716. *DOI: 10.3389/fimmu.2023.1264716*
- **Mulder PPG**, Hooijmans CR, Vlig M, Middelkoop E, Joosten I, Koenen HJPM, Boekema BKHL. Kinetics of Inflammatory Mediators in the Immune Response to Burn Injury: Systematic Review and Meta-Analysis of Animal Studies. *In Press at Journal of Investigative Dermatology*. **2023**. *DOI: 10.1016/j.jid.2023.09.269*
- **Mulder PPG,** Raktoe RS, Vlig M, Elgersma A, Middelkoop E, Boekema BKHL. Full Skin Equivalent Models for Simulation of Burn Wound Healing, Exploring Skin Regeneration and Cytokine Response. *Journal of Functional Biomaterials*. **2023**, 14, 29. *DOI: 10.3390/jfb14010029*
- **Mulder PPG**, Vlig M, Fasse E, Matthea MM, Pijpe A, van Zuijlen PPM, Joosten I, Boekema BKHL, Koenen HJPM. Burn-Injured Skin is Marked by a Prolonged Local Acute Inflammatory Response of Innate Immune Cells and Pro-Inflammatory Cytokines. *Frontiers in Immunology*. **2022**, 13, 1034420. *DOI: 10.3389/fimmu.2022.1034420*
- **Mulder PPG**, Koenen HJPM, Vlig M, Joosten I, de Vries RBM, Boekema BKHL. Burn-Induced Local and Systemic Immune Response: Systematic Review and Meta-Analysis of Animal Studies. *Journal of Investigative Dermatology*. **2022**, 142, 3093-3109. *DOI: 10.1016/j.jid.2022.05.004*
- **Mulder PPG**, Vlig M, Boekema BKHL, Stoop MM, Pijpe A, van Zuijlen PPM, et al. Persistent Systemic Inflammation in Patients With Severe Burn Injury Is Accompanied by Influx of Immature Neutrophils and Shifts in T Cell Subsets and Cytokine Profiles. *Frontiers in Immunology*. **2021**, 11, 621222. *DOI: 10.3389/fimmu.2020.621222*

Scientific publications unrelated to this thesis

- Korkmaz HI, Sheraton VM, Bumbuc RV, Li M, Pijpe A, **Mulder PPG**, Boekema BKHL, de Jong E, Papendorp SGF, Middelkoop E, Sloot PMA, van Zuijlen PPM. An in silico modeling approach to understanding the dynamics of the post-burn immune response. *Submitted*.
- Affandi AJ, Grabowska J, Olesek K, Venegas ML, Barbaria A, Rodríguez E, **Mulder PPG**, Pijffers HJ, Ambrosini M, Kalay H, O'Toole T, Zwart ES, Kazemier G, Nazmi K, Bikker FJ, Stöckl J, van den Eertwegh AJM, de Gruijl TD, Storm G, van Kooyk Y, den Haan JMM. Selective tumor antigen vaccine delivery to human CD169+ antigen-presenting cells

using ganglioside-liposomes. *Proceedings of the National Academy of Sciences (PNAS)*. **2020**, 117, 27528-27539. *DOI: 10.1073/pnas.2006186117*

- Koppen BC, **Mulder PPG**, de Boer L, Riool M, Drijfhout JW, Zaat SAJ. Synergistic microbicidal effect of cationic antimicrobial peptides and teicoplanin against planktonic and biofilm-encased Staphylococcus aureus. *International Journal of Antimicrobial Agents*. **2019**, 53, 143-151. *DOI: 10.1016/j.ijantimicag.2018.10.002*
- Badoux P, Euser SM, Bruin JP, **Mulder PPG**, Yzerman EPF. Evaluation of the bioNexia Legionella test including the impact of incubation time extension for the detection of Legionella pneumophila serogroup 1 antigen in urine. *Journal of Clinical Microbiology*. **2017**, 55, 1733-1737. *DOI: 10.1128/JCM.02448-16*

Popular science publications related to this thesis

- **Mulder PPG**, Boekema BKHL. Immuunrespons bij Brandwonden: Systematische Review en Meta-analyse van Experimenteel Onderzoek. *WCS Nieuws*. **2022** December.
- **Mulder PPG**, Vlig M, Boekema BKHL. Nabrander: De Ontstekingsreactie na het Oplopen van Brandwonden. *WCS Nieuws*. **2022** March.

SCIENTIFIC CONTRIBUTIONS

Oral presentations

- Congress Nederlandse Vereniging Experimentele Dermatologie in Lunteren 2023. “Full Skin Equivalents for Simulation of Burn Wound Healing and Inflammatory Response”.
- Wetenschapsdag Nederlandse Brandwonden Stichting in Amersfoort 2023. “Begrijpen van dysregulatie in de immuunrespons na brandwondenletsel: op weg naar therapeutische behandeling”.
- Congress Netherlands Society for Biomaterials and Tissue Engineering in Lunteren 2022. “Full Skin Equivalents to Simulate Burn Wound Healing and Inflammation”.
- Symposium Nederlandse Vereniging voor Brandwondenzorg & Kreisprijs uitreiking in Beverwijk 2022. “Ontstekingsreactie bij Brandwonden”.
- Symposium Nederlandse Brandwonden Stichting in Beverwijk 2022. “Het Belang van Fundamenteel Onderzoek”.
- Congress European Tissue Repair Society in Lyon 2022. “Full Skin Equivalents to Simulate Burn Wound Healing and Inflammation”.
- Congress European Burns Association in Turin 2022. “Burn Injury Causes Long-Lasting Influx of Neutrophils, Release of Pro-Inflammatory Cytokines and Shifts in T cell Composition in Blood and Burn Tissue from Patients”.
- Radboudumc RIMLS PhD Retreat in Veldhoven 2022. “Modeling the Burn-Induced Immune Response”.

- Wetenschapsdag Nederlandse Brandwonden Stichting in Wijk aan Zee 2022. “Beter Begrijpen van de Immuunrespons na het Oplopen van Brandwonden voor de Ontwikkeling van een *in vitro* Model.
- Congress European Wound Management Association in Paris 2022. “Persistent Inflammation in Burn Patients is Accompanied by Influx of Neutrophils and Shifts in T cell Subsets and Cytokines Profiles”.
- Wetenschapssessie Nederlandse Brandwonden Stichting online 2022. “Beter Begrijpen van de Immuunrespons na het Oplopen van Brandwonden”.
- Congress Global Scar Society G-SCARS online 2021. “Persistent Systemic Inflammation in Burn Patients is Accompanied by Influx of Immature Neutrophils and Shifts in T Cell Subsets and Cytokine Profiles”.
- Congress European Wound Management Association online 2021. “Burn-Induced Immune Response in Animals”.
- Congress International Society for Burn Injuries online 2021. “Persistent Systemic Inflammation in Burn Patients is Accompanied by Influx of Immature Neutrophils and Shifts in T Cell Subsets and Cytokine Profiles”.
- Wetenschapssessie Nederlandse Brandwonden Stichting online 2021. “Ontwikkeling van een Verbeterd Brandwondenmodel voor Onderzoek naar Ontstekingsprocessen en Wondheling”.
- Congress Nederlandse Vereniging voor Immunologie online 2020. “Persistent Systemic Inflammation in Burn Patients is Accompanied by Influx of Immature Neutrophils and Shifts in T Cell Subsets and Cytokine Profiles”.
- Wetenschapsdag Nederlandse Brandwonden Stichting in Amersfoort 2020. “Systemische en Lokale Ontstekingsreacties na Verbranding: Van Kennis naar Model”.

Poster presentations

- Congress European Tissue Repair Society in Lyon 2022.
- Congress European Burns Association in Turin 2022.
- Congress New Frontiers (online) 2021.
- Radboudumc RIMLS PhD Retreat (online) 2021.
- SEMM PhD Networking Days in Milan 2020.
- Congress Nederlandse Vereniging voor Immunology in Noordwijkerhout 2019.
- Congress ENABLE + New Frontiers in Lent 2019.

Grants, awards and nominations

- Research Grant for Postdoc Project of 3 years “Understanding dysregulation in the immune response after burn injury: the road to therapeutic intervention” by the Dutch Burns Foundation 2023.

- 1st Prize “Kreis Prize for Best Young Researcher in the Field of Burns of 2021-2022” by Dutch Society for Burn Care (NVBZ) 2022.
- Travel Grant for the European Tissue Repair Society Congress in Lyon 2022.
- 5th Prize “Young Investigator Award” by the European Tissue Repair Society 2021.
- Grant for Systematic Review Project by ZonMw 2020.
- Travel Grant for the PhD Networking Days SEMM in Milan 2020.

Social outreach and media appearances

- **News article** Noord-Hollands Dagblad, Haarlems Dagblad, Leidsch Dagblad 2022. “Brandwondenprijs voor studie naar ontstekingsreacties”.
- **News article** Noord-Hollands Dagblad 2022. “Grenzen verleggen in de wereld van brandwonden”.
- **Article** InFocus jaargang 45 nummer 1 2022. Brandwondenonderzoeker Patrick Mulder ging mee als Staflid met Scarwars”.
- **Article** Nederlandse Brandwonden Stichting Website 2021. “Maak Kennis met Patrick Mulder”.
- **Laymen talk** ENABLE Pubtalks for the public in Nijmegen 2019. “What happens inside your body after a burn injury?”.
- **Laymen talk** Wetenschapsmiddag voor Brandwond Ervaringsdeskundigen NBS in Beverwijk 2019. “Brandwondmodellen voor onderzoek naar wondgenezing en evaluatie van geneesmiddelen”.

PHD PORTFOLIO

Department: Laboratory of Medical Immunology, Department of Laboratory Medicine
PhD period: 01/02/2019 – 01/05/2023
PhD Supervisor(s): Prof. I Joosten
PhD Co-supervisor(s): Dr. HJPM Koenen, Dr. BKHL Boekema

Training activities	Hours
Courses	
- General Introduction by Radboudumc (2019)	6
- Introduction "In the Lead" by Radboudumc (2019)	15
- Workshop Adobe Illustrator by Radboudumc (2019)	2
- Systematic Review of Animal Studies by SYRCLE (2019)	8
- Poster Pitching by Radboud University (2019)^	28
- Project Management for PhDs by Radboud University (2019)	56
- Masterclass on Data Visualization by Dutch Chemometrics Society (2019)	8
- Introduction in Using R by Radboudumc (2019)	8
- Scientific Integrity by Radboudumc (2020)	20
- Workshop Flow Panel Design by BD (2020)	8
- R Data Analysis by TenWise (2021)	28
- Next Step in My Career by Radboudumc (2021)	20
- Basic Analyses in R by Data Science Partners (2022)	32
Seminars	
- Research Meetings Laboratory Medical Immunology (weekly; 2019-2023)^	60
- Research Meetings Association of Dutch Burn Centres (weekly; 2019-2023)^	60
- Symposia Wetenschapsdag Dutch Burns Foundation *(4×;* 2019-2023)^	40
- Symposia Dutch Society of Burn Care (NVBZ) (3×; 2019-2023)^	30
- Burn Care Club Red Cross Hospital Beverwijk (monthly; 2019-2023)	12
- Radboud Research Rounds (3×; 2019-2020)^	6
- Radboud Research Integrity Rounds (3×; 2019-2020)	6
- Symposia Dutch Society for Immunology (NVVI) (2×; 2019,2021)	32
- Research Meetings for Burn Survivors (2×; 2019,2022)^	6
- Symposium Animal Free Innovations Amsterdam UMC (2019)	6
- Symposium Reproductive Immunology Network Netherlands (2020)	4
- Research Meeting Plastic Surgery Amsterdam UMC (2020)^	4
- Webinar "Children with Burn Injuries" by Amsterdam UMC (2021)	2
- Symposium Dutch Burns Foundation 50 years (2022)^	8
- Webinar "How to prevent bad scarring" by G-SCARS (2023)	2

Conferences	
- PhD Retreat Radboudumc (2019)	16
- Dutch Society for Immunology (NVVI) in Noordwijkerhout (2019)^	16
- New Frontiers + ENABLE in Nijmegen (2019)^	24
- SEMM PhD Networking Days in Milan (2020)^	16
- PhD Retreat Radboudumc Online (2020)	8
- Dutch Society for Immunology (NVVI) Online (2020)^	16
- International Society for Burn Injuries (ISBI) Online (2020)^	28
- European Wound Management Association (EWMA) Online (2021)^	16
- New Frontiers Online (2021)^	8
- PhD Retreat Radboudumc Online (2021)^	8
- Global Scar Community (G-SCARS) Online (2021)^	8
- European Wound Management Association (EWMA) in Paris (2022)^	30
- PhD Retreat Radboudumc in Veldhoven (2022)^	17
- European Burns Association (EBA) in Turin (2022)^	38
- European Tissue Repair Society (ETRS) in Lyon (2022)^	30
- Biomaterials and Tissue Engineering (NBTE) in Lunteren (2022)^	22
Other	
- Member PhD Council RIMLS Radboudumc (2019-2021)	60
- Member PhD Network in the Netherlands PNN LOUP (2019-2021)	60
- Organizer/staff Camp for Young People with Burn Scars NBS (2021-2023)	60
Teaching activities	
Lecturing	
- Lecture for nurses of the Burn Center and Intensive Care (3×; 2022)^	7
Supervision of internships / other	
- Co-supervisor BSc Internship Lisa Popma (2019)	20
- Co-supervisor BSc Internship Evi Warmerdam (2019-2020)	20
- Supervisor MSc Internship Rosa Rentenaar (2020)	40
- Supervisor MSc Literature Thesis Myrthe van der Zwan (2020)	20
- Supervisor BSc Internship Myrthe Witbaard (2020-2021)	60
- Supervisor BSc Internship Leonore Mastenbroek (2021-2022)	60
- Supervisor BSc Internship Lotte Rozemeijer (2022-2023)	60
Total	**1,285**

^ indicate oral and poster presentations.

DATA MANAGEMENT PLAN

Data was collected and stored following FAIR principles to increase findability, accessibility, interoperability and reuse of the datasets. Primary and secondary data obtained during this PhD project were collected and stored in the electronic labjournal (OneNote, Microsoft) and at the local server of the Association of Dutch Burn Centres in Beverwijk. The labjournal contains an overview of all performed experiments, including research questions, discussion, conclusions, succeeding steps and references to the location of raw data. Servers were well-secured by and backed up every day by the Information Technology Department of the Dutch Burns Foundation. All data archives on the server are accessible by the associated staff members. Data will be stored for at least 15 years after finalization of this project (January 31st, 2023).

Part of the optimization experiments for flow cytometry of single cells isolated from burn wound and skin tissue (described in **Chapter 5**) were performed at the Laboratory of Medical Immunology, Department of Laboratory Medicine, Radboudumc. These data were stored at both the server of the Association of Dutch Burn Centres and the local server of the Radboudumc. Published data generated or analyzed in this thesis are part of published articles and its additional files are available from the associated corresponding authors on request.

The collection and analysis of blood samples from burn patients and healthy volunteers, described in **Chapter 4**, were conducted in accordance with the principles of the Declaration of Helsinki. The study protocol with numbers “NL54823.094.15” (for patient samples) and “NL54823.094.15” (for volunteer samples) was approved by the METc of the VU Medical Center (Amsterdam, the Netherlands).

Burn wound tissue was obtained from patients who underwent eschar debridement as part of their treatment at the Burn Center of the Red Cross Hospital in Beverwijk, the Netherlands. Healthy skin samples were used from abdominal, leg or arm reconstructions wherein excess skin was removed were obtained from adult patients who underwent elective surgery (excision of excess abdominal leg or arm skin) at the Department of Plastic and Reconstructive Surgery of the Red Cross Hospital. Consent for the use of these anonymized, post-operative residual tissue samples was received through the informed opt-out protocol of the Red Cross Hospital, which was in accordance with the national guidelines (https://www.coreon.org/) and approved by the institutional privacy officers. Subjects were actively informed of this procedure and were able to easily withdraw at any point. The privacy of the participants was secured by use of encrypted and unique individual subject codes.

ACKNOWLEDGEMENTS – DANKWOORD

Dit was hem dan, het einde van een lange, bijzondere reis. Het was regelmatig lastig, het kostte veel tijd en energie, maar het was altijd razend interessant en leerzaam. Ik kijk terug op een fantastische tijd en ben enorm trots op alles wat we hebben bereikt. Men vergelijkt een PhD wel eens met een marathon: “It is not a race, it is a marathon”. Ik besloot, met mijn onderzoekers-geest, om beide te doen. Het vergt beide een enorme inspanning, je voelt je aan het einde gesloopt maar het is een enorme prestatie die veel voldoening geeft. Het is bijzonder wat je als mens kunt bereiken met de juiste instelling. Zo inspirerend dat ik dit ook regelmatig terugzie bij patiënten en ervaringsdeskundigen.

“If you want to go fast, go alone. If you want to go far, go together”. Dit werk was niet tot stand gekomen zonder de inzet, hulp en betrokkenheid van mijn promotieteam, paranimfen, collega’s, de manuscriptcommissie, de opponenten, sponsoren en natuurlijk mijn dierbare vrienden en familie. Mijn dank aan jullie allen is groot! Daarnaast spreek ik ook graag mijn dank uit aan alle betrokken ziekenhuismedewerkers, patiënten, ervaringsdeskundigen, collectanten, donateurs en medewerkers van de Nederlandse Brandwonden Stichting. Een aantal mensen wil ik graag in het bijzonder aanspreken.

Allereerst mijn promotor, professor **Irma Joosten**. Ontzettend bedankt voor de tomeloze inzet, adviezen, oplettendheid en begeleiding. Ondanks de overgang naar je welverdiende pensioen, heb je je altijd met volle interesse ingezet voor ons project. Jouw scherpe blik, aanwijzingen en kennis hebben het project en de daaruit volgende artikelen zichtbaar naar een hoger niveau gebracht. Opmerkingen die regelmatig de ronde deden waren bijvoorbeeld “wat is nou precies je onderzoeksvraag?” of “wat is je boodschap?”. Je bracht mij, en soms ook de anderen, terug naar basis als dat nodig was en dat hielp ons altijd om verder te komen. Met jouw adviezen zorgde je ervoor dat er focus bleef en dat de artikelen een duidelijke boodschap kregen.

Bouke Boekema en **Hans Koenen**, jullie zijn beide fantastische copromotoren en hebben altijd voor mij klaar gestaan. Door jullie hulp is niet alleen het project een succes geworden, maar heb ik mijzelf ook persoonlijk kunnen ontwikkelen. Bouke, jouw rol werd gedurende het project steeds groter en ik zie je niet alleen als collega en begeleider, maar ook als hardloopmaatje en vriend. Ons zijproject (systematic review) liep behoorlijk uit de hand, maar hier zijn wel 2 prachtige artikelen uit voortgekomen waar ik met veel trots op terugkijk. Hans, op jou heb ik altijd kunnen rekenen voor raadgeving, steun en vertrouwen. Je zorgde ervoor dat we over alle belangrijke punten nadachten en jouw kennis over het immuunsysteem en de gebruikte technieken is op talloze momenten onmisbaar geweest. Bedankt ook voor een gezellige tijd in Nijmegen waar ik mij altijd

welkom heb gevoeld en waar ik veel heb geleerd. Het is dan ook erg fijn dat je bij het vervolgproject betrokken blijft!

Leidinggevende van de VSBN, professor **Esther Middelkoop**, je bent voor ons allen een prachtig voorbeeld als onderzoeker. Jouw unieke positie slaat een brug tussen klinisch en preklinisch onderzoek. Je rol bij ons project is steeds groter geworden en zette je zichtbaar in om het tot een succes te maken. Mijn dank is groot voor al je hulp en alle kansen die je mij gegeven hebt op ons mooie laboratorium. Op naar een succesvol vervolg!

Onmisbare spelers in het projectteam waren **Marcel Vlig** en **Anouk Elgersma**. Het was voor mij dan ook een makkelijke keuze om jullie te vragen als paranimfen. Mijn dank en bewondering voor al jullie harde werk is groot. Marcel, jouw praktisch inzicht, hulp en creatieve oplossingen kwamen altijd goed van pas en hebben ervoor gezorgd dat het project goed en vlot is verlopen. Daarnaast weet je de stemming er ook altijd goed erin te houden op de werkvloer! Anouk, het is indrukwekkend hoeveel tijd en energie jij aan het huidmodel hebt besteed en dankzij jou hebben we een prachtig model om onderzoek mee te doen. Ik ben blij en dankbaar dat ik op jou heb kunnen rekenen voor hulp en advies voor het maken van de modellen en alles daaromheen. Jullie zijn zeer prettige collega's en ik werk graag met jullie samen.

Magda Ulrich, ik wil je ontzettend bedanken voor je inzet en betrokkenheid bij het opzetten van ons project. Je deed dit werk met veel passie en hebt ons goed op weg geholpen. Ik hoop dat je nu volop geniet van een welverdiend pensioen.

Ik wil graag mijn fijne, gezellige collega's van de **VSBN** bedanken voor alle steun en al het plezier op de werkvloer. **Marlies Kobesen**, **Miranda Jekhmane**, **Kim Schilders**, **Rajiv Raktoe**, **Madalena Gomes**, **Gizem Cosar** en alle overige VSBN'ers, jullie zijn fantastisch. Marlies ik waardeer al het werk dat je doet, je adviezen en alle gezelligheid die je met je meebrengt. Miranda en Kim, jullie zijn later bij het team gekomen maar het voelde al snel alsof jullie er al lang bij horen en het is fijn om met jullie te werken. Miranda bedankt ook voor je scherpe blik op de tekst in het proefschrift. Met Rajiv heb ik ook altijd veel kunnen lachen, of het op het lab nou mee zat of tegen zat. We deelden ook vaak de treffende PhD memes met elkaar die we met een lach en een traan bekeken.

Het beschreven werk is ook mogelijk gemaakt door de inzet van alle studenten die met ons hebben samengewerkt in de vorm van stageprojecten. **Lisa Popma**, **Evi Warmerdam**, **Rosa Rentenaar**, **Myrthe van der Zwan**, **Myrthe Witbaard**, **Leonore Mastenbroek** en **Lotte Rozemeijer** enorm bedankt voor al jullie harde werk. Ik vond

het erg leuk om jullie te begeleiden tijdens jullie stages en ben erg trots op de resultaten die we samen hebben bereikt.

Mijn collega's van het **Radboudumc** wil ik graag bedanken voor de leuke en leerzame tijd. Ik had een onvergetelijke en gezellige tijd gehad in Nijmegen met dank aan professor **Marien de Jonge**, **Esther Fasse**, **Bram Cranenbroek, Marilen Benner**, **Dorien Feyaerts**, **Yessica Rodriguez Rosales**, **Sija Landman**, **Xuehui He**, **Pieter Langerhorst** en de andere **LMI**'ers. Het eerste jaar vloog voorbij, maar ik heb het erg naar mijn zin gehad met jullie. Wat fijn dat ik op Esther en Bram kon rekenen voor hulp als de experimenten ineens groter werden dan van tevoren gedacht. **Mark Gorris** bedankt voor al je hulp met de Vectra. **Marije Koenders** wil ik graag bedanken voor haar rol als mentor en voor de fijne gesprekken. **Rob de Vries** en **Carlijn Hooijmans** bedankt voor de begeleiding van de systematic reviews. Uiteindelijk een enorm project geworden met een prachtig en bruikbaar resultaat.

Daarnaast had ik ook een leuke tijd gehad bij **Promovendi Netwerk Nederland** en de **RIMLS PhD council** met professor **René Bindels**, **Bert van der Reijden**, **Clasien Oomen**, **Judith Ariens**, **Anouk Becker**, **Sophie Raterman**, **Francesca Tiso**, **Iris te Paske**, **Iris Brummelhuis**, **Bastiaan Privé**, **Pepijn Thomas**, **Xander Staal**, **Romy Bouwmeester**, **Judith Schaart**, **Luca Meesters** en **Iris van der Hoorn**.

Mijn dank gaat uit naar de collega's van het **Brandwondencentrum** van het **Rode Kruis Ziekenhuis**: professor **Paul van Zuijlen**, **Anouk Pijpe**, **Matthea Stoop**, **Evelien de Jong**, **Stephan Papendorp**, **Annebeth de Vries**, **Daniëlle Rijpma**, **Maxime Cuijpers**, **Robin Verwilligen** en de andere onderzoekers. Jullie betrokkenheid en klinische inzichten waren onmisbaar bij deze onderzoeken. Bedankt voor de fijne samenwerking. Daarnaast wil ik graag alle artsen, chirurgen, verpleegkundigen, overige medewerkers en patiënten bedanken voor hun inzet en deelname aan onze onderzoeken. Zonder jullie werk is onderzoek doen onmogelijk en ik waardeer jullie inzet dan ook zeer.

Collega's van **Amsterdam UMC Ibrahim Korkmaz** en **Britt van der Leeden** bedankt voor de fijne tijd en de mooie, gezellige momenten op werk en tijdens de congressen. Leuk om met jullie te sparren over nieuwe ideeën en samen nieuwe experimenten uit te proberen.

Ik wil mijn collega's van de **Nederlandse Brandwonden Stichting** graag bedanken voor de gezelligheid op de werkvloer. Wat heb ik leuke en bijzondere momenten beleefd jullie. Van mooie, diepgaande gesprekken tijdens de ochtendwandelingen en fietstochten met **Rob Baardse** tot het organiseren van activiteiten voor jongeren met brandwonden samen met **Marjorie Holtus**, **Aram van Jaarsveld**, **Marion de Koning**,

Jan-Kees Zuiker, **Marije van Leeuwen**, **Ahmet Kaptan** en **Sigrid van Gerven**. Wat zijn de **ScarWars** weekenden toch bijzonder, zo indrukwekkend en mooi om lotgenoten op zo'n manier bij elkaar te zien. De gesprekken en momenten met de deelnemers en stafleden relativeren en hebben een diepe indruk achtergelaten in mij als mens.

Britt Lents, dankjewel voor je betrokkenheid en hulp bij onze aanvraag. **Carine van Schie**, **Kees Hoogewerf**, **Bianca Prinse**, **Adinda Mieras** en **Karlijn Joosten** dankjulliewel voor de gezellige momenten op de werkvloer en tijdens de congressen. Prachtig om te zien hoe jullie je inzetten om de brandwondenzorg beter te maken. Dank ook aan mijn semi-collega's van de **ETB-BISLIFE** voor de gezellige momenten bij het koffie automaat.

De volgende mensen wil ik graag bedanken omdat jullie voor mij een bron van inspiratie waren: professor **Douglas Storey**, **Bas Zaat**, **Bjorn Herpers**, **Alex de Vos**, **Alsya Affandi** en **Joke den Haan**. Ik heb veel van jullie geleerd tijdens mijn stageprojecten en wil jullie graag bedanken voor de steun om aan een PhD traject te kunnen beginnen. Alsya bedankt voor het boek "PhD" ter voorbereiding op dit avontuur.

Door de gezellige en leuke momenten met goede vrienden heb ik dit altijd goed vol kunnen houden. Mijn dank is groot voor al het vertrouwen en alle liefde van mijn fantastische vrienden. Wat hebben wij een geweldig leuke vriendengroep met **Jasper Moonen, Mechteld Overwater, Ard Hospers** en **Hannah van Lint**. Jullie waren er altijd voor mij en wat hebben wij waanzinnig leuke momenten met jullie beleefd thuis, op (motor)vakantie of waar dan ook.

Gezellig en leuk was het ook altijd met mijn oude huisgenoot en goede vriend **Chris Flipse** en lieve vriendin **Sharlaine Sowdagar-Flipse**. Wat hadden jullie een prachtige bruiloft. **Indira Tjaden** en **Bob Muller** wat heb ik met jullie leuke, gezellige avonden beleefd. Ik heb ook altijd op de steun kunnen rekenen van mijn goede vriend **Nick Croon**. Even lekker naar de film of gamen voor wat afleiding of een gezellige bbq-en in onze tuin. **Stijn Klarenbeek**, **Lieve van der Donk**, **Melvin Bernard** en **Wessel Krom** mogen ook niet ontbreken in deze lijst. Met Stijn ben ik de uitdaging aangegaan om de Marathon van Leiden te lopen. Dit lukte ons beide in 4 uur en 15 minuten. Wat was het zwaar, maar wat een ongelooflijke prestatie. Ik kijk met veel trots terug op deze bijzondere ervaring!

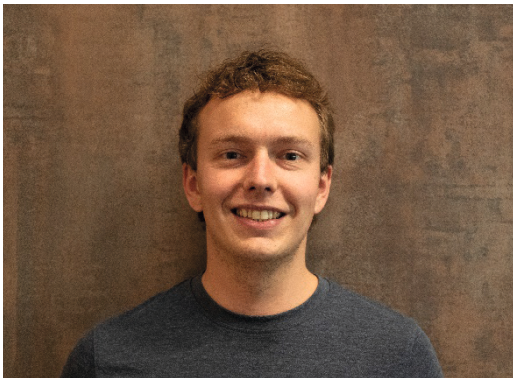
Cavemen **Ingmar van Hengel**, **Bruce Koppen**, **Thomas Brouwers**, **Roland Ritsma** en **Sander Keizer** bedankt voor de gezellige tijd. Zo leuk dat we elkaar nog altijd regelmatig zien na onze stage in de donkere cave van het AMC in 2016. Ik kijk ook terug op een leuke

vakantie in Napels. Bruce, ik zal ook nooit vergeten dat wij samen ons eerste artikel hebben geschreven.

Dit werk was niet mogelijk geweest zonder de steun en het vertrouwen van mijn lieve familie. Dank aan mijn lieve ouders **Koos Mulder** en **Anja Mulder**, mijn broers **Marthijn Mulder** en **Jeroen Mulder** en **Miranda Geerlings** voor alle liefde en steun tijdens dit avontuur. Ik wil mijn lieve schoonfamilie **Herman Geluk**, **Margreet Geluk** en **David Geluk** graag ontzettend bedanken voor alle hulp, belangstelling en ondersteuning. Wat een drukke maar leuke tijd hebben we gehad met de verhuizing en verbouwing van ons prachtige huis. Mijn dank gaat ook uit naar alle andere familieleden en aanhang.

Zoals ook met publiceren zijn vooral de eerste en de laatste persoon belangrijk: mijn lieve vriendin **Sophie Geluk**. Je bent mijn rots in de branding, mijn steun en toeverlaat en mijn grote liefde. Dit werk zou zonder jou nooit zo goed verlopen zijn. Het was niet altijd gemakkelijk, soms zaten we er even doorheen en ook voor jou waren dit niet de makkelijkste jaren. Toch hebben we elkaar door deze tijd heen geholpen en is onze band sterker geworden. Je kon mij niet altijd volgen als ik het over die fascinerende cellen en ontstekingsreacties had, maar dat maakte niet uit. Waar het om gaat is dat je er altijd voor me bent geweest en dat we samen een mooi leven hebben opgebouwd met een prachtig huis. Ook hebben we fantastische reizen gemaakt door onder andere Frankrijk, Italië, Oostenrijk en de Balkan. Ik houd ontzettend veel van je en ben je dankbaar voor al je hulp. Op een mooi leven samen!

ABOUT THE AUTHOR



Patrick Petrus Gijsbertus Mulder was born in 1995 in De Zilk. It was already at high school that he became passionate about medicine and biology. After high school, he decided to go to the University of Applied Sciences in Leiden for a Bachelor's program on Biology and Medical Laboratory Science in 2012. During the third year of his study he was involved in an international program for which he traveled to Canada to perform an internship in the group of professor Douglas Storey at the University of Calgary. He there studied *P. aeruginosa* infection during cystic fibrosis. To conclude his Bachelor's program in 2016, he performed an internship to learn more about infection diagnostics at the Regional Laboratory for Public Health in Haarlem followed by a research internship in the group of dr. Bas Zaat at the Department of Medical Microbiology & Infection Prevention of the Amsterdam Medical Center (AMC) in Amsterdam. In Amsterdam he investigated the synergistic interaction between synthetic antimicrobial peptides and antibiotics and wrote his first research paper together with Bruce Koppen.

He continued studying and followed a Master's program in Biomedical Sciences at the VU University in Amsterdam in 2016, specializing in immunology and infectious diseases. He combined this program with a part-time job, first as a cook at Italian restaurant Woodstone in Haarlem and later as a microbiology analyst at the Regional Laboratory for Public Health. For his first internship he conducted a research project in the dr. Alex de Vos at the Center of Experimental and Molecular Medicine of the AMC in Amsterdam. There, he studied interactions between immune receptor DC-SIGN and *K. pneumoniae*. For his final internship, he investigated uptake and internalization of various liposome vaccines by sentinel macrophages as potential treatment for cancer. This research took place in the group of dr. Joke den Haan at the Department of Molecular Cell Biology & Immunology of the VU Medical Center in Amsterdam.

After his Master's program, Patrick started a combined position at SMS-oncology (now Allucent) in Schiphol as an associate contract manager and clinical trial assistant in 2018. In 2019, he took the opportunity to start a PhD project at the Association of Dutch Burn Centres (ADBC) and the Radboudumc, focusing on the immune response to burn injury. During his PhD, Patrick was a member of the PhD council of the Promovendi Network of Netherlands and of the PhD council of the Radboudumc Institute for Molecular Life Sciences. He volunteers at the Dutch Burns Foundation as staff member for the ScarWars camp for young people with burn scars.

Patrick loves to run and therefore it was no surprise that he challenged himself to run the Leiden Marathon, which he completed in 2023 in 4 hours and 15 minutes. He often takes the motorcycle to go to work, but sometimes he runs or walks. Patrick will continue his research on the immune response to burn injury using patients samples and in vitro skin models as a postdoctoral researcher at the ADBC.

ADBC
Association of
Dutch Burn Centres

Radboudumc
university medical center

Radboud University