



Pro-Inflammatory Chemokines and Cytokines Dominate the Blister Fluid Molecular Signature in Patients with Epidermolysis Bullosa and Affect Leukocyte and Stem Cell Migration

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Hereditary epidermolysis bullosa (EB) is associated with skin blistering and the development of chronic non-healing wounds. Although clinical studies have shown that cell-based therapies improve wound healing, the recruitment of therapeutic cells to blistering skin and to more advanced skin lesions remains a challenge. Here, we analyzed cytokines and chemokines in blister fluids of patients affected by dystrophic, junctional, and simplex EB. Our analysis revealed high levels of CXCR1, CXCR2, CCR2, and CCR4 ligands, particularly dominant in dystrophic and junctional EB. In vitro migration assays demonstrated the preferential recruitment of CCR4⁺ lymphocytes and CXCR1⁺, CXCR2⁺, and CCR2⁺ myeloid cells toward EB-derived blister fluids. Immunophenotyping of skin-infiltrating leukocytes confirmed substantial infiltration of EB-affected skin with resting (CD45RA⁺) and activated (CD45RO⁺) T cells and CXCR2⁺ CD11b⁺ cells, many of which were identified as CD16b⁺ neutrophils. Our studies also showed that abundance of CXCR2 ligand in blister fluids also creates a favorable milieu for the recruitment of the CXCR2⁺ stem cells, as validated by in vitro and in-matrix migration assays. Collectively, this study identified several chemotactic pathways that control the recruitment of leukocytes to the EB-associated skin lesions. These chemotactic axes could be explored for the refinement of the cutaneous homing of the therapeutic stem cells.

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INTRODUCTION

Heritable epidermolysis bullosa (EB) is a group of mechano-bullous disorders characterized by skin fragility. Based on the level of tissue separation within the dermoepidermal basement membrane zone (BMZ), EB is divided into four categories: simplex (EBS), junctional (JEB), dystrophic (DEB), and Kindler syndrome. Each subtype of EB is associated with mutations in specific genes encoding proteins associated with the mechanical stability of the BMZ. Despite the diversity of the

phenotypic manifestations, all EB forms are accompanied by tissue separation and development of blisters and erosions after trauma to the skin. In physiological conditions, healing starts from coagulation of blood in ruptured blood vessels and release of different signaling molecules that recruit various cell types to the site of injury. This leads to an acute local inflammatory response, activation of tissue repair, regeneration, and restoration of tissue integrity.

A plethora of cytokines have been shown to participate in the dynamic wound healing process, including one superfamily of small (8–15 kD) proteins called chemokines (Efron and Moldawer, 2004). Once secreted from damaged tissue, chemokines orchestrate wound healing-associated inflammation, angiogenesis, and recruitment of extracellular matrix-producing cells. Wound healing studies on rodent models demonstrated that aberrant chemotactic signaling leads to the unresolved inflammation, chronic wounds, accumulation of extracellular matrix, scarring, and necrosis (Luster et al., 1998; Rennekampff et al., 2000). At present, however, only limited information is available regarding cytokines and chemokines in EB-affected skin.

In this study, we examined chemotactic signatures in blister fluid (BF) collected from patients affected by different types of EB. We identified chemotactic signals that can potentially contribute to impaired blister healing and define chemotactic axes improving the recruitment of therapeutic stem cells to

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Abbreviations: ADSC, adipose-derived stem cell; BF, blister fluid; BMZ, basement membrane zone; DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; MMP, matrix metalloproteinase; PBMC, peripheral blood mononucleated cell

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EB-affected skin. This work highlights the inflammatory and immune activities in the blistering skin of patients with EB, thus, providing potential markers of disease activity and treatment effect.

RESULTS

Plasma and BF of EB-affected patients contain proinflammatory chemokines and cytokines

Chemokines were analyzed in plasma and BF collected from patients with EB (DEB [n = 59], JEB [n = 6], EBS [n = 10], and dominant DEB pruriginosa [n = 2]) using antibody arrays. The clinical characteristics of patients with EB are summarized in [Supplementary Table S1](#) online. Of 38 chemokines, high levels of CXCL7, CCL5, CCL19, and CCL4 were detected in the plasma. Of all, only CCL11, CXCL8, CCL15, CXCL7, and CXCL12 showed a statistically significant difference ($P < 0.05$) in plasma of patients with EB when compared with healthy controls ([Figure 1a](#)).

In the majority of BF from EB-affected patients, high levels of CCL27, CCL24, Gro-family (CXCL1, CXCL2, CXCL3), CXCL8, CXCL10, CCL2, CCL4, CCL15, CXCL7, and CCL5 were detected ([Figure 1b](#)) independent of age, gender, and blister location (data not shown). A side-by-side comparison of EB-derived fluids and control samples collected from frostbite or burn-induced blisters of healthy donors showed a similar chemotactic pattern overall ([Figure 1b](#)). When chemotactic content of the advanced blisters (>98 hours from onset) was compared with early blisters (≤ 24 hours from onset), the majority of early DEB and JEB blisters contained two to three times lower levels of chemokines than advanced blisters ([Figure 1c](#), [Supplementary Figure S1](#) online). On the contrary, early EBS blisters contained significantly higher levels of CXCL5, Gro family chemokines, CXCL8, and CCL2 chemokines than advanced lesions. CCL15, CCL17, and CCL20 were also consistently higher in early EBS than in other EB types ([Figure 1c](#)). To validate array data, several identified chemokines were quantified by Multi-Analyte ELISArray. Analysis confirmed high levels of CXCL8 (up to 1,000 pg/ml), Gro-family chemokines (CXCL1/2/3) (up to 400 pg/ml), CCL2, CCL4, and CCL5 in EB-associated BF ([Figure 1d](#)).

Elevated levels of cytokines such as IL-1 β , IL-2, IL-6, and reduced levels of tumor necrosis factor- α have been reported in the serum of patients with DEB and EBS, suggesting that EB could be classified as a systemic inflammatory disease rather than a skin-limited disorder ([Annicchiarico et al., 2015](#)). Cytokine antibody array analysis showed that BF of all EB types share a similar profile: of 88 examined cytokines, adiponectin (ACRP30), angiogenin, and IL-6 were detected at the highest levels ([Figure 2](#)). Levels of BDNF, EGF, ICAM-1, IGFBP-1, -2, -6, MSP α , and osteoprotegerin were also consistently high in all EB types. BF also contained proinflammatory cytokines, including IL-1, IL-6, tumor necrosis factor- α , IL15; however, some of these cytokines were counterbalanced by antagonistic soluble receptors, including IL-1ra and sTNF-R1 and sTNF-R2 ([Figure 2](#)).

BF-derived chemokines modulate leukocyte migration in vitro

Elevated levels of several chemokines in BF suggested that inflammatory cells expressing a restricted set of chemokine

receptors could be mobilized to blistering skin and contribute to EB pathology. Thus, CXCR1- and CXCR2-expressing cells could be recruited to blistered skin by CXCL1/2/3/7/8, whereas CCR2⁺ could be recruited by CCL2, CCR4⁺ cells by CCL5, CCL17, and CCL22, and CCR3⁺ cells by CCL5, CCL11, and CCL24 ligands ([Figure 1d](#)). To better characterize leukocyte recruitment and contribution to EB pathology and to define chemotactic axes for the recruitment of therapeutic stem cells to EB-affected skin, human peripheral blood mononucleated cells (PBMC) isolated from healthy donors were used for the in vitro migration in Transwell chambers. A dose- and time-dependent migration of leukocytes was observed toward BF ([Figure 3a, 3b](#)). At 60 minutes, PBMC migration was substantially enhanced by adding BF into lower chambers. At 120 minutes, a fivefold increase in migration was observed relative to control. Adding BF into both upper and lower chambers inhibited PBMC migration ([Figure 3b](#)).

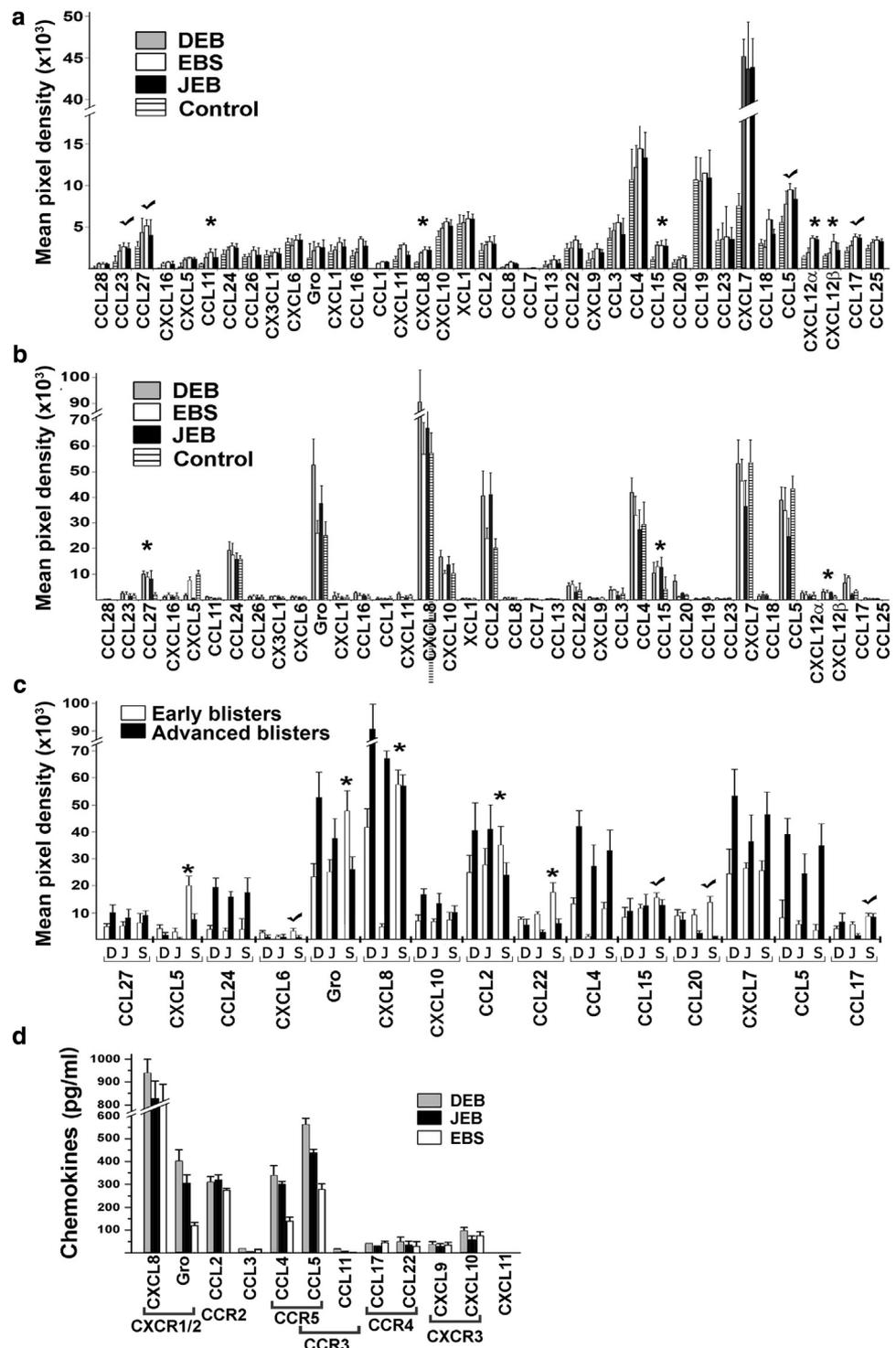
To define chemotactic axes directing leukocyte migration, expression of lead chemokine receptors for BF-associated chemokines was further examined by FACS. Lymphocytes that migrated to the lower chambers in response to BF during the first 60 minutes predominantly expressed CCR4 and CCR5 receptors for CCL2/17/22 and CCL4/5 BF-derived chemokines, respectively ([Figure 3c, 3e](#)). The majority of migrating myeloid cells expressed CXCR2, CCR2, and CXCR1, which are receptors for the most abundant BF chemokines, including CCL2, CXCL1/2/3, CXCL7, and CXCL8 ([Figure 3d, 3e](#)). To identify fast-responding leukocytes, migration of lead chemokine receptor-positive cells was analyzed at 30 minutes. Based on side scatter/fluorescence data, the majority of migrating CXCR2⁺ and CXCR1⁺ cells were myeloid ([Figure 3f](#)). This population was also highly positive for CCR2, whereas migrating lymphocytes were mostly positive for CCR4. Migrating CCR3⁺ and CCR5⁺ cells were also detected but to a considerably lesser extent ([Figure 3f](#)). Taken together, these findings suggest that circulating leukocytes could be preferentially recruited to EB-affected blistering skin via engagement of CXCR1, CXCR2, CCR2, and CCR4 receptors.

Chemokines enhance the recruitment of CXCR2⁺ leukocytes to EB-affected skin

The recruitment of leukocytes to EB-affected skin was further validated on the tissue array containing samples of three EB types (DEB, n = 15; EBS, n = 8; JEB, n = 5). Indirect immunofluorescence analysis confirmed a three- to fivefold increase in skin-infiltrating CD45⁺ leukocytes in all EB samples. A majority of the infiltrating cells were represented by CD45RO⁺, CD45RA⁺T cells, and CD11b⁺ myeloid cells ([Figure 4a, 4b](#)). Clusters of CD45 infiltrating cells were identified as CXCR2⁺ leukocytes, which were more predominant in blistering than nonblistering skin in all EB types ([Figure 5a, 5b](#)). Moreover, in recessive DEB skin, at least half of the CD11b⁺ cells were identified as CXCR2⁺ cells with at least two-thirds of them represented by CD16b⁺CXCR2⁺ neutrophils ([Figure 5c, 5d](#)). Overall, this analysis confirmed our in vitro data on leukocyte recruitment by BF-derived chemokines and suggested that only few chemokine receptors, including CXCR2, can direct leukocyte migration to EB-affected skin.

Figure 1. Proteomic screens of chemokines in plasma and blister fluids of EB-affected patients. (a–c)

Antibody array analysis of the chemokines in (a) plasma and (b, c) blister fluids of EB-affected patients. Asterisks indicate chemokines the levels of which are significantly different ($P < 0.05$) in EB samples relative to control. Check marks indicate consistently high chemokines. (a) Blood plasma from healthy donors was used as a control. (b) Blister fluids from frost-bite and burn-induced blisters of healthy donors were used as a control. The data were collected from independent arrays with duplicate measurements for each chemokine using all samples listed in [Supplementary Table S1](#). Data are presented as a mean pixel density \pm SD. EB types are indicated in the insets. (c) Comparison of the selected chemotactic molecules in early and advanced blisters of patients with EB. EB forms (DEB [D], JEB [J], and EBS [S]) and chemokines are listed below the columns. (d) Multi-Analyte ELISA analysis of common human chemokines in selected DEB ($n = 18$), JEB ($n = 6$), and EBS ($n = 6$) BF samples. The values are presented as the mean chemokine concentration (pg/ml) \pm SD. Detected chemokines and their cognate receptors are shown below the columns. BF, blister fluid; DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; SD, standard deviation.



BF-derived chemokines induce migration of CXCR1⁺ and CXCR2⁺ therapeutic stem cells in collagenous matrix

Several clinical investigations involving systemic transplantation of potentially therapeutic stem cells have reported inefficient engraftment of the cells to the cutaneous tissue (Nagy et al., 2011; Petrof et al., 2015; Wagner et al., 2010). With the aim of improving cutaneous homing of transplanted stem cells, we recently investigated chemotactic signals in a recessive DEB mouse model and identified several potential

chemotactic axes directing stem cells to the recessive DEB skin (Alexeev et al., 2016). On the basis of these data, we hypothesize that adipose-derived stem cells (ADSC) expressing CXCR1 and/or CXCR2 receptors on the cell surface could be effectively recruited to EB-affected skin. However, our recent studies demonstrated that only a small percentage (4–6%) of ADSC are CXCR1- and CXCR2-positive (Alexeev et al., 2016). To generate human ADSC that uniformly express candidate receptors, we stably

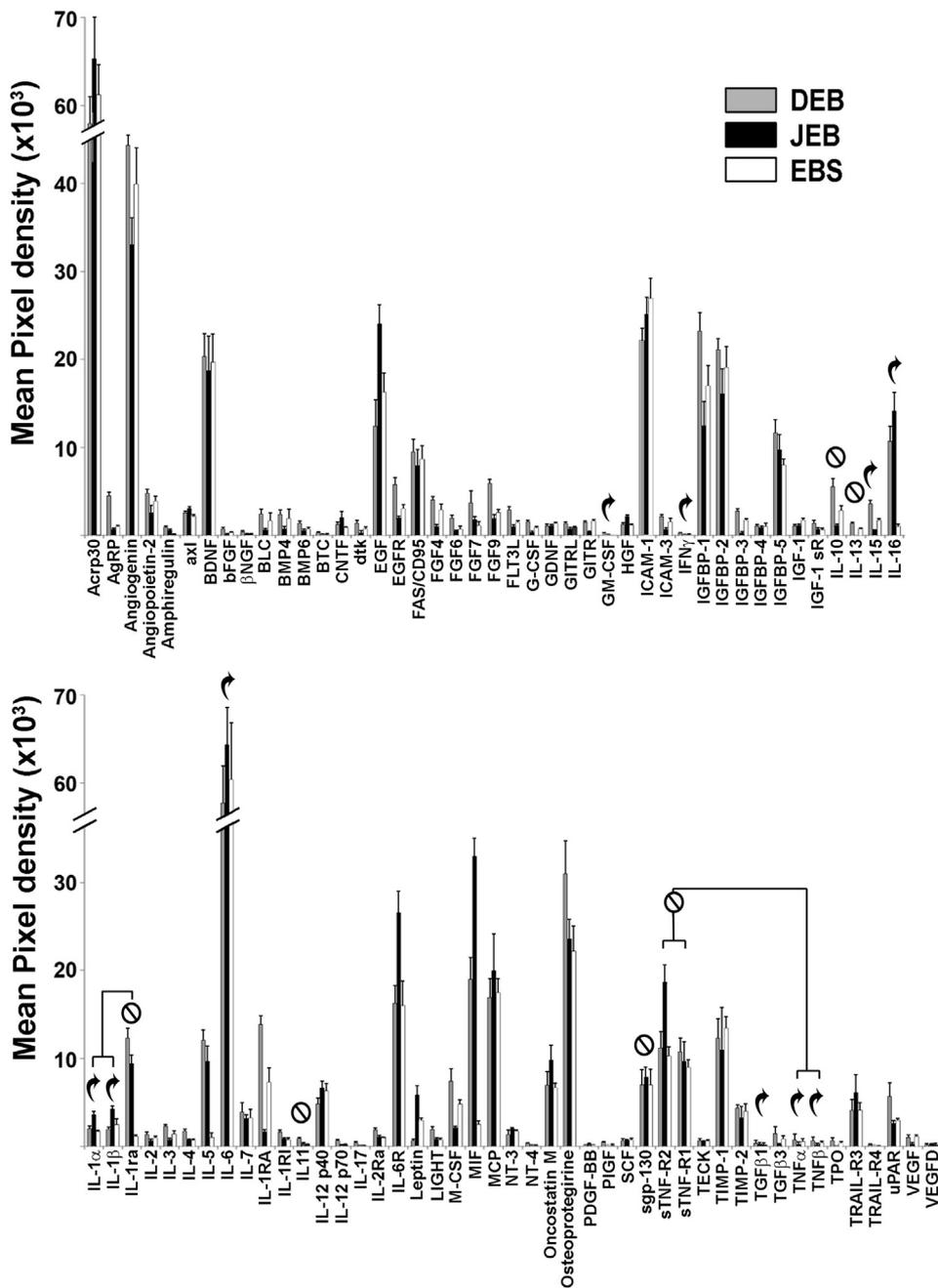


Figure 2. Proteome analysis of cytokines in blister fluids of EB-affected patients. The 88 most common cytokines were assessed in BF by protein array analysis. Cytokines are listed below the columns. Data are presented as a mean pixel density \pm SD. EB types are indicated in the inset. Curved arrows are placed above typical proinflammatory cytokines and crossed circles are placed above typical anti-inflammatory cytokines. BF, blister fluid; DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; SD, standard deviation.

transduced ADSC with plasmids encoding human CXCR1 and CXCR2 and showed robust expression of receptors on the surface of up to 70% of cells (Supplementary Figure S2 online). Then, CXCR1⁺ and CXCR2⁺ cells were used for in vitro migration assay in Transwell chambers with EB-derived BF or culture media (control). More than 30% of CXCR1⁺ cells migrated to the lower chamber in response to BF (Figure 6a, 6b). CXCR2-driven migration was more pronounced and BF concentration-dependent: more than 70% of CXCR2⁺ ADSC migrated to the lower chamber in response to BF-derived chemokines (Figure 6a, 6b).

Directional migration of the ADSC was further validated by the in vitro migration assay in collagenous matrix where stem cells and BF-containing collagenous layers were separated by

at least 5-mm-thick collagenous matrix. Forced expression of CXCR1 and CXCR2 led to a directional migration of the ADSC toward the BF-containing layer throughout the collagenous matrix against gravity (Figure 6c). No substantial migration of the cells was detected in the absence of BF (not shown) or when unselected ADSC were used (Figure 6c). Taken together, these studies showed that CXCR1 and CXCR2 chemokine receptors mediate directional migration of ADSC toward chemotactic gradients provided by BF-derived chemokines.

DISCUSSION

Chemokines play an important role in wound healing by orchestrating an intricate sequence of cellular events that are

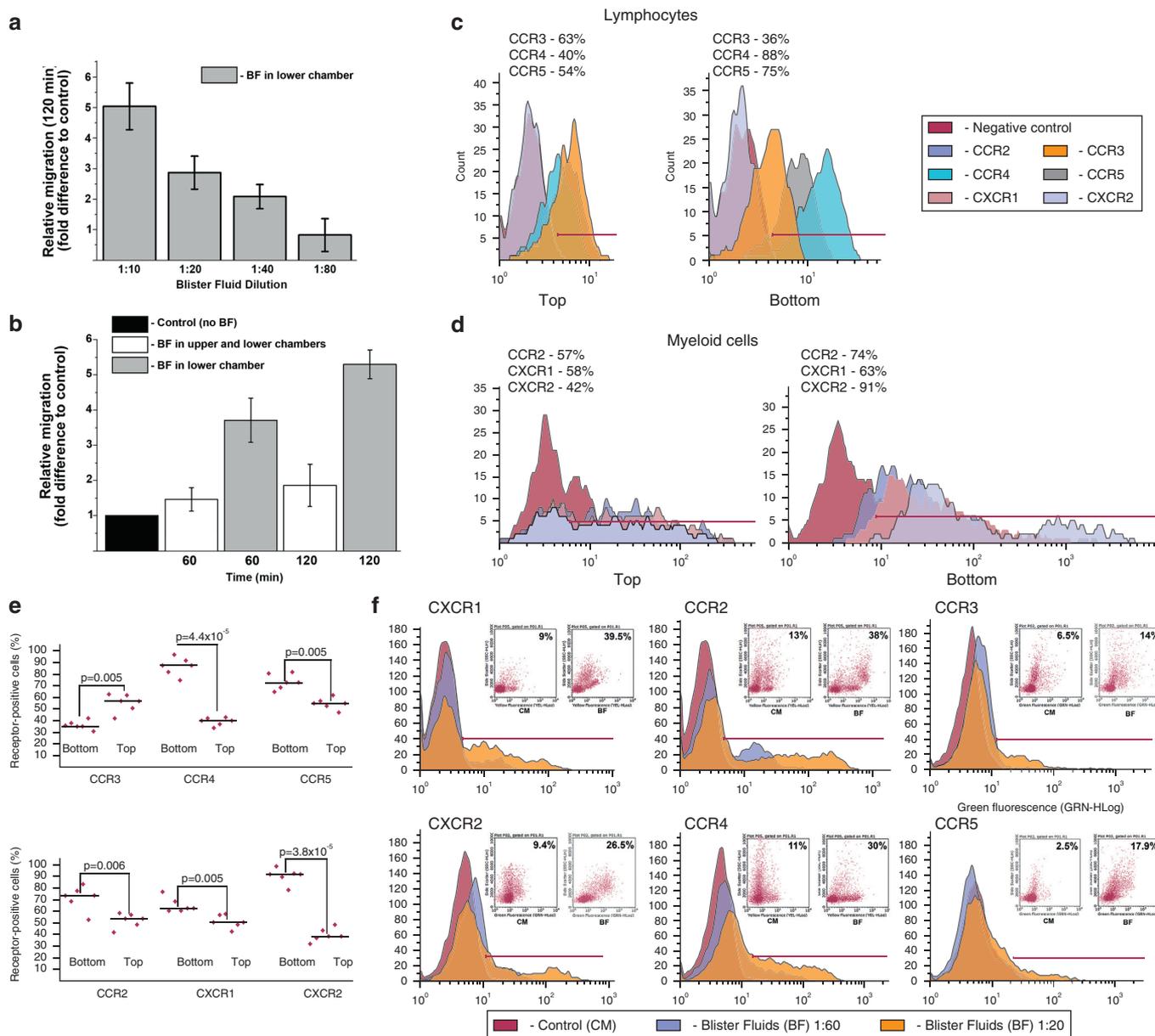


Figure 3. EB blister fluid-derived chemokines affect leukocyte migration in vitro. (a) Concentration and (b) time-dependent BF-induced leukocyte migration in Transwell migration chambers. Dilution of BF and time indicated below the columns. Conditions are shown in the panel insets. The data were collected from three independent experiments in triplicate and presented as migration (fold difference) relative to control (culture media in both chambers) \pm SD. (c, d) FACS-based analysis of selected chemokine receptors on BF-responding (c) lymphocytes and (d) myeloid cells. Representative profiles illustrate chemokine receptor-positive cells retained in the top chamber (Top) or migrated to the lower chamber and the underside (Bottom) of the Transwell migration chamber within 60 minutes. Detected chemokine receptors are shown in the insets. The percentage of receptor-positive cells is shown above the profiles. (e) Scatter plots showing the percentage and median of chemokine receptor-positive translocated (Bottom) and sessile (Top) cells depicted in panels (c) and (d). Statistical significance (*P* value) is shown above the plots. (f) Short-term analysis of selected chemokine receptor-positive populations migrated in Transwell chambers in response to BF at 1:20 and 1:60 dilutions (as indicated) within 30 minutes. Detected chemokine receptors are shown above representative histograms. The insets show the distribution of receptor-positive populations (side scatter/fluorescence) exposed to culture media (CM) and blister fluid (BF). The percentage of migrated chemokine-receptor-positive population is shown in the insets. Histogram markers define a threshold of chemokine-receptor positive cells. BF, blister fluid; EB, epidermolysis bullosa; SD, standard deviation.

divided into inflammatory, proliferative, and remodeling phases. The function of certain chemokines in wound healing (e.g., CXCL8) is well characterized, whereas information about others is only now emerging (Hasegawa et al., 2013; Kroeze et al., 2009, 2012; van den Broek et al., 2014). There is still a substantial gap in understanding the role of chemokines in the healing of trauma-induced skin blisters and particularly in hereditary blistering disorders. A better

understanding of these molecules in EB could provide opportunities for new therapeutic approaches aimed at reducing blister formation and improving cell-based therapies.

To date, several studies on EB patient-derived material have demonstrated increased levels of IL-1 β and IL-6 in plasma and CXCL8 (IL-8) in BF of patients with DEB and EBS. Increased levels of CCL2, CCL19, and CCL20 were observed

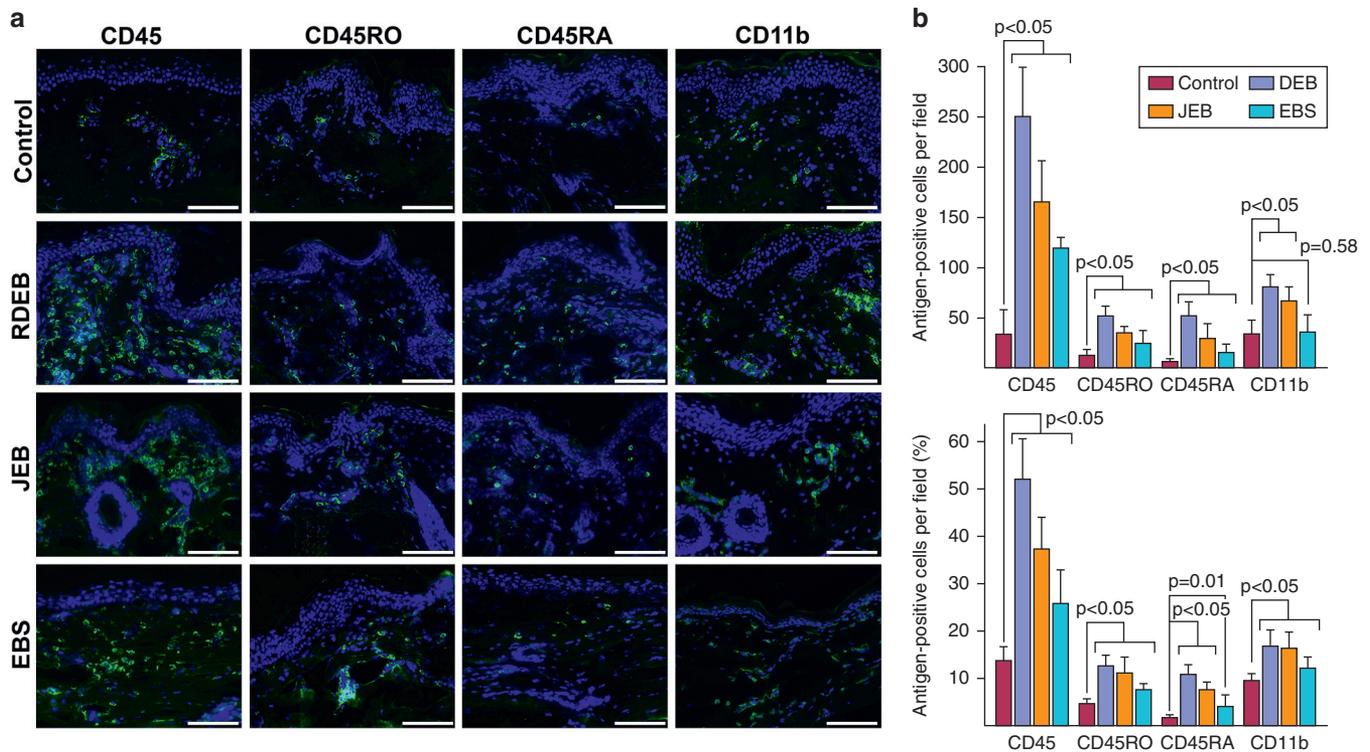


Figure 4. EB-affected skin is infiltrated with T lymphocytes and myeloid cells. (a) Immunophenotyping of infiltrating leukocytes in normal (control) and nonblistering EB-affected skin using CD45, CD45RO, CD45RA, and CD11b antibodies (green). EB types appear to the left of the micrographs. Detected antigens appear on the top. Blue: DAPI nuclei staining. Scale bar = 100 μ m. (b) Quantitation of skin-infiltrating CD45RO⁺ and CD45RA⁺ T cells and CD11b⁺ myeloid cells. Analysis was performed on five random sections from three independent biopsies of control and EB-affected skin. Data are presented as the number and percentage of antigen-positive cells per microscopic field \pm SD. Infiltration was compared using *t*-test. *P* value \leq 0.05 was considered statistically significant. DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; RDEB, recessive DEB; SD, standard deviation.

in the skin of EBS mice (Annicchiarico et al., 2015; Heinemann et al., 2011; Lettner et al., 2013; Roth et al., 2009). Our prior studies also identified reduced transcription of CCL27, CSF3, CX3CL1, and CXCL14 and higher expression of CXCL18 in the nonblistering skin of JEB and DEB transgenic mice (Alexeev et al., 2013). Proteomic screens of chemotactic molecules in BF of DEB blistering mouse skin showed consistently high levels of CCL6, chemerin, CCL8, CCL9/CCL10, CCL12, CXCL1, CXCL2, CXCL12, CXCL5, and CDF (Alexeev et al., 2016). As presented here, the analysis of human BF showed a somewhat similar pattern. High levels of the CXC chemokines containing Glu-Leu-Arg (ELR) motif (Gro family, CXCL5, CXCL7, and CXCL8) were detected in early blisters. In acute wound healing, these chemokines are responsible for the first step of the inflammatory phase, rapid recruitment of neutrophils (Van Damme et al., 1997), which begins within minutes to hours of wounding and typically lasts for 4 days (Romagnani et al., 2004). Contrary to the acute wound healing, advanced blisters and EB-affected skin contained high levels of the ELR chemokines (with the exception of CXCL5) and showed persistent infiltration with neutrophils. These observations suggest an abnormal initiation of blister healing.

The initial stage of the inflammatory phase of wound healing ends with the disappearance of neutrophils from the wound site through apoptosis, followed by the recruitment of myeloid cells through CCL2, CCL3, CCL4, CCL5, and CCL7

chemokines during the first week after wounding (Engelhardt et al., 1998; Jackman et al., 2000; Wetzler et al., 2000). Consistent with these data, we also detected higher levels of CCL5, CCL2, and CCL4 in advanced blisters compared with early blisters. Similar to chronic wounds (Schwarz et al., 2013), persistently high levels of these chemokines could be disadvantageous for blister healing.

Because ELR chemokines, particularly CXCL8, act as stimulators of neoangiogenesis and re-epithelialization (Engelhardt et al., 1998), it is plausible that consistently high levels of these chemokines accompanied by high level of angiogenin in BF may lead to unbalanced blister healing and development of chronic wounds in patients with EB. Angiogenin possesses both ribonuclease and angiogenic activities (Gao and Xu, 2008), and it hydrolyzes RNA and modulates protein synthesis. In addition, it binds to a 42 kDa smooth muscle-type alpha-actin to activate proteolytic cascades leading to degradation of the laminin and fibronectin layers of the basement membrane (Tello-Montoliu et al., 2006) and allowing endothelial cells to penetrate perivascular tissue. Although we did not find any significant differences in angiogenin level in different forms of EB by ELISA (Supplementary Figure S3 online), it is plausible that a consistently high level in BF is one of the precipitating factors in DEB- and JEB-associated squamous cell carcinoma development and, along with ELR chemokines, in the development of chronic wounds.

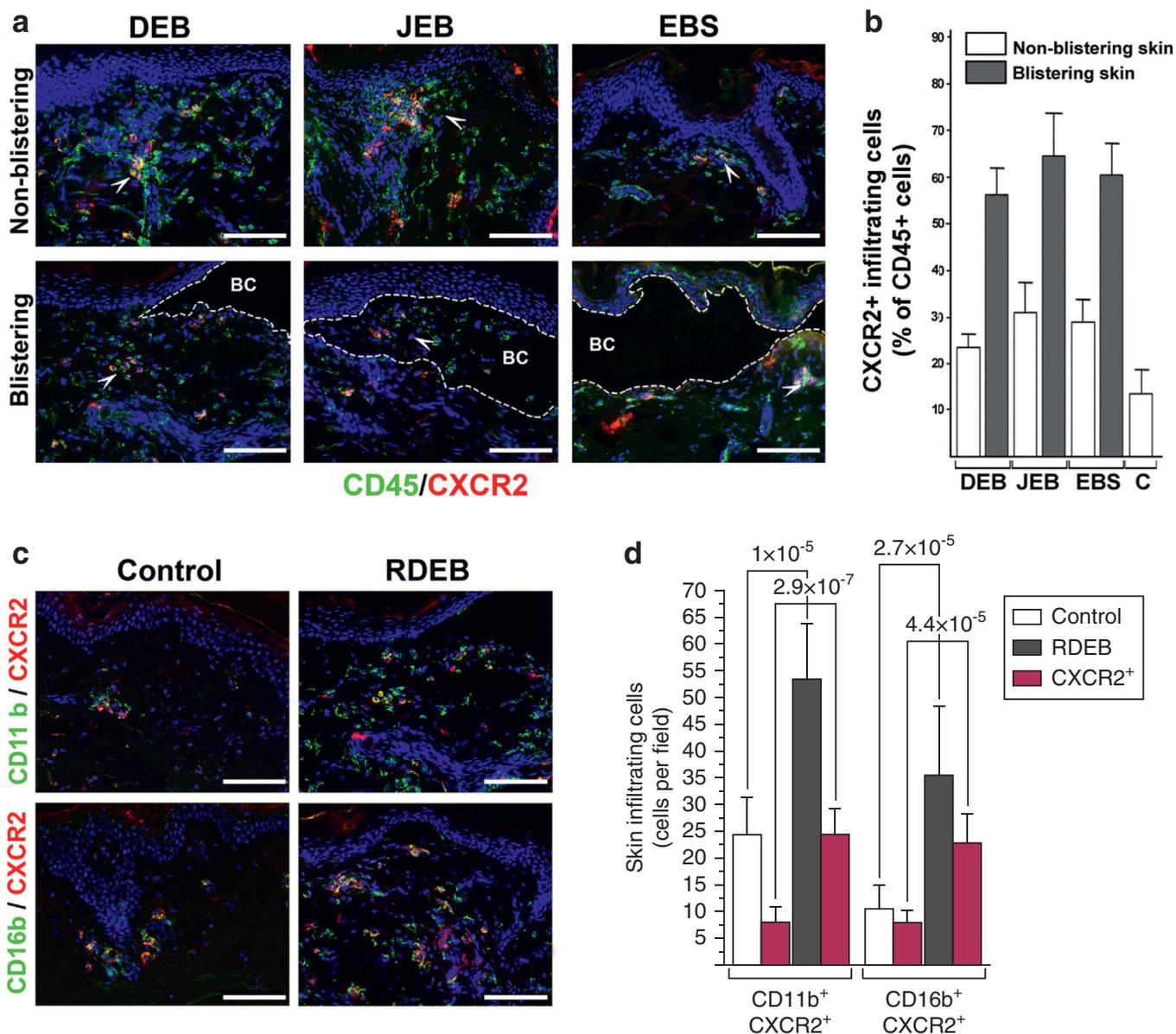
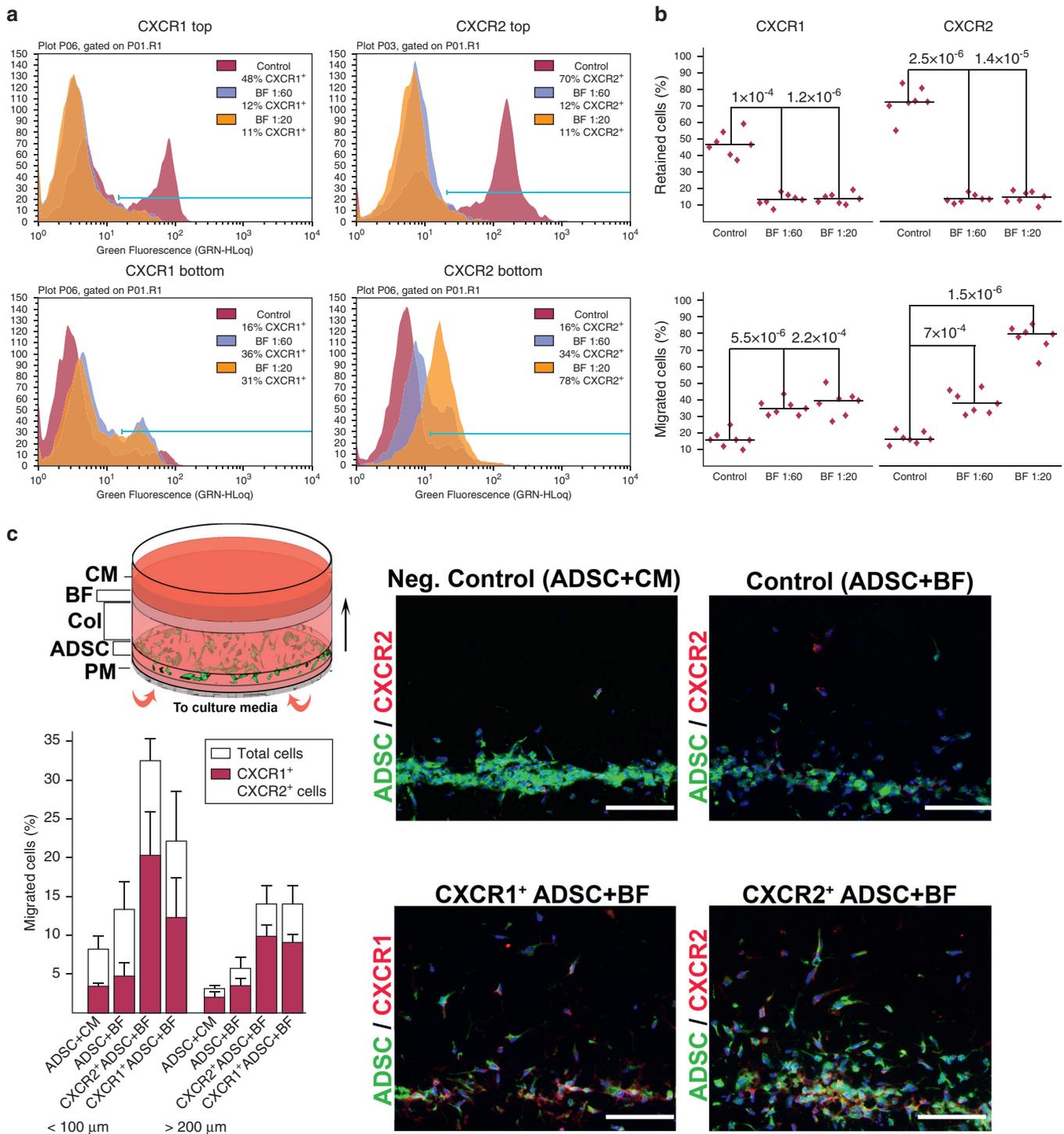


Figure 5. EB-affected skin is infiltrated with CXCR2⁺ leukocytes. (a) Infiltration of the DEB-, JEB-, and EBS-affected blistering and nonblistering skin with CD45⁺ and CXCR2⁺ leukocytes. EB types are indicated above representative images. Detected antigens (in corresponding color) are shown at the bottom of the panel. The blister cavity (BC) is outlined by a dotted line. White arrowheads point to representative groups of CD45⁺ CXCR2⁺ leukocytes. (b) Quantitation of lymphocytic infiltrate in control (C) and EB-affected skin was performed on 10 random microscopic fields of blistering and nonblistering skin for each EB type, averaged and presented as a percentage of CD45⁺ cells per microscopic field ± SD. Skin phenotype is shown in the insets. All samples achieved statistical significance when compared with control skin ($P < 0.05$). (c) Characterization of skin-infiltrating CXCR2⁺ cells in control and RDEB-affected skin. Detected antigens (in respective colors) are shown to the left of the panels. On all representative images: Blue: DAPI nuclear staining; scale bar = 100 μm. Single channel images are presented in [Supplementary Figures S4, S5](#) online. (d) Quantitation of CXCR2⁺-infiltrating leukocytes in control and RDEB-affected skin (shown in the insets). The data are presented as a mean number of antigen-positive cells (shown below the columns) per microscopic field ± SD. Statistical significance (P value) is shown above the columns. DEB, dystrophic epidermolysis bullosa; EB, epidermolysis bullosa; EBS, epidermolysis bullosa simplex; JEB, junctional epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; SD, standard deviation.

In the remodeling phase of wound healing, chemokines could be responsible for excessive degradation of extracellular matrix through the induction of matrix metalloproteinase (MMP) expression by skin resident cells and through the sustained recruitment of neutrophils known to secrete various MMPs.

It is particularly evident in chronic wounds exhibiting neutrophil-dependent impairment in re-epithelialization and increased extracellular matrix degradation ([Dovi et al., 2003](#);

[Martin et al., 2003](#)). Neutrophil-derived proteases such as cathepsin G, elastase, and protease 3 cleave elastin, fibronectin, laminin, vitronectin, and collagen IV ([Witko-Sarsat et al., 2000](#)), and they interfere with re-epithelialization and the formation of a new BMZ in the dermoepidermal junction ([Briggaman et al., 1984](#)). Neutrophil-secreted MMPs (MMP8 [collagenase-2], MMP9 [gelatinase B], and MMP2 [gelatinase A]) also interfere with BMZ formation by cleaving fibrillar collagens (MMP8) and type IV collagen



(MMP2 and 9). This is underscored by high levels of active MMP8 and high gelatinase activity in chronic wounds (Bullen et al., 1995; Moor et al., 2009; Nwomeh et al., 1998, 1999; Wysocki et al., 1993). Also, these MMPs can cleave and activate CXCL5 and CXCL8 (Tester et al., 2007) and process CCL2 to generate CCR1, 2, and 3 antagonists that inhibit leukocyte recruitment (McQuibban et al., 2002). Although our analysis demonstrated that several cytokines and chemokines could be associated with EB severity, prognostic value of these molecules requires further biostatistical evaluation and modeling in a larger cohort of patients.

Our analysis also revealed a high level of adiponectin (ACRP30) in EB-derived BF. This protein hormone modulates several metabolic processes, including glucose regulation and fatty acid oxidation (Diez and Iglesias, 2003). Because adiponectin level correlates with body fat percentage (Ukkola and Santaniemi, 2002), its presence in BF may also have a prognostic/evaluative value for patients with EB, particularly children, who could be chronically malnourished with signs of subcutaneous fat depletion (Gruskay, 1988).

The analysis presented is of particular importance to the development of cell-based therapies for the treatment and management of EB. Recent clinical trials demonstrated that allotransplantation of bone marrow-derived stem cells into patient with DEB alleviated some disease-associated symptoms attributed to paracrine effects rather than donation of the therapeutic protein by donor cells to the BMZ (Nagy et al., 2011; Petrof et al., 2015; Wagner et al., 2010). In fact, the inefficient recruitment of the adult stem cells to damaged skin has been documented in wound healing studies (Ojeh et al., 2015; Shingyochi et al., 2015). Previously, we analyzed BF in mouse models mimicking DEB (Fritsch et al., 2008; Heinonen et al., 1999) and identified several potential chemotactic axes for the enhanced recruitment of therapeutic stem cells to blistering mouse skin (Alexeev et al., 2016). Although a side-by-side comparison of chemokines in mouse and human BF showed substantial differences, it is apparent that BF of both species contain high levels of CXCR1 and CXCR2 ligands and these chemokine receptors could be harnessed to drive migration of the therapeutic stem cells to blistering skin. Our current analysis of BF-induced human stem cell migration in vitro is consistent with this notion. Proteome data are also supportive of the recent findings showing elevated CXCL12 levels in serum of EB-affected patients (Ujii et al., 2017). Yet, the CXCL12-CXCR4 chemotactic axis was mostly associated with dissemination of cancers to the lymph node, bone, lung, brain, and liver (Sun et al., 2010; Teicher and Fricker, 2010). In the skin, CXCL12 expression was associated with skin-infiltrating dendritic cells, pericytes, endothelial cells, and fibroblasts (Avniel et al., 2006; Rezk et al., 2017), as well as with emigration of dendritic cells from the cutaneous tissue (Kabashima et al., 2007). In fact, data presented by Ujii et al. also demonstrate that CXCL12 expression in EB-affected skin is mostly associated with a skin infiltrating cell. Therefore, the role of CXCL12 in the recruitment of cells to the skin remains to be further elucidated.

As discussed previously (Alexeev et al., 2013, 2016), migration of cells to peripheral tissues is a two-step process

requiring (i) extravasation of cells from the blood stream, and (ii) migration of cells within the peripheral tissue (skin). Usually, separate sets of chemokines regulate these two processes. Extravasation of memory T cells from dermal blood vessels is mediated by CCR4 ligands (CCL17, CCL22, and CCL5) (Tubo et al., 2011), whereas migration of cells within the tissue requires activation of other receptors (e.g., CCR10) by epithelia-derived CCL27 (Alexeev et al., 2013). Separation of extravasation and in-tissue migration chemotactic signals helps restrict migration of circulating cells to the injured sites. Only few chemokines, including CXCL8, can mediate both processes by inducing rolling, diapedesis, and intracutaneous migration (Kohda et al., 2002). Taking into account the high levels of CXCL7 (another CXCR2 ligand) in the plasma of EB-affected patients, it is also plausible that CXCL7 activates rolling of CXCR2⁺ cells, whereas other CXCR2 ligands mediate intracutaneous migration. These findings, along with our recent animal studies (Alexeev et al., 2016), strongly support the hypothesis that a CXCR2⁺ population of stem cells could be effectively recruited to EB-affected skin and is advantageous for cell-based EB therapy via systemic transplantation.

Collectively, a robust analysis of BF- and plasma-associated chemokines and cytokines revealed a number of EB skin-associated chemotactic gradients, which may contribute to EB pathology by altering recruitment of leukocytes and creating a favorable milieu for the progression of the skin blisters to nonhealing wounds. This study also revealed several chemotactic axes involving CXCR1/2 and CCR4 ligands that could be further exploited for the effective recruitment and homing of the therapeutic stem cells to EB-affected skin and the improvement of the cells-based therapies.

MATERIALS AND METHODS

BF and skin from patients with EB

BF, blood, and skin biopsies from patients with EB were obtained with support from Dystrophic Epidermolysis Bullosa Research Association Chile and Dystrophic Epidermolysis Bullosa Research Association Mexico according to ethical committee rules and individual country's regulations. Clinical features of the patients are presented in [Supplementary Table S1](#). The study was not considered as human subject research.

Chemokine and cytokine antibody arrays

Human Chemokine Antibody Arrays C1 and Human Cytokine Antibody Array C1000 (RayBiotech, Norcross, GA) were used to detect chemokines and cytokines in plasma and BF according to manufacturer protocols. The analysis of the obtained data was performed with RayBiotech Analysis Tools software.

ELISA assay

Multi-Analyte (Qiagen, Valencia, CA) and Angiogenin (RayBiotech) ELISA assays were used to detect most common chemokines and angiogenin as devised by manufacturers.

Cell culture

ADSC were isolated from human foreskin and characterized and cultured according to a previously established protocol (Alexeev et al., 2014). PBMC were purchased from Stem Cell Technologies (Cambridge, MA) and cultured in RPMI1640 media supplemented with 10% FBS (Thermo/Fisher, Grand Island, NY).

FACS analysis

Cells were incubated with fluorochrome-conjugated chemokine receptor-specific antibodies (BioLegend, San Diego, CA; [Supplementary Table S2](#) online) (Alexeev et al., 2014). Expression of chemokine receptors was assessed on the Guava EasyCyte system and analyzed using GuavaSoft 2.7 software (Millipore, Billerica, MA). Gating of different leukocyte populations was performed based on the assessment of side scatter versus forward scatter.

Histological and immunofluorescence analyses

Frozen tissues were cryosectioned and 7 μm sections were fixed in 4% paraformaldehyde and used for indirect immunofluorescence analysis using antigen-specific primary antibodies ([Supplementary Table S2](#)). Immunocomplexes were detected with fluorochrome-labeled secondary antibodies (Thermo/Fisher). Slides were covered with Fluorosafe reagent (Millipore) and analyzed by fluorescent microscopy.

Nucleofection

Nucleofection of plasmids was performed using the Amaxa Nucleofector II system (Lonza, Basel, Switzerland) with Kit V and T-027 program.

In vitro migration in Transwell chambers and collagenous matrix

PBMC (3×10^6 cells) or ADSC (1×10^6 cells) were loaded into the top chamber of the 8- μm pore size Transwell chambers (Corning Costar, Scranton, PA). Control or BF-containing culture media was placed into a lower chamber. Migrated cells were collected from the lower chambers and the undersides at indicated times, enumerated and used for FACS analysis. For in-matrix migration, ADSC were fluorescently labeled with Vibrant DiO dye (Thermo/Fisher) and plated in collagen gel on the bottom of the Transwell chamber. A separating collagenous layer was created on top. The migration unit was completed by creating BF-containing collagen gel on the top. The migration unit was placed into the Transwell plate and incubated for 4 days under a standard tissue culture condition. After fixation in formalin and freezing, 10 μm cryosections were used for analysis.

More detailed information is available as [Supplementary Materials and Methods](#) online.

Statistical analysis

Comparison of the data was performed using Student's two-tailed *t*-test; $P < 0.05$ was considered statistically significant.

A more detailed description of experimental procedures is given in the [Supplementary Materials and Methods](#).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <http://dx.doi.org/10.1016/j.jid.2017.07.002>.

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