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## Aberrant recruitment of leukocytes defines poor wound healing in patients with recessive dystrophic epidermolysis bullosa.

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## Original Article

# Aberrant recruitment of leukocytes defines poor wound healing in patients with recessive dystrophic epidermolysis bullosa



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## ABSTRACT

**Background:** Poorly healing wounds are one of the major complications in patients suffering from recessive dystrophic epidermolysis bullosa (RDEB). At present, there are no effective means to analyze changes in cellular and molecular networks occurring during RDEB wound progression to predict wound outcome and design better wound management approaches.

**Objectives:** To better define mechanisms influencing RDEB wound progression by evaluating changes in molecular and cellular networks.

**Methods:** We developed a non-invasive approach for sampling and analysis of wound-associated constituents using wound-covering bandages. Cellular and molecular components from seventy-six samples collected from early, established and chronic RDEB wounds were evaluated by FACS-based immuno-phenotyping and ELISA.

**Results:** Our cross-sectional analysis determined that progression of RDEB wounds to chronic state is associated with the accumulation (up to 90 %) of CD16<sup>+</sup>CD66b<sup>+</sup> mature neutrophils, loss of CD11b<sup>+</sup>CD68<sup>+</sup> macrophages, and a significant increase (up to 50 %) in a number of CD11c<sup>+</sup>CD80<sup>+</sup>CD86<sup>+</sup> activated professional antigen presenting cells (APC). It was also marked by changes in activated T cells populations including a reduction of CD45RO<sup>+</sup> peripheral memory T cells from 80 % to 30 % and an increase (up to 70 %) in CD45RA<sup>+</sup> effector T cells. Significantly higher levels of MMP9, VEGF-A and cathepsin G were also associated with advancing of wounds to poorly healing state.

**Conclusions:** Our data demonstrated that wound-covering bandages are useful for a non-invasive sampling and analysis of wound-associated constituents and that transition to poorly healing wounds in RDEB patients as associated with distinct changes in leukocytic infiltrates, matrix-remodeling enzymes and pro-angiogenic factors at wound sites.

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## 1. Introduction

Hereditary recessive dystrophic epidermolysis bullosa (RDEB) is a mechanobullous disease characterized by the fragility of the skin and development of blisters and erosions following minor stress to the skin. The disease is caused by mutations in the *Col7A1* gene, which result in a lack or dysfunction of type VII collagen (Col7), the exclusive structural component of anchoring fibrils, at the dermal-epidermal junction (DEJ) [1]. Separation of skin layers leads to the development of lesions ranging from skin blisters to chronic ulcerated wounds. RDEB skin wounds are often associated with numerous complications, including pain and itch, loss of

**Abbreviations:** RDEB, Recessive Dystrophic Epidermolysis Bullosa; DC, dendritic cells; APC, antigen presenting cells; LC, Langerhans cells; MMPs, matrix metalloproteinases; TIMP-1, tissue inhibitor of metalloproteinase 1; IL, interleukin; VEGF, vascular endothelial growth factor; MVD, micro/vessel density; DFU, diabetic foot ulcer; FACS, fluorescence activated cell sorting.

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fluids and nutrients, infection, sepsis, dehydration, extensive scarring, deformities of the hand and feet, and cancer, thus, presenting a major health-related burden for RDEB patients [2].

At present, there are no objective means to predict the wound outcome in RDEB. Wound healing is a well-orchestrated process with rather distinct phases of hemostasis, inflammation, proliferation and maturation [3]. Prolonged and de-regulated inflammatory stage is often associated with compromised healing and progression to chronic wounds [4], which are often observed in RDEB [5]. In RDEB patients, skin lesions frequently occur at sites of most mechanical stress (knees, feet, hands, and elbows). RDEB wounds also often colonized with pathogenic bacteria, leading to excessive inflammation and affecting healing [6]. Although substantial clinical data regarding RDEB wounds have been accumulated over the years, there is still a substantial gap in our understanding of the molecular and cellular events associated with wound healing and the development of chronic wounds in RDEB skin, in part due to the lack of non-invasive protocols to distinguish wound stage and prognosis of healing.

To understand molecular networks controlling wound healing in RDEB skin, we developed a non-invasive approach for sampling and analysis of inflammatory and immune activities at wound sites using wound-covering bandages recovered from skin of RDEB patients. This study highlights inflammatory and immune activities in RDEB wounds, thus providing potential markers of disease activity and treatment effect.

## 2. Materials and methods

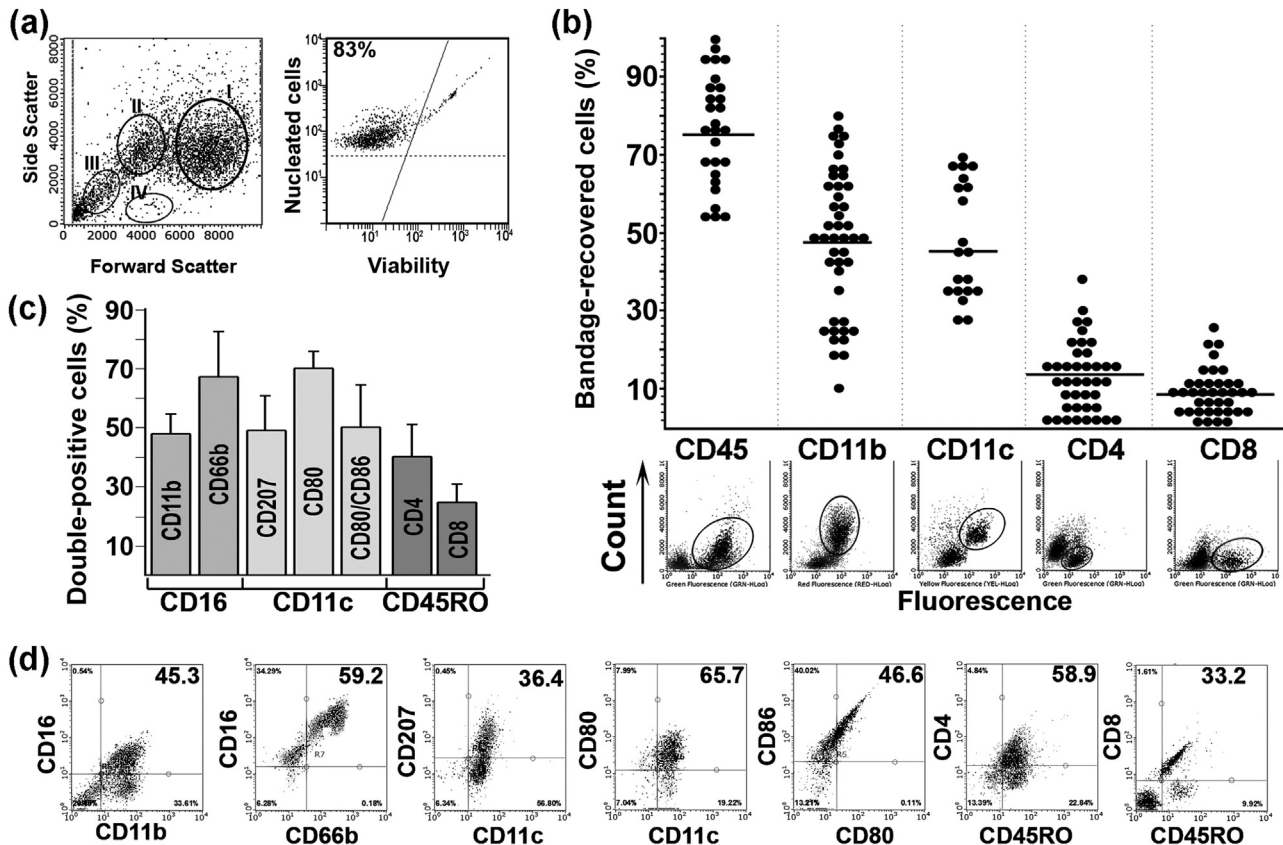
More detailed methods are described in Supplementary Materials and Methods.

### 2.1. Subjects and samples

All patients received counseling pertaining to the study, and written informed consents were obtained from all patients or their legal guardians. Totally, seventy-six wound-covering bandages were collected from early (1–5 days old;  $n = 22$ ), established (1 week old;  $n = 23$ ; 2 weeks old;  $n = 9$ ) and chronic ( $\geq 3$  weeks old;  $n = 22$ ) wounds.

### 2.2. Collection and preparation of wound-covering bandages

Non-occlusive wound dressings ( $\sim 40 \times 40 \text{ mm}^2$ ) were removed from the center of the wounds and placed in ice-cold DMEM transport media (Corning, Corning, NY) supplemented with 0.5 % Fetal Bovine Serum (FBS) (R&D Systems, Minneapolis, MN), antibiotics (Primocin; Invivogen, San Diego, CA), and proteinase inhibitors (Pierce, ThermoFisher, Waltham, MA). Bandages containing ointments were excluded. All wounds had no documented active bacterial infections. Cellular and soluble components were recovered from bandages within 24–48 h after collection.



**Fig. 1.** FACS-based analysis of bandage-recovered cellular constituents of RDEB wounds. (a) Scatter plot evaluation of cellular populations of distinct size and complexity. Percentage of viable cells recovered from bandages is shown in the upper left quadrants of the viability plot. (b) Dot-plot evaluation of bandage-recovered cells expressing specific leukocytic markers, as indicated below the plots. Each dot represents an average of duplicate measures per individual sample. Number of dots corresponds to a number of bandages used in the analysis. Annotated bars show mean percentages of marker-specific cells. Representative scatter plots with outlined marker-specific populations are shown below the dot plots. (c) Columns showing mean percentages of marker-specific populations  $\pm$  SD defined by staining with subset-specific markers shown on and under the columns. (d) Representative scatter plots showing distribution and percentages (upper right quadrant) of specific subsets in bandage-recovered leukocytes. Detected antigens are shown to the left and under the corresponding plots. In all scatter plots, detected antigens are shown on axes. Percentages of double-positive cells are shown in upper right quadrants.

2.3. Recovery of cellular bandage-derived components

Biological material from bandages was removed with cell scrapers and suspended in transport media. Clarified cell pellets were washed with Phosphate Buffered Saline (PBS) and resuspended in DMEM media containing 10 % FBS and Primocin. Cell viability was assessed by FACS using Guava ViaCount reagent (Millipore, Burlington, MA) on the Guava EasyCyte system (Millipore).

2.4. Soluble bandage-derived components

Bandage transport media was collected from pelleted cells and clarified using sequential filtration on 1 μm and 0.22 μm filters. Media was separated into high molecular weight (HMW) and low molecular weight (LMW) fractions on Ultracel-100 K and Ultracel-3 K concentration units (Millipore).

2.5. FACS-based immuno-phenotyping of bandage-derived leukocytes

About 5 × 10<sup>4</sup> cells per sample were incubated with fluorophore-labeled leukocyte marker-specific antibodies (BioLegend, San Diego, CA) (Table S1). FACS was done on Guava EasyCyte System and analyzed using GuavaSoft 2.7 software (Millipore). Gating of specific population was based on forward versus side scatter or labeling of cells with population-specific antibodies.

2.6. ELISA-based quantitation of cytokine release

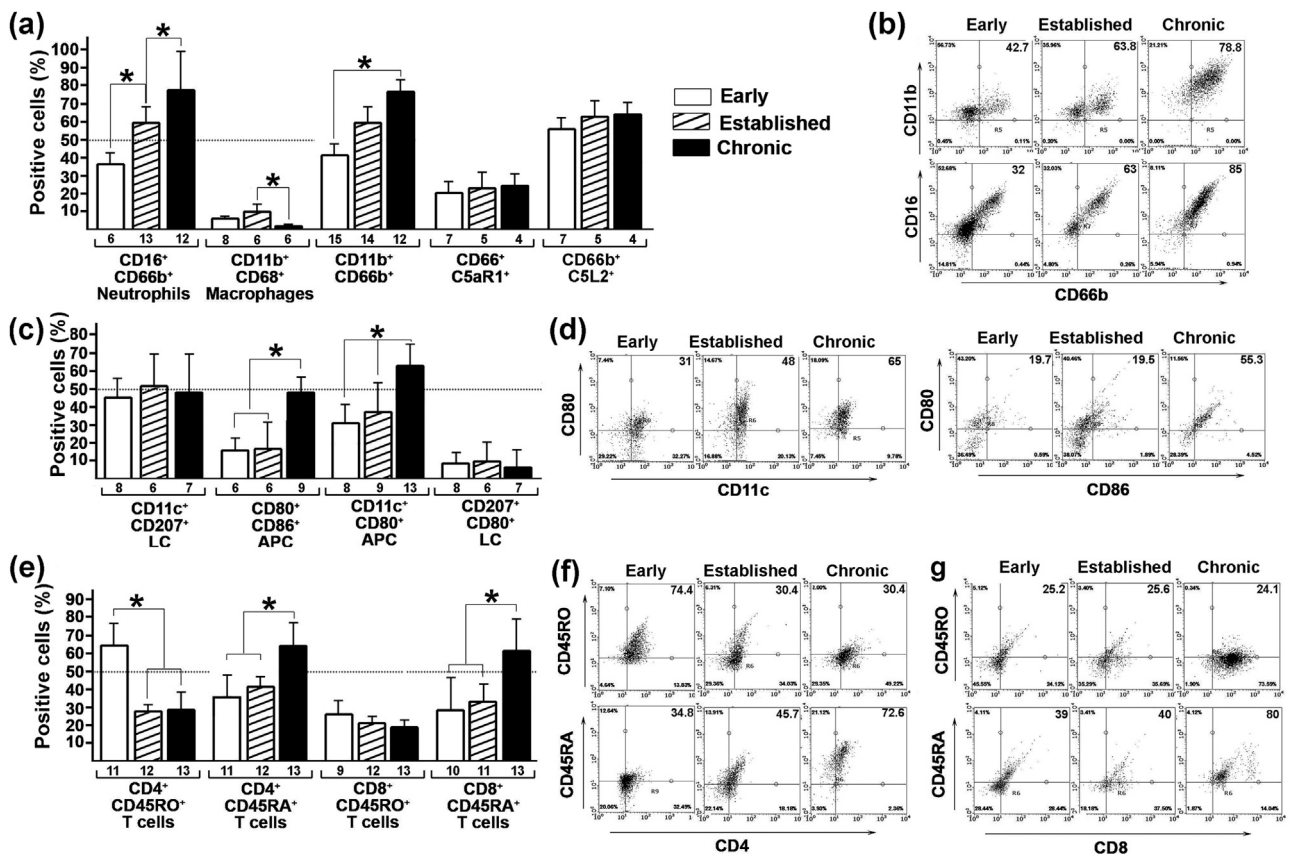
Single-Analyte Quantikine ELISA (MMP2, MMP9, TIMP-1, IL-2, IL-6, CXCL8, and TGFβ; R&D Systems) was performed according to manufacturer protocol.

2.7. Immunofluorescence analyses

Healthy and RDEB skin samples were embedded in Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA, Inc., Torrance, CA), frozen, and cryosectioned. Seven-micron sections fixed in 4 % paraformaldehyde were incubated with CD31 primary antibodies (BD Biosciences, San Jose, CA) following detection of immunocomplexes with fluorophore-labeled secondary antibodies. Blood vessel density (BVD) was determined by direct counting of CD31-positive blood vessels on at least 3 independent microscopic fields from 8 control and 23 RDEB samples.

2.8. Statistical analysis

Comparison of the data was performed using Student 2-tailed *t*-test. A P-value of < 0.05 was considered statistically significant.



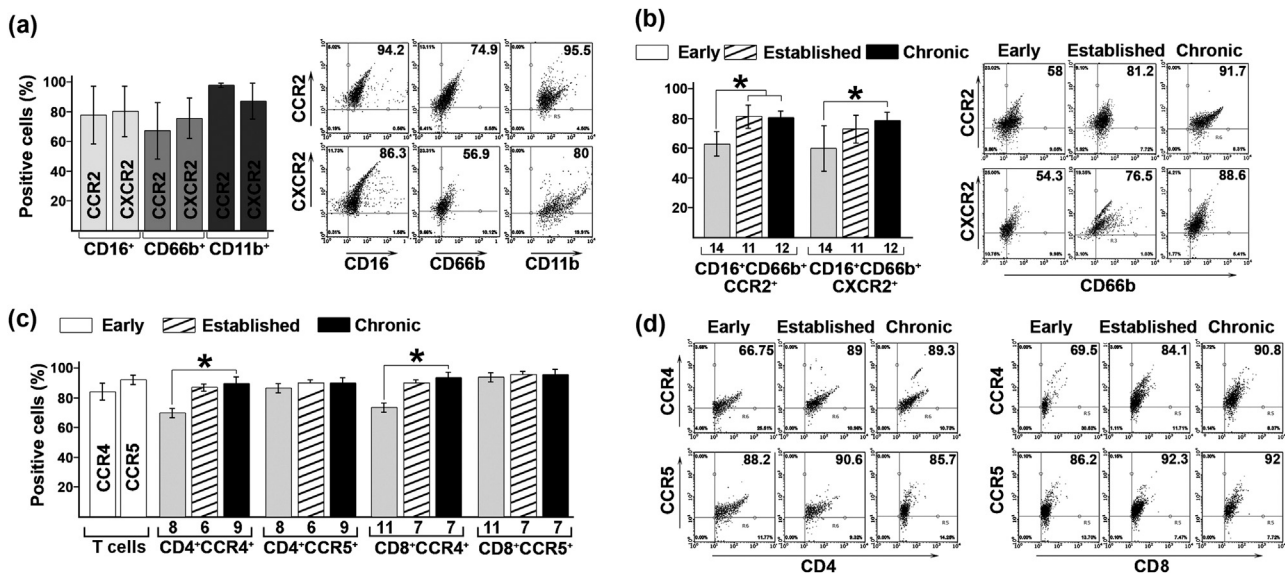
**Fig. 2.** FACS analysis of specific cellular subsets in RDEB wounds. (a) Column charts and (b) representative density pots illustrating progressive accumulation of CD16<sup>+</sup>CD66b<sup>+</sup> and CD11b<sup>+</sup>CD66b<sup>+</sup> mature neutrophils and dissipation of CD11b<sup>+</sup>CD68<sup>+</sup> macrophages in chronic wounds. (c) Column charts and (d) representative density plots illustrating accumulation of CD11c<sup>+</sup>CD80<sup>+</sup>CD86<sup>+</sup> APC in chronic wounds. (e) Column charts and (f, g) representative density plots depicting reduction of CD45RO<sup>+</sup>CD4<sup>+</sup> and CD8<sup>+</sup> peripheral memory T cells and accumulation of CD45RA<sup>+</sup> effector T cells in chronic wounds. Wound type (early, established, chronic) is indicated in the key in panel (a). In all column charts, detected antigens are shown below the columns. Y-axis: percent of double-positive cells. The data are presented as mean percentage ± SD. Statistical significance (*p* < 0.05) is indicated by asterisk. Number of analyzed bandages per wound type per combination of antigens is shown under the columns. In all scatter plots, detected antigens are shown on axes. Percentages of double-positive cells are shown in upper right quadrants.

### 3. Results

Invasive methods, such as skin punch biopsies, are not clinically recommended for RDEB patients. For analysis of wound bed-associated infiltrates, we established non-invasive sampling approach utilizing wound-covering dressings. Cells isolated from bandages, which stayed on the wounds for 24–48 h, showed 3–4 distinct cell populations of varying sizes and complexity and an average viability of 80 % (Fig. 1a). Cells from dry or more than 5-day-old bandages showed decreased viability (less than 30 %), no distinct cell populations (Fig. S1a, b), and were excluded from all analyses. Initial fluorescence activated cell sorting (FACS)-based immuno-phenotyping showed that on average 75 % of recovered cells were represented by CD45<sup>+</sup> cells (Fig. 1b). Gating on specific populations showed that population I and IV were mostly comprised of CD45<sup>+</sup> leukocytes, presumably myeloid and T cells, respectively. The remaining 25 % of CD45<sup>-</sup> bandage-associated cells were mostly represented by fully differentiated and anucleated keratinocytes (population II) (Fig. S1c). Bandage-recovered cells associated with population III were able to produce fibroblastic cultures, which differentiate into alpha smooth muscle actin ( $\alpha$ SMA) expressing cells when maintained as a sparse culture (Fig. S1d–g). Further immuno-phenotyping showed that about 47 % of leukocytes expressed CD11b myeloid cell marker, 45 % of CD11c antigen presenting cell (APC) marker, and about 10 % expressed CD8 and CD4 T cell markers (Fig. 1b). About 50 % of CD11b<sup>+</sup> cells expressed CD16 myeloid cell marker, and about 70 % of these cells were positive for CD66b granulocyte neutrophil activation marker (Fig. 1c, d). In addition, 49 % of CD11c<sup>+</sup> expressed CD207 (Langerin) marker of Langerhans cells (LC). On average, 70 % of all CD11c<sup>+</sup> cells expressed CD80 APC activation marker, 50 % of which co-express CD86. Activated/effector populations were noted in CD4<sup>+</sup> and CD8<sup>+</sup> bandage-derived T cells. Cumulative data showed that about 40 % of CD4<sup>+</sup> and 25 % of CD8<sup>+</sup> T cells express CD45RO marker of antigen-experienced effector memory T cells (Fig. 1c, d). About 60 % of CD4<sup>+</sup> and 75 % of CD8<sup>+</sup> cells expressed CD45RA marker of naive and effector cells (data not shown).

Considering broad distribution of CD11b<sup>+</sup>, CD11c<sup>+</sup>, and T cell populations (Fig. 1b), we further investigated whether percentages of specific cell types correlate with wound progression. All wounds were categorized into three groups, early, established and chronic, as defined by the age of the wounds. FACS-based evaluation showed a progressive and significant accumulation of CD11b<sup>+</sup>CD66b<sup>+</sup> cells at chronic wounds. In early wounds, neutrophils represented about 20 % of total leukocytes, whereas in 3 weeks and older wounds these cells represented up to 90 % of total leukocytes (Fig. 2a, b). Further immuno-phenotyping confirmed that this population is represented by CD16<sup>+</sup>CD66b<sup>+</sup> mature neutrophils (Fig. 2b). Accumulation of neutrophils in chronic wounds could be associated with dysfunctional healing and correlate with the loss of complement component 5a C5aR1 and C5L2 receptors [7]. However, examination of 5a C5aR1 and C5L2 expression on CD66b<sup>+</sup> cells showed no significant reduction of both receptors on neutrophils recovered from either early or chronic wounds. FACS-based evaluation showed that an average 60 % and 20 % of CD66b<sup>+</sup> neutrophils express C5L2 and C5aR1 receptors, respectively, in all wound types (Fig. 2a). Interestingly, CD11b<sup>+</sup>CD68<sup>+</sup> macrophages were present at low fractions in all examined wounds (Fig. 2a). Overall percentage of macrophages among wound-bed associated leukocytes was higher in established wounds (up to 10 %) but dropped significantly down to 2.5 % in chronic wounds (Fig. 2a).

All CD11c<sup>+</sup> APC were equally represented by CD207<sup>+</sup> LC and CD207<sup>-</sup> DC populations in all wound types (Fig. 2c). Intriguingly, progression of wounds from early to chronic was associated with the accumulation of CD11c<sup>+</sup>CD80<sup>+</sup> activated APC. Early wounds contained about 30 % of CD80<sup>+</sup> APC, whereas chronic wounds showed up to 70 % of activated APC (Fig. 2c, d). In all wound types, there were no significant changes in CD207<sup>+</sup> LC activation. These findings were further confirmed by evaluation of both CD80 and CD86 activation markers. About 17 % of total APC expressed both makers in early and established wounds, while this percentage was increased up to 35 % in chronic wounds. These data showed that accumulation of the activated APC in chronic wounds is mostly due



**Fig. 3.** FACS analysis of chemokine receptors on RDEB wound-associated leukocytes.

(a) Column charts and representative profiles depicting expression of CXCR2 and CCR2 chemokine receptors on CD16<sup>+</sup>, CD66b<sup>+</sup>, and CD11b<sup>+</sup> myeloid cell populations. Detected chemokine receptors are on the columns. (b) Column charts and representative profiles depicting CCR2 and CXCR2 expression on CD16<sup>+</sup>CD66b<sup>+</sup> mature neutrophils in early, established and chronic wounds. (c) Column charts illustrating expression of CCR4 and CCR5 chemokine receptors on a total population of wound-associated T cells and on CD4<sup>+</sup> and CD8<sup>+</sup> T cells in early, established and chronic wounds. (d) Representative density plots depicting CCR4 and CCR5 expression of CD4 and CD8-gated T cell populations in early, established and chronic wounds. In all column charts, the data are presented as mean percentage  $\pm$  SD. Statistical significance ( $p < 0.05$ ) is indicated by asterisk. Number of analyzed bandages per wound type per combination of antigens is shown under the columns. In all scatter plots, detected antigens are shown on axes. Percentages of double-positive cells are shown in upper right quadrants.

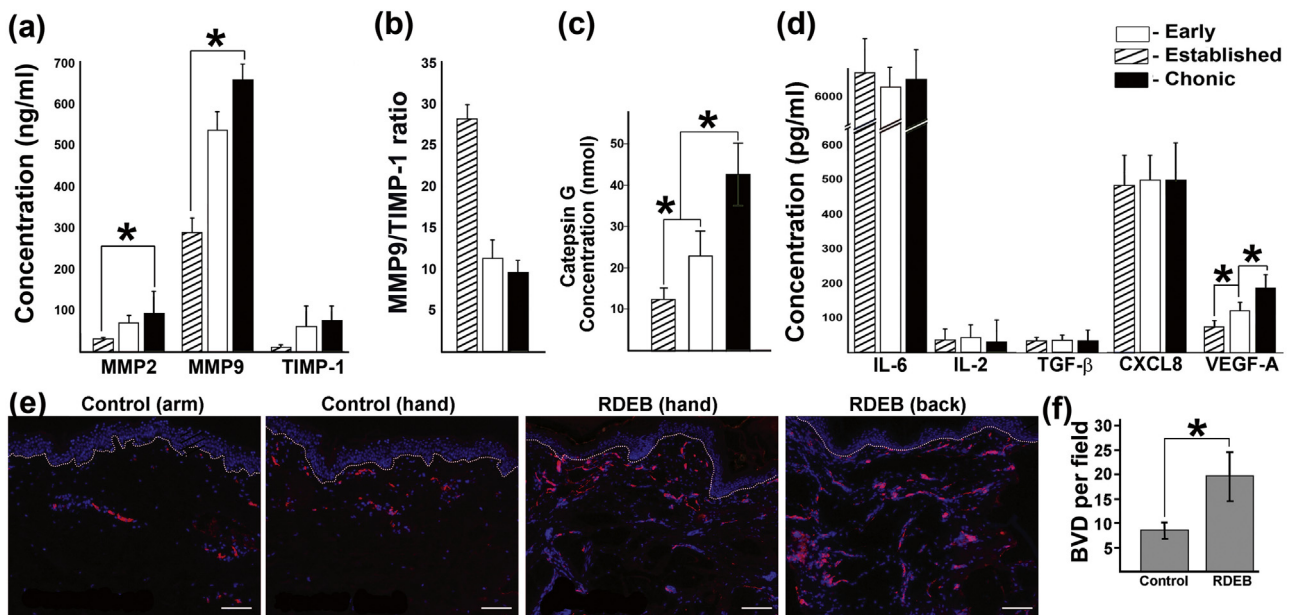
to the accumulation of activated DC. Evaluation of the T cells showed that early wounds were populated with CD4<sup>+</sup>CD45RO<sup>+</sup> effector memory and CD4<sup>+</sup>CD45RA<sup>+</sup> naïve T cells (Fig. 2e–g). Progression of wounds was associated with a substantial decrease of the effector memory T cells and increase of the CD4<sup>+</sup>CD45RA<sup>+</sup> effector T cells in chronic wounds (Fig. 2c). Similar trend was observed for CD8<sup>+</sup> T cells.

We previously reported that high levels of CCR2, CXCR1, CXCR2, and CCR4 ligands dominate early RDEB blisters, and that these chemokine receptors support directional migration of leukocytes *in vitro* [8]. Evaluation of these receptors on bandage-derived leukocytes showed that about 80 % of cells expressed both CCR2 and CXCR2 receptors (Fig. 3a, b). Most consistent receptor expression was detected on CD11b-gated cell population. Substantially greater variations in percentages of CCR2<sup>+</sup> cells were seen in CD66b-gated cells. Nevertheless, analysis of CD66b<sup>+</sup> population showed a significant accumulation of both CCR2<sup>+</sup> and CXCR2<sup>+</sup> cells in established and chronic wounds (Fig. 3a, b). Although such accumulation was detected in a rather large number of samples (n = 9 per wound type), several samples collected from one patient showed a substantially low number of CCR2<sup>+</sup> and CXCR2<sup>+</sup> granulocytes in all wound types and, particularly, in chronic wounds (Fig. S2).

Previously, we also reported that CCR4<sup>+</sup> and CCR5<sup>+</sup> lymphocytes migrate in response to RDEB-derived blister fluids *in vitro* [8]. As an accumulation of the activated/effector T cells was observed in chronic wounds, we evaluated whether these chemokine receptors could facilitate migration of the T cells to the wound bed. When assessing a T-cell gated population, about 83 % and 91 % of cells were identified as CCR4<sup>+</sup> and CCR5<sup>+</sup>, respectively. Percentages of CCR5<sup>+</sup> T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) were consistently high in all wound types (Fig. 3c, d). In early wounds, about 70 % of CD4<sup>+</sup> and

86 % of CD8<sup>+</sup> T cells express CCR4 on cell surface. In established and chronic wounds, these percentages were significantly increased up to 86 % and 91 %, respectively (Fig. 3c, d).

Wound repair results from a highly complex interaction of cellular and biochemical events. To date, many investigations described proteins and signaling molecules that contribute to the disordered healing, as for example in diabetic foot ulcers (DFU) [9,10], yet, these molecular networks remain poorly defined in RDEB wounds. Considering a known contribution of matrix metalloproteinases (MMPs) to chronic wound pathogenesis [11], we assessed MMP2 and MMP9 in a low molecular weight (LMW) fraction. Quantitative cytokine-specific ELISA showed that both MMPs are present at wound sites, and that concentration of MMP9 is 5–10 times greater than MMP2. Moreover, significantly higher MMP9 levels were detected in established and chronic wounds as compared to early lesions (Fig. 4a). Consistent with our prior studies [8], we also detected low levels of tissue inhibitor of metalloproteinases 1 (TIMP-1) in all wounds. When evaluating MMP9/TIMP-1 ratio, another predictor of wound healing [12], we found that early RDEB wounds had the highest MMP9/TIMP-1 ratio (Fig. 4b). Considering that mature neutrophils produce MMPs and other matrix remodeling enzymes, we also assessed cathepsin G content. Concentration of this serine protease was significantly higher in chronic wounds as compared to early wounds (Fig. 4c). Healing of skin wounds depends on different biochemical mediators such as growth factors and cytokines. To evaluate suitability of bandage-derived samples for the analysis of these soluble factors, we also measured interleukin 6 (IL-6) and IL-2, two cytokines known to contribute to wound healing. ELISA-based quantitation showed that both interleukins are present in all examined wounds with IL-6 levels in nanogram range and IL-2 in picogram range (Fig. 4d). No significant difference was detected



**Fig. 4.** Evaluation of bandage-recovered soluble factors in early, established and chronic wounds and analysis of angiogenesis in RDEB skin samples. (a) ELISA-based quantitation of MMP2, MMP9, and TIMP-1 in a soluble fraction recovered from bandages covering early, established, and chronic wounds. Data are presented as a mean concentration (ng/mL) of triplicate measures  $\pm$  SD. (b) Column chart depicting average MMP9/TIMP-1 ratio in early, established and chronic wounds. Data are presented as fold difference  $\pm$  SD. (c) Column charts showing cathepsin G concentration in early, established and chronic wounds as determined by cathepsin G assay. Data are presented as mean concentration  $\pm$  SD. (d) ELISA-based quantitation of selected cytokines and growth factors (indicated below the columns) in early, established and chronic wounds (as indicated in the key). Data are presented as mean concentration (pg/mL)  $\pm$ SD. In all column charts, statistical significance ( $p < 0.05$ ) is indicated by asterisk. At least, 4 independent samples per wound type per detected protein were used. (e) Representative micrographs illustrating elevated number of CD31<sup>+</sup> blood vessels in RDEB intact (undamaged) skin. Source of skin samples (genetically normal control and RDEB) are indicated above the micrographs. Red – CD31<sup>+</sup> blood vessels; blue – DAPI nuclear staining. The basement membrane zone is outlined by dotted line. Scale bar – 100  $\mu$ m. (f) Analysis of blood vessel density (BVD) per section of the skin. Data are presented as an average number of blood vessels per section  $\pm$  SD. Eight independent control and 23 RDEB samples were used for the assessment. Statistical significance ( $p < 0.05$ ) is indicated by asterisk.

between the cytokine levels in different wounds. Similarly, there were no significant differences in soluble TGF- $\beta$  and a pro-inflammatory/pro-angiogenic chemokine CXCL8 (IL-8) in all wound types. Assessment of vascular endothelial growth factor (VEGF)-A, a known angiogenic factor which contributes to normal wound healing [13], showed significant difference in chronic wounds, as compared to early lesions (Fig. 4d).

Because excessive angiogenesis and elevated VEGF levels contribute to hypertrophic scarring [14], we examined micro vessel density (MVD) in biopsies obtained from 23 RDEB patients and 8 controls. Indirect immunofluorescence detection of CD31<sup>+</sup> blood vessels showed that RDEB skin has more blood capillaries than normal skin (Fig. 4e, f). Together with high level of pro-angiogenic CXCL8 and elevated VEGF-A in chronic wounds, these findings suggest that increased angiogenesis in RDEB skin may contribute to poor wound healing and hypertrophic scarring of the skin.

#### 4. Discussion

The concept of acute vs. chronic wounds has changed over the past few years. Generally, it was accepted that a 21-day arbitrary limit for re-epithelization defined acute wounds. Currently, chronic wounds could be defined as not healing at the expected rate, or stalled in one of the wound healing phases. However, for various disorders and diseases, including RDEB, “expected rate” is not well-defined. Molecular and cellular networks associated with development of poorly healing/chronic wounds in RDEB remain uncharacterized, and there are no reliable biomarkers to evaluate or predict the fate of RDEB wounds. Because punch biopsies are not medically advisable for RDEB patients, we tested whether wound-covering dressings could be used for a non-invasive recovery of both cellular and molecular constituents of the RDEB wounds. In the past, biopsies of the skin adjacent to wound sites, wound debridement, wound exudates, peripheral blood, and smears from wound bed were used for biochemical assessment of wound status [9,15–18]. Although valuable, all these approaches have method-specific limitations. In comparison, our approach allows recovery of cellular and molecular components directly from the wound, immuno-phenotyping of wound-derived cells, isolation of specific cell types for functional assays, and establishment of selected cell lines.

Evaluation of cellular components showed that the majority of wound bed-associated cells are represented by various leukocytes, and fully differentiated keratinocytes and fibroblastic cells. Considering a crucial role of innate immunity in wound healing, it was not surprising that the majority of wound-associated leukocytes were represented by CD11b<sup>+</sup> myeloid cells. However, most of CD11b<sup>+</sup> cells, particularly in chronic wounds, were identified as CD16<sup>+</sup>CD66b<sup>+</sup> mature neutrophils. As neutrophils play an important role on early stages of wound healing and are usually cleared at the end of the inflammatory stage [19], their accumulation at the RDEB wounds indicates on deregulation of the early inflammatory stage. At present, the cause for such deregulation remains unknown. Nevertheless, sustained recruitment and predominance of activated neutrophils in chronic RDEB suggest that these wounds could be similar to the lesions of other neutrophilic dermatoses, such as pyoderma gangrenosum (PG) [20]. Although further comparative evaluation of soluble factors affecting neutrophilic infiltrates in RDEB and PG are required, it is plausible that treatments with anakinra or canakinumab that were successful in treating PG [21–24] or topical steroids could be used for management and treatment of chronic RDEB wounds. Based on recent animal data showing partly impaired macrophage response in DEB mice [25] and our data showing a low percentage of CD11b<sup>+</sup>CD68<sup>+</sup> macrophages in all types of RDEB

wounds, it is also plausible that macrophages cannot eliminate neutrophils from the wound bed at the end of the inflammatory phase. Down-modulation of C5aR1 expression on CD66b<sup>+</sup> neutrophils in chronic wounds may also indicate that neutrophils have reduced ability to clear bacterial infection [7]. Although our current analysis did not show any significant changes in receptor expression on CD66b<sup>+</sup> neutrophils in different wound types, it is possible that relatively low percentage (up to 30 %) of C5aR1<sup>+</sup>CD66b<sup>+</sup> cells could implicate on neutrophil's functional activity. Although a more rigorous analysis is required, persistent accumulation of mature neutrophils in chronic RDEB wounds indirectly supports this notion.

Independently of neutrophil's functional status, our current data demonstrated that constitutive recruitment of myeloid cells and neutrophils to RDEB wounds could be mediated by CCR2/CXCR2-driven chemotaxis. These findings are consistent with our prior data showing an abundance of CCR2/CXCR2 ligands in early RDEB lesions [8]. Although the source of these ligands in early and chronic RDEB wounds remains undefined, it is reasonable that systemic or localized blocking of CCR2 and/or CXCR2 receptors using small molecule inhibitors [26,27] could reduce neutrophil-associated detrimental effects and accelerate wound closure.

An accumulation of neutrophils in established and chronic RDEB wounds was accompanied by the elevated levels of MMP9 and cathepsin G. These findings correlate extremely well with prior data showing that increased MMP9 levels can predict poor wound healing in DFU [9,12]. An imbalance in MMP9 and TIMP-1 proteins results in excessive ECM degradation and higher MMP9/TIMP-1 ratio defines poor wound healing [28]. Our data showing that ratio in early RDEB wounds ( $29.6 \pm 3.1$ ) is compatible with that in severe neuropathic DFU ( $34.81 \pm 12.87$ ) [12] suggest that healing capacity in 1–5 days old lesions is already diminished. Although insignificant increase in TIMP-1 levels was detected in established and chronic wounds, its overall level remained low, and MMP9/TIMP-1 ratio ( $11.4 \pm 3.2$ ) remained compatible with unhealed DFU ( $18.88 \pm 6.8$ ) [12]. Similarly, high activity of cathepsin G in chronic wounds suggests that this protease alongside with MMP9 and other enzymes degrades multiple fibrillar collagen and extracellular matrix components, thus, preventing migration of keratinocytes and re-epithelialization of wounds. Although further longitudinal evaluation of these enzymes will be necessary, it is likely that targeted inhibition of matrix remodeling enzymes could be a viable approach to enhance RDEB wound healing.

Our analysis also revealed an accumulation of activated CD80<sup>+</sup>CD86<sup>+</sup> DC in chronic wounds with both markers expressed on up to 50% of all CD11c<sup>+</sup> cells. These findings suggest an on-going acquisition of antigens by the APC, and activation of the adaptive immune response. Although CD80 and CD86 molecules can substitute for each other in the initial activation of resting CD4<sup>+</sup> T cells, CD86 could be more important for initiating T-cell responses, while CD80 could be more significant for their maintenance [29]. Considering a frequent colonization of the RDEB wounds with bacteria and fungi [6,30], it is possible that wound-associated APC play an active role in acquisition of bacterial and/or fungal antigens, T cell priming, and activation. This notion is supported by the presence of effector memory (CD45RA<sup>-</sup>CD45RO<sup>+</sup>) and naïve (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) T cells at early wounds and accumulation of CD45RA<sup>+</sup>-effector T cells in chronic wounds. Considering an increased percentage of CCR4<sup>+</sup> T cells in advanced wounds (Fig. 3) and an important role of this receptor in skin-homing [31], it is plausible that CCR4 mediates recruitment of T cells to the wounds.

It is well-established that RDEB wounds are prone to fibrotic scarring in which TGF- $\beta$ 1 plays a major role [32]. However, pathogenesis of hypertrophic RDEB scars could also depend on



altered cytokine and chemokine signaling that supports inflammation, elevated activity of matrix remodeling enzymes, and misbalanced production of growth factors. By assessing selected bandage-recovered soluble proteins (IL-2, IL-6, CXCL8, TGF- $\beta$ 1, and VEGF-A), we found that IL-6 is present at a relatively high level in RDEB wounds. These findings are in agreement with prior animal and human studies showing that delays in diabetic wound healing may be associated with increased IL-6 [33], and that IL-6 levels are higher in human diabetic chronic wounds than in healing wounds [34]. Considering animal data showing that IL-6 supplementation is associated with enhanced leukocyte recruitment, collagen production and angiogenesis [35], it is possible that high IL-6 levels in RDEB wounds create a favorable milieu for poor wound healing. Additional studies will be necessary to evaluate the role of this cytokine in RDEB pathology.

High levels of pro-antigenic CXCL8 in RDEB blister fluids [8], elevated VEGF-A level in chronic wounds, and high vascularization of the intact RDEB skin [36] suggest that there is excessive angiogenesis in wounded RDEB skin. This characteristic sets RDEB wounds apart from diabetic, pressure, and arterial ulcers, where lack of angiogenesis, hypoxia and ischemia contribute to poor healing. Because elevated angiogenesis was linked to fibrotic scarring [14], it is plausible that angiogenesis contributes pathogenic scarring of RDEB skin. These findings also suggest that targeting of angiogenesis *via* treatment with interferon alpha2b or bevacizumab, a humanized anti-VEGF antibody used to treat various malignancies, may reduce hypertrophic scarring and improve healing of RDEB wounds [37,38].

In summary, presented cross-sectional evaluation showed that progression of early wounds to chronic state is associated with the elevated infiltration with mature neutrophils, significant reduction of macrophages, and activation of APC- and T cell-mediated adoptive immunity. Also, our data showed that excessive, neutrophil-derived matrix remodeling enzymes, MMP9 and cathepsin G, could prevent wound healing, whereas elevated levels of VEGF-A and higher vascular density in RDEB skin and wounds may support inflammation and negatively affect wound healing.

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### Declaration of Competing Interest

The authors have no conflict of interest to declare.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdermsci.2020.10.009>.

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