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14. ABSTRACT

Hereditary Epidermolysis Bullosa (EB) is a mechanobullous disease characterized by the fragility of the skin. Separation of skin layers, a hallmark of the disease, leads to the development of skin lesions ranging from skin blisters to chronic ulcerated wounds, which represent the major health-related burden for EB patients as these lesions are associated with infection, sepsis, dehydration, deformities, and cancer. Our previous findings along with several clinical observations strongly suggest that detailed characterization of molecular and cellular events impairing wound healing is crucial to improve stem cell-based and combination treatment modalities. The mechanisms that control the fate of the EB wounds remain poorly understood. Our overarching hypothesis is that an altered secretome along with microbial infection at EB wounds lead to the establishment of pro-inflammatory feedback loops and inhibition of wound healing and that targeting of chronic skin lesions with pharmacological treatments can restore aberrant wound healing and skin integrity. These questions were tested by investigating molecular and cellular mechanisms underlying unresolved wound healing, characterizing microbial dynamics and immunogenicity of the wound colonizing bacteria, and developing pharmacological approaches for EB treatment and management. By using samples from three major EB types, we identified type-specific changes in wound healing. We also delineated cellular populations and pathways preventing wound healing that were amenable for therapy development. In conjunction with state-of-the-art microbiome sampling and bio-informatics, our studies accelerated understanding of the differences between chronic and healing wounds. Conducted analysis of wound progression helped to define precipitating factors that predefine EB wound aberrations. Completion of the proposed studies provided mechanistic information about dynamic changes in molecular and cellular interaction and microbiota communities during progression and healing of EB skin wounds – data critical to tailor EB treatments and development of novel therapeutic intervention strategies to accelerate wound healing, reduce inflammation, and, possibly, fibrosis. Our completed studies thus have potential to significantly impact EB treatment and management.

15. SUBJECT TERMS
Epidermolysis Bullosa, wound healing, microbiome

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1. INTRODUCTION

Epidermolysis bullosa (EB), a heterogeneous group of mechanobullous disorders, is characterized by fragility of the skin. Tissue separation manifesting as blistering of the skin and mucous membranes in different variants of EB takes place at the level of the cutaneous basement membrane zone (BMZ) at the dermal-epidermal junction (DEJ). Despite extensive studies of EB genetics and testing various therapeutic approaches to cure the disease, only palliative care revolving around judicious use of protective garments, bandages, and antibiotic creams is available to EB patients. Separation of skin layers following minor trauma to the skin is a hallmark of the disease. It leads to the development of blisters, erosions and non-healing wounds, which are associated with numerous complications including infection, sepsis, dehydration, deformities, and cancer. Although many EB skin blisters and erosions progress to skin wound, at present, there is no objective measure to predict whether a wound will heal or become chronic. Our previous findings indicate that blistering and wounded EB skin is characterized by an excessive production of several pro-inflammatory chemokines and deregulated recruitment of the leukocytes, particularly neutrophils, to the damaged skin. This is accompanied by the activation of adaptive and innate immunity triggered by wound colonizing bacteria and release of extracellular matrix (ECM) remodeling enzymes from the recruited neutrophils. In turn, secreted enzymes in blister fluids and wounds continuously degrade ECM and generate ECM-derived damage associated molecular patterns (DAMPs) that activate toll-like receptors (TLRs) on fibrocytes, and create a pro-inflammatory feedback loop. In case of a skin injury, when physical segregation between the host and microbiota is destroyed, the immune system could be overwhelmed by the volatile situation - when pathogens and commensals share the same inflamed environment and may negatively affect wound heling, particularly in EB-affected skin compromised by the abnormal DEJ. As adequate T cell responses are essential for immune-mediated protection against microbiota, we suggest that a high number of activated T cells at wound sites can either exhaust or establish tolerance toward wound-colonizing bacteria. Together, these events create a favorable milieu for the persistent inflammation, excessive digestion of the ECM, abrogation of keratinocyte motility, wound re-epithelization, and fibrosis. To date, no study has investigated these dynamics in EB blisters and wounds. Our current studies are designed to assess the complexity of the molecular and cellular interactions influencing the development of non-healing wounds on EB genetic background, and to delineate therapeutic intervention approaches. Specifically, we suggest: (1) to examined changes occurring in secretome and cellular infiltrates during wound progression and characterize contribution of specific cell types to the establishment of chronic wounds; (2) to analyze the dynamics of bacterial and fungal communities during wound progression and healing and defined microbial biomarkers predictive of the wound outcome; and (3) to test novel pharmacological treatment options to accelerate wound healing in chronic wounds *in vivo.*

2. KEYWORDS

BMZ - Basement Membrane Zone
CHOP – Children's Hospital of Philadelphia
CTL - Cytotoxic T Lymphocytes
DAMPs - Damage Associated Molecular Patterns
DEJ - Dermal-Epidermal Junction
DFU - Diabetic Foot Ulcers
EB - Epidermolysis Bullosa
ECM - Extracellular Matrix
ELISA - Enzyme-linked Immunosorbent Assay
FACS - Fluorescence Activated Cell Sorting
JEB - Junctional Epidermolysis Bullosa
HMW - High Molecular Weight
IFNγ - Interferon gamma
LMW - Low Molecular Weight
OUT - Operationale Taxonomic Unit
PCR- Polymerase Chain reaction

PD - Phylogenetic Distance
RDEB - Recessive Dystrophic Epidermolysis Bullosa
RDEB-SSC - RDEB-Associated Squamous Cell Carcinoma
TLRs - Toll-like Receptors

3. ACCOMPLISHMENTS
OVERAL PROJECT SUMMARY

Poorly healing wounds are one of the major complications in patients suffering from epidermolysis bullosa (EB). At present, there are no effective means to analyze changes in cellular and molecular networks occurring during EB wound progression to predict wound outcome and design betted wound management approaches. To better define mechanisms influencing EB wound progression by evaluating changes in molecular and cellular networks, in Specific Aim1,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 EB wounds were evaluated by FACS-based immuno-phenotyping and ELISA. Our cross-sectional and longitudinal analysis determined that progression of RDEB wounds to chronic state is associated with the accumulation (up to 90%) of $CD16^{+}CD66b^{+}$ mature neutrophils, loss of $CD11b^{+}CD68^{+}$ macrophages, and a significant increase (up to 50%) in a number of $CD11c^{+}CD80^{+}CD86^{+}$ activated professional antigen presenting cells (APC). It was also marked by changes in activated T cells populations including a reduction of $CD45RO^{+}$ peripheral memory T cells from 80% to 30% and an increase (up to 70%) in $CD45RA^{+}$ effector T cells. Significantly higher levels of MMP9, VEGF-A and cathepsin G were also associated with advancing of wounds to poorly healing state. 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 EB patients as associated with distinct changes in leukocytic infiltrates, matrix-remodeling enzymes, and pro-angiogenic factors at wound sites.

EB patients develop poorly healing skin wounds that are frequently colonized with microbiota. To evaluate the dynamics of the microbiota colonizing early, established, and chronic EB wounds, in Specific Aim 2, we conducted high throughput sequencing to define multiple metrics of the microbiome, including diversity, stability, and relative abundance of potential pathogens and identified mirobiomic features associated with disease clinical outcomes. We found that progression of RDEB wounds to chronic state is associated with reduced abundance of specific taxa and overall reduced diversity of bacterial communities. Specifically, it is characterized by disappearance of *Corynebacterium*, *Propionobacterium*, and several other taxa and accumulation of pre-dominantly *Staphylococcus* and *Pseudomonas* species in chronic wounds. Because T cells play an important role in clearing such pathogens, we defined the status of adaptive T cell-mediated immunity in wounds. We found that RDEB wounds and epithelial cells are most frequently infected with *Staphylococcus* sp. and *Pseudomonas* sp. The wound-associated T cells contain populations of $CD4^{+}$ and $CD8^{+}$ peripheral memory T cells that respond to soluble microbial antigens by proliferating and secreting interferon gamma (IFNγ). Also, $CD8^{+}$ cytotoxic T lymphocytes recognize *S. aureus*-infected RDEB keratinocytes and respond by producing interleukin-2 (IL-2) and IFNγ and degranulating and cytotoxically killing infected cells. Prolonged exposure of RDEB-derived T cells to microbial antigens *in vitro* does not trigger PD1-mediated T cell exhaustion but induces differentiation of the $CD4^{high}$ population into $CD4^{high}CD25^{+}FoxP3^{+}$ regulatory T cells. Collectively, our data demonstrated that adaptive T cell-mediated immunity could clear infected cells from wound sites, but these effects might be inhibited by PD1/Treg-mediated immuno-suppression.

Having obtained a detail characterization of early, established and chronic EB wounds, in Specific aim 3 using K14-Lamc2 conditional transgenic animals, we developed an inducible animal model suitable for analysis of wound healing in environmental milieu that mimics wounded human EB skin. Using this model, we confirmed that constitutive recruitment and accumulation of mature neutrophils at EB wound sites are detrimental for proper wound healing. We also showed that abrogation of the chemotactic recruitment of neutrophils with CXCR1/2 dual inhibitor, Ladarixin, facilitates wound re-epithelization and closure of the early and established wounds. Our data also demonstrated that activation of TLR-4 signaling at wound sites leads to the elevated

recruitment of neutrophils and stalled wound healing. Based on our data, we propose that this effect could be mediated by TLR-4-mediated NF-Kb signaling that is well known to up-regulate Gro-family chemokines and CXCL8 expression. Our animal studies showed that topical treatment of the wounds with TLR-4 inhibitor, TAK-242, enhances wound re-epithelization and closure. Collectively, our wound healing studies in the developed animal model that mimics human JEB showed that systemic inhibition of the CXCR1/2-mediated neutrophil/leukocyte recruitment to the wounds provides favorable outcome and accelerated wound healing. These studies also showed that topical application of TLR-4 inhibitors could also enhance healing of EB skin wounds.

KEY RESEARCH ACCOMPLISHEMNTS

Specific Aim 1. To conduct longitudinal analysis of secretome in epidermolysis bullosa (EB) healing and non-healing wounds and define the role of leukocytic and fibrocytic infiltrates.

Major Task 1: Cross-sectional and longitudinal evaluation of secretome in healing and non-healing EB wounds.

Subtask 1: **S**ample collection and evaluation of wounds in EB-affected patients. Timeline - 18 months (1-18).
Per SOW: Wound-dressing bandages will be collected during routine re-dressing of the wounds at Dr. Salas's clinique. Demographic data, EB type, age of the wound will be collected by attending physicians at Dr. Salas's clinique. At least 40 patients with each type of wound (fresh, established or chronic) will be used for cross-sectional analysis. At least 40-50 patients with each type of wound (fresh, established or chronic) will be used for longitudinal analysis with 8 to 12 repeated measures per wound during the observation period of about one year and a half. To rigorously investigate any potential sex bias, all experiments will be done in male and female cohorts.

Subtask 2: Analysis of secretome activity in early, established and chronic wounds. Timeline - 18 months (1-18).
Per SOW: Bandage transport media from wound dressings will be collected, clarified and concentrated for secretome analysis. Composition of EB secretomes will be evaluated by Multiplex ELISA assays. Secretome activity of various EB wounds on recruitment of various inflammatory cells will be evaluated using various FACS-based protocols.

Milestone(s) Achieved: A well-characterized analysis of secretome of EB-associated wounds. Acquisition of statistically significant data. All tasks are accomplished, data published.

Major Task 2: Cross-sectional and longitudinal analyses of cellular infiltrates in EB healing and non-healing wounds

Subtask 1: Characterization of cellular infiltrates at the sites of healing and non-healing EB skin wounds. Timeline - 18 months (1-18).
Per SOW: Wound-associated bandage-derived cells will be isolated concurrently with the secretome using cell-

Subtask 2: Analysis of neutrophils in EB wounds. Timeline - 6 months (18-24).
Per SOW: Neutrophil functionality in healing and non-healing EB wounds will include neutrophil morphology, apoptosis, phagocytic capacity, production of proteases and anti-microbial peptides using various assays. specific techniques. Evaluation of wound-associated cell populations will be done using FACS-based protocols.

Subtask 3: Assessment of fibrocytes at wound sites. Timeline - 6 months (18-24).
Per SOW: Fibrocyte dynamics in healing and non-heling wounds will be characterized by FACS using sub-type specific markers; fibrocyte-secreted pro-inflammatory mediators will be evaluated by Multiplex-based multi-analyte ELISAs; TLR4 activation by fibrocyte-derived DAMPs will be analyzed by Western blot, RT-PCR and ELISA assays.

Milestone(s) Achieved: A well-characterized analysis of cellular infiltrates of EB-associated wounds. Acquisition of statistically significant data. All tasks are accomplished, data published.

Specific Aim 1 Major Milestones: We tested whether wound-covering dressings could be used for a non-invasive recovery of both cellular and molecular constituents of the EB wounds. Our approach allowed 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 $CD11b^+$ myeloid cells. However, most of $CD11b^+$ cells, particularly in chronic wounds, were identified as $CD16^+CD66b^+$ 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, their accumulation at the 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 wounds suggest that these wounds could be similar to the lesions of other neutrophilic dermatoses, such as pyoderma gangrenosum (PG). Although further comparative evaluation of soluble factors affecting neutrophilic infiltrates in EB and PG are required, it is plausible that treatments with anakinra or canakinumab that were successful in treating PG or topical steroids could be used for management and treatment of chronic EB wounds. Based on recent animal data showing partly impaired macrophage response in DEB mice and our data showing a low percentage of $CD11b^+CD68^+$ macrophages in all types of EB 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 $CD66b^+$ neutrophils in chronic wounds may also indicate that neutrophils have reduced ability to clear bacterial infection. Although our current analysis did not show any significant changes in receptor expression on $CD66b^+$ neutrophils in different wound types, it is possible that relatively low percentage (up to 30%) of $C5aR1^+CD66b^+$ cells could implicate on neutrophil's functional activity. Independently of neutrophil's functional status, our data demonstrated that constitutive recruitment of myeloid cells and neutrophils to 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 lesions. Although the source of these ligands in early and chronic wounds remains undefined, it is reasonable that systemic or localized blocking of CCR2 and/or CXCR2 receptors using small molecule inhibitors could reduce neutrophil-associated detrimental effects and accelerate wound closure.

An accumulation of neutrophils in established and chronic 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. An imbalance in MMP9 and TIMP-1 proteins results in excessive ECM degradation and higher MMP9/TIMP-1 ratio defines poor wound healing. Our data showing that ratio in early EB wounds is compatible with that in sever neuropathic DFU 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 remained compatible with unhealed DFU. 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 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 $CD80^+CD86^+$ DC in chronic wounds with both markers expressed on up to 50% of all $CD11c^+$ 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 $CD4^+$ T cells, CD86 could be more important for initiating T-cell responses, while CD80 could be more significant for their maintenance. Considering a frequent colonization of the RDEB wounds with bacteria and fungi, 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 ($CD45RA^{-}CD45RO^{+}$) and naïve ($CD45RA^{+}CD45RO^{-}$) T cells at early wounds and accumulation of $CD45RA^{+}$-effector T cells in chronic wounds. Considering an increased percentage of $CCR4^{+}$ T cells in advanced wounds and an important role of this receptor in skin-homing, it is plausible that CCR4 mediates recruitment of T cells to the wounds.

It is well-established that EB wounds are prone to fibrotic scarring in which TGF-β1 plays a major role. However, pathogenesis of hypertrophic 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-β1, and VEGF-A), we found that IL-6 is present at a relatively high level in EB 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, and that IL-6 levels are higher in human diabetic chronic wounds than in healing wounds. Considering animal data showing that IL-6 supplementation is associated with enhanced leukocyte recruitment, collagen production and angiogenesis, it is possible that high IL-6 levels in EB wounds create a favorable milieu for poor wound healing. Additional studies will be necessary to evaluate the role of this cytokine in EB pathology.

High levels of pro-antigenic CXCL8 in EB blister fluids, elevated VEGF-A level in chronic wounds, and high vascularization of the intact EB skin suggest that there is excessive angiogenesis in wounded EB skin. This characteristic sets EB 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, it is plausible that angiogenesis contributes pathogenic scarring of EB 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.

In summary, presented cross-sectional/longitudinal 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 EB skin and wounds may support inflammation and negatively affect wound healing

Specific Aim 2. To characterize microbial dynamics and immunogenicity of the wound colonizing bacteria.

Major Task 3: Analysis of bacterial microbiome in new, established and chronic wounds.
Subtask 1: Sample collection. Timeline - 12 months (1-16).
Per SOW: Wound swabs will be collected concurrently with wound-dressing bandages during routine re-dressing of the wounds at Dr. Salas's clinique using the Levine non-invasive technique. Demographic data, EB type, age of the wound/blisters will be collected by attending physicians at Dr. Salas's clinique. At least 40-50 patients with each type of wound (fresh, established or chronic) will be used for bacterial microbiome studies.

Subtask 2: Sample processing and analysis. Timeline - 12 months (13-24).
Per SOW: DNA isolation and sequencing using the Illumina MiSeq platform will be done at the CHOP microbiome Center as a service. Data analysis will be done using R Statistical Package as well as non-parametric Wilcoxon rank-sum tests, Spearman correlations and Kruskal-Wallis tests for all computations.

Milestone(s) Achieved: A well-characterized analysis of bacterial communities of EB-associated wounds. Acquisition of statistically significant data. All tasks are accomplished, data published.

Major Task 4: Analysis of fungal microbiome in new, established and chronic wounds.
Subtask 1: Sample collection. Timeline - 12 months (1-12).

Per SOW: Samples collected for microbiome studies will also be used for fugal assessment. At least 40-50 patients with each type of wound (fresh, established or chronic) will be used for fungal studies.

Subtask 2: Sample processing and analysis. Timeline - 12 months (13-24).
Per SOW: Partly, DNA extracted for microbiome assessment will be used for fungal mycobiome analysis. The fungal component will be sequenced using the Illumina MiSeq platform at CHOP microbiome Center as a service. The Shannon diversity index, Simpson diversity index (1-Dominance), Faith's phylogenetic distance (PD), and number of observed species (richness) will be calculated using the QIIME 1.8.0 alpha_diversity.py script.

Milestone(s) Achieved: A well-characterized analysis of fungi communities of EB-associated wounds. Acquisition of statistically significant data. All tasks are accomplished, data published.

Major Task 5: Analysis of microbiota immunogenicity and adaptive T cell immunity at wound site.
Subtask 1: Assessment of microbiota-specific T cells in early, established and chronic wounds.
Timeline – 12 month (13-18).
Per SOW: Functional profile of bacteria-specific T cells will be evaluated by Multiplex ELISA assays.

Subtask 2: Analysis of the microbiota-induced immunosuppression. Timeline -12 (18-24).
Per SOW: Differentiation and activation of the bandage-derived T cells at different stages of wound progression will be assessed by FACS. Cytokine production and secretion by activated T cells will be analyzed by ELISA and ELISpot assays.

Milestone(s) Achieved: A well-characterized role of T cell immunity in controlling microbiota in RDEB-associated wounds. All tasks are accomplished, data published.

Specific Aim 2 Major Milestones: Completion of this aim allowed us to characterize bacterial and fungi communities in EB wounds, trace changes within these communities during wound progression, delineate potential microbiota biomarkers predictive of wound outcomes, defining the role of T cell immunity in controlling microbiota in chronic wounds, and establish the basis for the re-activation of the adaptive immune responses to bacterial and fungal infection.

Microbial burden plays an important role in impaired healing and development of infection-related complications in EB. To evaluate the dynamics of the microbiota colonizing early, established, and chronic EB wounds, we conducted high throughput sequencing to define multiple metrics of the microbiome, including diversity, stability, and relative abundance of potential pathogens and identified mirobiomic features associated with clinical outcomes. EB microbiomes were determined by sequencing of hypervariable regions VI through V3 of the 16S ribosomal RNA (rRNA) gene. We found that progression of wounds to chronic state is associated with reduced abundance of specific taxa and overall reduced diversity of bacterial communities. Specifically, it is characterized by disappearance of *Corynebacterium*, *Propionobacterium*, and several other taxa and accumulation of pre-dominantly *Staphylococcus* and *Pseudomonas* species in chronic wounds. PCR-based analysis of the most common EB wound contaminants showed that *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Pseudomonas aeruginosa* are the most common wound contaminants in lesions. Respectively, these species and, particularly *Staphylococcus sp.,* could be the primary targets for therapeutic intervention. It is expected that a more effective adaptive immunity may reduce microbiota burden and the involvement of innate immunity, such as neutrophils. Also, this data may point to EB-specific biomarkers and offer alternative treatments such as probiotics to restore bacterial community dynamics instead of the lone antibiotics. In the analysis of fungal communities, we acquired tools to bin taxa into "pathogens" and "allergens" categories because most of filamentous fungi often identifies as common allergenic molds.

The role of innate and adaptive immunity in controlling microbiota in EB-associated wounds remains incompletely understood. Recently, animal studies demonstrated that poor control of infection in RDEB wounds could be associated with altered macrophage activity due to lower levels of cochlin in the circulation. However, it is plausible that inaptitude of the immune system to control wound infection could be caused by other post-infectious immunopathology in EB skin. We previously demonstrated that blister formation coincides with

increased levels of CXCL2, CCL2, CCL4, and CCL5 and with elevated recruitment of granulocytes and T cells. Furthermore, we showed in Specific Aim 1that progression of wounds to an established and chronic state is accompanied by reduction of macrophages, continuous recruitment of mature neutrophils, accumulation of antigen-experience APC, and differentiation of peripheral memory T cells into effector T cells. Based on our current knowledge, we proposed that wound-associated APC play a role in the acquisition of microbial antigens, T cell priming, and activation as it was shown for naïve T cells that differentiate into effector and memory T cell pools and support B cell-mediated antibody response and innate immunity to clear bacterial contamination. Obtained data showed that wounds contain antigen-experienced $CD4^+$ T cells that proliferate and secrete IFNγ, a potent activator of polymorphonuclear neutrophils (PMN) in response to microbial antigens. Higher number of IFNγ-producing T cells in established RDEB wounds correlates extremely well with our prior data demonstrating accumulation of differentiated PMN to these advanced lesions. T cell-derived IFNγ is also heightens macrophage response to microbial products and is a potent inducer of macrophage M1 polarization. However, paucity of the macrophages in EB advanced skin lesions detected in our prior studies and impaired macrophage response in infected RDEB wounds suggest that T cell-derived IFNγ primarily activates PMN in EB lesions. Reduction of IFNγ-producing T cells in chronic EB wounds suggests inadequate IFNγ-mediated PMN stimulation and their functional impairment in chronic lesions.

Besides detecting $CD4^+$ Th cells, we have identified a small population of $CD8^+$ CTL among wound-associated T cells. Although the role of CTL in harnessing bacterial infection in the skin, and particularly in EB wounds, remains poorly defined, we suggest that bacterial Ag-specific CTL could target host cells in which bacteria evade intracellular destruction. Such a situation has most frequently been described for professional phagocytes, such as macrophages. Bacteria have diverse mechanisms for avoiding autophagy, including escaping to cytoplasm where they can survive and replicate. Such escape leads to the introduction of bacterial antigens into the cytoplasmic compartment and MHC class I-mediated presentation, making bacterial Ag "visible" to the CTL. Although this mechanism was described for some bacterial and protozoan infections more than 30 year ago, only recently have we started to learn about the intracellular behavior of the majority of bacterial species *in vivo*. Thus, several studies have shown that keratinocytes could engulf bacterial particles and that bacteria (e.g., *S. aureus*) could escape into the cytoplasm by inhibiting autophagosome maturation. Among others, *S. aureus,* as one of the most frequent skin colonizers, was associated with progression of keratinocyte skin tumors. Clinical studies have shown that this bacteria frequently infects pre-malignant actinic keratosis and cutaneous SCC. Our data corroborate these findings in showing significantly higher susceptibility of the RDEB SCC to *S. aureus* infection and suggesting that malignant transformation of the cells could modulate bacterial engulfment. Although the exact mechanism of *S. aureus* engulfment by SCC remains to be further elucidated, it is plausible that some sphingolipids that are associated with advanced stage of SCC play an important role in bacterial engulfment via "lipid zippering", a mechanism described for *P. aeruginosa* infection. Moreover, identified association of the *S. aureus* particles with both $LAMP2^+$ phagolysosomes and $LC3^+$ autophagosomes suggests that, in RDEB keratinocytes, bacteria could be routed to degradation or escape to the cytoplasm. In the latter scenario, adaptive immunity could recognize bacterial antigens presented in the context of MHC I by specific T cell receptors developed against MHC-antigenic peptide complex. This concept is directly supported by our data showing typical T cell responses toward *S. aureus*-infected keratinocytes. Of particular note are elevated CTL activity toward infected cells and significant induction of both IFNγ and IL-2 after exposure of T cells to infected keratinocytes. This is contrary to the lack of IL-2 response to soluble microbial antigens, where only IFNγ induction and secretion were significant. Considering that *S. aureus*-derived super-antigens could activate T cells in orders of magnitude above conventional process, it is plausible that a full array of T cell activities could be engaged only against MHC I-mediated presentation of bacterial Ag and that exposure of peripheral memory T cells to soluble microbial Ag is limited to IFNγ production to support PMN activity.

In chronically infected wounds, T cells are often exposed to persistent antigenic load and inflammatory signals, resulting in over-stimulation and T cell exhaust and/or Treg-mediated immuno-suppression. Our experiments showing transient induction of CD69, persistent expression of PD-1 in T cells exposed to microbial Ag and infected keratinocytes, and a lack of CD57 induction suggest that PD-1-mediated exhaustion may not be the primary mechanism of the T cell inhibition. Nevertheless, presented data showing that the $CD4^{high}$ T cells differentiate into $CD25^+FoxP3^+$ Treg cells upon exposure to pooled microbial and *S. aureus* Ag but do not

differentiate in experiments involving infected keratinocytes suggest that soluble microbial Ag at wounded sites could induce Treg and provide a primary immuno-inhibitory effect. Because the $CD4^{high}$ population almost uniformly expresses PD-1, it is plausible that PD-1 expression synergizes with Treg-mediated inhibition and enhances suppression of the $CD8^+$ T cell responses, as it was shown in chronic viral infection and neoplasms. Our findings showing that wound-derived T cells could present their anti-microbial activity *ex vivo* and that PD-1^+ Treg cell differentiation during the same period suggest that synergistic Treg-PD-1-mediated inhibition of T cell immunity could be a conservative mechanism that extends to cutaneous bacterial infection. These findings also indicate that revitalization of exhausted T cells or inhibition of Treg differentiation could re-invigorate anti-bacterial T cell-mediated immunity in EB wounds and, potentially, prevent bacterial survival in the host and RDEB-associated SCC progression via immune-mediated elimination of infected malignant cells. Activation of T cells by microbial antigens and inhibition of this response after prolong exposure is also supported by secretion of pro-inflammatory chemokines and cytokines by *S.aureus* antigen-exposed RDEB T cells. This is illustrated by reduced secretion of the immune-inhibitory cytokine IL-10 during first 2.5 days of antigen exposure and induction of this cytokine during consecutive 2 days. This process coincides with secretion of T cell-derived CCL11. It is known that CCL11 is expressed by skin-residing T cells and that it is chemotactic to eosinophils that participate in tissue repair and remodeling. Very recent studies have shown that CCL11 increases the proportion of $CD4^+CD25^+FoxP3^+$ Treg cells. This data is in agreement with our findings showing differentiation of EB-derived T cells toward $FoxP3^+$ Treg upon prolong exposure to microbial /*S. aureus* antigens.

Although acknowledging that *ex vivo* and *in vitro* experimental settings do not recapitulate the complexity of molecular and cellular interactions at wound sites, our studies demonstrated that microbial/*S. aureus*-specific T helper and cytotoxic T cells are present in wounds and that CTL could target infected keratinocytes. Our data also indicate that prolong exposure of wound-associated T cells to soluble microbial antigens and other molecules (e.g. CCL11) could trigger differentiation of Treg cells that could inhibit T cell-mediated immunity at wound sites.

Specific Aim 3. To develop therapeutic strategies aimed at the inhibition of pro-inflammatory signals to restore healing of chronic EB wounds.

Major Task 6: Development and characterization of the wound-heling EB animal model.
Subtask 1: *In vivo* characterization of wound-induced skin in JEB mice. Timeline – 6 months (18-24).
Per SOW: JEB mice (n=60) will be used for morphologic and immunofluorescence evaluations of wound-induced skin lesions.

Milestone(s) Achieved: Established the basis to test pharmacological treatment in experimental EB-specific wound healing model. Task is accomplished.

Major Task 7: Targeting of inflammatory response in chronic EB wounds.
Subtask 1: Inhibition of CXCR1/2 signaling with Ladarixin (LDX). Timeline – 6 months (25-30).
Per SOW: JEB mice (n=90) will be used to define whether targeted inhibition of CXCR2 signaling by LDX abrogates/ reduces recruitment of neutrophils to wounded skin and restores/accelerates wound healing.

Subtask 2: Targeting of the pro-inflammatory TLR4 signaling with TAK-242. Timeline – 6 months (30-35).
Per SOW: JEB mice (n=90) will be used to define whether TLR4 inhibition by TAK-242 facilitates resolution of the inflammatory phase and accelerates wound healing.

Milestone(s) Achieved: Evaluated pharmacological approaches to improve healing of the EB-associated skin lesions. Acquisition of statistically significant data. All tasks are accomplished, manuscript in preparation.

Specific Aim 3 Major Milestones: Our data obtained upon completion of Specific Aims 1 and 2 indicated that inflammatory milieu of EB-associated wounds results in the continuous recruitment of neutrophils. Progressive accumulation of these cells to wound sites was associated with progression of wounds to chronic state and

inhibition of wound healing. In this aim, we tested whether inhibition of the chemotaxis-mediated recruitment of neutrophils to EB skin wounds facilitates wound closure. We have used a transgenic K14-LamC2 conditionally knock out mice in which removal of doxycycline from the diet results in the inhibition of *Lamc2* gene expression, disappearance of mature Laminin 3,3,2 from the BMZ, and separation of skin layers mimicking junctional form of EB. We developed a blister suction technique suitable for consistent and concurrent induction of blisters in Dox-deprived adult K14-LamC2 animals (Fig. 1a). The established protocol allowed rapid induction of blister of the similar size and permitted rapid recovery of the Dox-deprived K14-LamC2 mice after procedure (Fig. 1b). After establishment of the blisters, blister cups were removed and wounds were allowed to establish. Initial characterization of the wounds showed an accumulation of the granulocyte neutrophils to the wound bed during the first week after induction. Transition of the skin wounds in

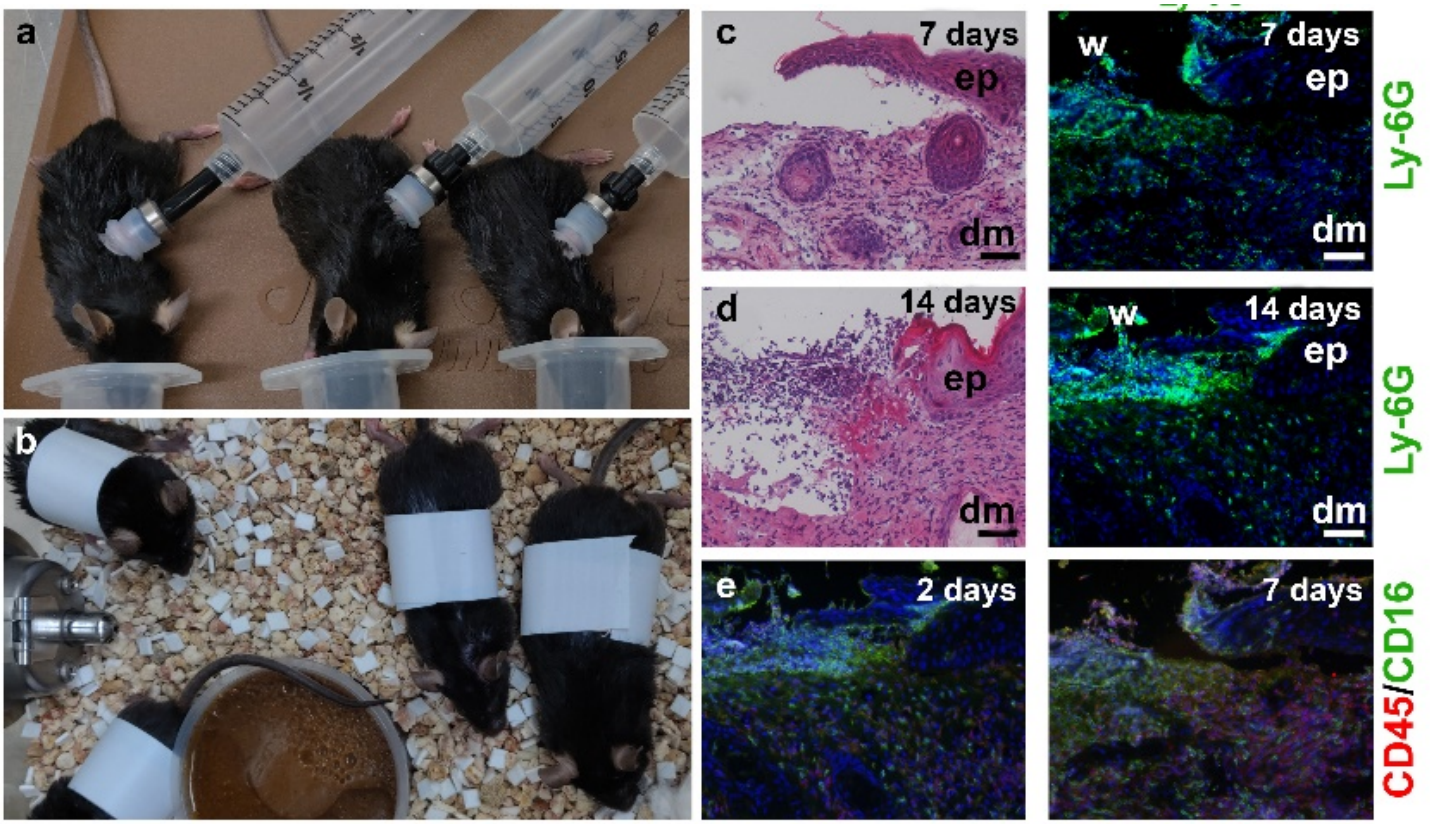

Fig. 1. induction of blisters and characterization of wounds in K14-LamC2 mice.
(a, b) Photographs illustrating application of the negative pressure to the skin using designed blister suction device (a) and bandaged mice recovered 5 min after procedure. (c, d) Micrographs of the wounds 7 and 14 days after induction (as indicated) showing inflammatory response (H&E staining) and accumulation of the Ly-6G$^+$ neutrophils (green) at wound sites detected by the indirect immunofluorescence. (e) Micrographs depicting rapid accumulation of the CD16+ mature neutrophils detected at wounds sites 2 days after wound induction. These cells persisted at wound site during wound progression to poorly healing state (7 days). Scale bar – 100 μm; ep – epidermis; dm – dermis. Detected antigens in respective colors are shown next to the panels.

Dox-deprived K14-Lamc2 mice to non-healing chronic state was accompanied by persistent recruitment Ly-6G$^+$ cells, which completely covered an unhealed wound by 2 weeks from induction (Fig. 1c, d). A more detailed characterization of the experimental wounds showed that CD16$^+$ mature neutrophils heavily infiltrate dermis at wound bed 2 days after induction and were present at the sites during transition to poorly healing state (7 days). These findings are consistent with our data showing persistent accumulation of mature neutrophils at poorly healing/chronic wounds in EB patients. These data also confirmed that selected animal model recapitulate poorly healing wounds in human EB patients and is suitable for the studies.

Considering continuous recruitment and accumulation of neutrophils at wound sites as a limiting factor that inhibits wound healing, we further validated whether targeted inhibition of the CXCR1/2- mediated

chemotaxis could improve healing of wounds in the established K14-Lamc2 model. After induction of the wounds, animals were treated systemically via intraperitoneal injection of Ladarixin, a dual CXCR1/2 inhibitor as described in our prior studies. Considering potential activation of the TLR4 signaling at wound sites by damage associated molecular patterns (DAMP) and consequent induction of the NF-Kb-mediated expression of

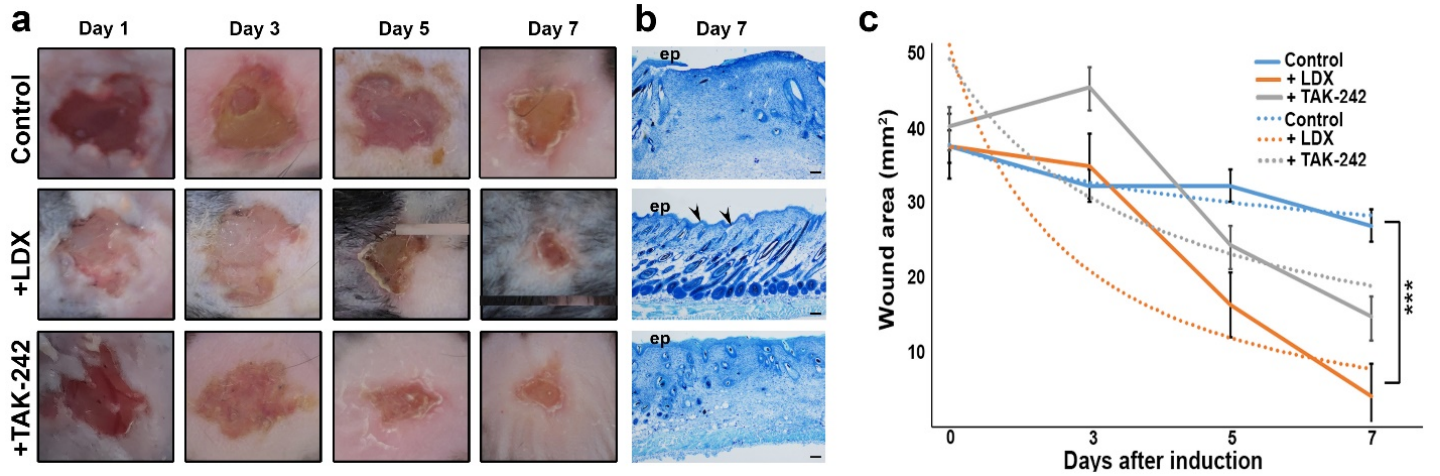

Fig. 2. Wound healing in LDX andTAK-242-treated mice. (a) Photographs of control and differently treated skin wound, as indicated to the left of the panel. (b) Representative micrographs of the cross-sections of the control and differently treated wounds illustrating lack of the re-epithelization of the control skin and re-epithelization of the LDX and TAK-242 treated skin. ep – epidermis. Arrows point to micro-blisters in the healed LDX-treated skin. Scale bar – 100 μm. (b) Graph illustration the dynamic of wound closure in control and treated wounds, indicated in the key. Solid lines – actual changes in wound area, dotted line - power trend lines depicting wound closure trends. Data is presented as an average ± SD. Statistical significance is indicated by asterisk.

CXCR1/2 ligands (e.g. CXCL8), in a separate cohort of wounded mice TLR4 was inhibited by topical application of the TAK-242 inhibitor. Wound healing was observed for 1 week. Based on wound size measurements, we determined that in the untreated, Dox-deprived mice K14-Lamc2 mice initiation of wound closure was detected but it was stalled 3 days after wound induction. At the end of the observation period, re-epithelization was not detected in Mock-treated (control) mice. Lack of epidermal layer or keratinocytes that migrated into the wounds was confirmed by histological evaluation. A substantial lymphocytic infiltrate was detected throughout the wounded site (Fig. 2a, b). On the contrary in LDX-treated cohort, a complete re-epithelization of the wounds was detected although the wounded site remained visible. A substantially reduced lymphocytic infiltrate was detected only in the upper dermis. Interestingly, treatment with LDX coincided with the induction of an anagen, a stage of active hair growth. To date, it is not known where there is a direct effect of the LDX to the hair cycle, although, complete re-epithelization was detected in most LDX-treated mice, some epidermal-dermal separation and micro blisters were detected in the healed skin (Fig. 2a, b). Treatment of wounds with topical TAK-242 also produced almost complete re-epithelization of the wounds in experimental mice. However, as compared to the LDX-treated animals, a substantial inflammatory infiltrate was detected in the healed regions and dermal-epidermal architecture was disorganized in TAK-242-treated animals (Fig. 2a, b). Healed skin also contained hair follicles in early anagen stage, suggesting that both LDX and TAK-242 induction of an anagen stage of the hair follicles was detected. Quantitative evaluation of changes of the wounded skin area showed that LDX treatment provided most effective and rapid wound closure, as compared to control and TAK-242-treated mice. Collectively, these experiments showed that systemic treatment of Dox-deprived K14-Lamc2 mice with epidermal wounds recapitulating human JEB with LDX was most effective in facilitating re-epithelization and wound closure. In similar experiments that we conducted in mice with established skin wounds, in which treatment was started at day 7 after induction, LDX was also most effective in reducing inflammation and facilitating wound healing (manuscript describing all obtained data is in preparation).

Collectively, our findings confirmed that CXCR1/2 mediated chemotactic axis plays an important role in the constitutive recruitment of the neutrophils to EB skin wounds and that systemic inhibition of CXCR1/2 with LDX blocks recruitment of neutrophils to the skin and facilitates re-epithelization and closure of the wounds. Our data also indicated that systemic CXCR1/2 inhibitors could be a first line of treatment of early and chronic wounds in EB patients. Currently, we are preparing another proposal aimed at the detailed pre-clinical evaluation of the CXCR1/2 inhibitors on animal models recapitulating different EB types.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT
Nothing to report

4. IMPACT
Completion of the proposed studies along with statistical modeling will lead to the computing of the 3-dimentional map of chronic wound development, where cellular, molecular and microbiome interactions will be mapped. This will create ample opportunities for design and development of novel intervention approaches prohibiting chronic wounds. In a short run, proposed studies will lay the groundwork for the clinical trials to test novel systemic and local treatments aimed at the restoration and enhancement of healing of the EB-associated skin lesions. Testing of at least two types of drugs on a relevant animal model will create the foundation for clinical trials aimed at systemic and local treatment of the EB patients to reduce chronic wound burden. Analysis of the microbiome and concurrent cellular infiltrates will also permit better selection of antibiotic and antiseptic creams/ointments to restore microbiota balance and decrease inflammatory response. It will also permit better selection of the antibiotics or probiotics to restore microbiota balance and decrease inflammatory response. In the long term, the obtained data will pave the path to the development of novel therapeutic approaches aimed at prevention and management of chronic wounds focused on reduction of inflammation and, potentially, fibrosis.

5. CHANGES/PROBLEMS
Nothing to report

6. PRODUCTS
Phillips T, Huitema L, Cepeda R, Cobos D, Perez R, Garza M, Ringpfeil F, Dasgeb B, Uitto J, Salas-Alanis JC, Alexeev V, Igoucheva O. Aberrant recruitment of leukocytes defines poor wound healing in patients with recessive dystrophic epidermolysis bullosa. Journal of Dermatological Science 2020; 100(3):209-216. https://pubmed.ncbi.nlm.nih.gov/33143962

Huitema L, Phillips T, Alexeev V, Tomic-Canic M, Pastar I, Igoucheva O. Intracellular escape strategies of Staphylococcus aureus in persistent cutaneous infections. Experimental Dermatology 2021; 30(10):1428-1439. https://pubmed.ncbi.nlm.nih.gov/33179358

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Alexeev V, Huitema L, Phillips T, Cepeda R, de los Cobos D, Perez RIM, Salas-Garza M, Fajardo-Ramirez OR, Ringpfeil F, Uitto J, Salas-Alanis JC, Igoucheva O. T cell activation and bacterial infection in skin wounds of recessive dystrophic epidermolysis bullosa patients. Experimental Dermatology 2022; 31(9):1431-1442.
https://pubmed.ncbi.nlm.nih.gov/35620886

Alexeev V, Huitema L, Phillips T, de los Cobos D, Matus Perez RI, Salas-Garza M, Fajardo-Ramirez OR, Ringpfeil F, Uitto J, Salas-Alanis JC, Grice E, Igoucheva O. Bacterial diversity in skin wounds of recessive dystrophic epidermolysis bullosa patients. 2022, under review.

7. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS (entire project)

Name: Olga Igoucheva
Project Role: Principal Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-9813-7184
Nearest person month worked: 10.2
Contribution to Project: PI worked directly with Drs. Alexeev, Uitto, Salas, Rosenbloom and Debes to characterize the profile of cells isolated from patient bandages using various approaches as described in the Aim 1 and 2; PI worked directly with Drs. Grice and Bittinger in comparing microbiome data analysis with endogenous cell profiles; and PI directed all animal stidies as outlined in Aim 3. PI managed and coordinated overall administrative activities and direction of the project and supervised all aspect of the study, including the design and implementation of the experiments, and the evaluation and interpretation of the experimental data. PI supervised directly the research associate and research technician.
Funding Support: NIH R21 grant 1R21AR079706-01A1

Name: Vitali Alexeev
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-0762-1833
Nearest person month worked: 4.8
Contribution to Project: Dr. Alexeev was involved in all aspect of the Aims 1, 2 and 3, including the design and implementation of the experiments and the evaluation and interpretation of the experimental data. Also, Dr. Alexeev had share some responsibilities with the PI such as supervision of the laboratory staff.
Funding Support: Hasumi Research fund

Name: Jouni Uitto
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0003-4639-807X
Nearest person month worked: 0.12
Contribution to Project: Dr. Uitto provided expertise in molecular genetics in general and in inheritable blistering skin disease, Epidermolysis Bullosa. Dr. Uitto also provided clinical expertise in the data assessment and interpretation throughout the entire project.
Funding Support: NIH R01 5R01AR072695-05; NIH R01 subcontract 5R01AI143810-03

Name: Gudrun Debes
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0003-4208-7362
Nearest person month worked: 0.12
Contribution to Project: Dr. Debes has provided expertise in leukocyte assessment of patient-derived samples and helped with characterization of leukocytic infiltrates and T cell involvement as stated in the Aims 1and 2.
Funding Support: Two NIH R01 grants

Name: Inna Chervoneva
Project Role: Co-Investigator
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0002-9104-4505
Nearest person month worked: 0.12
Contribution to Project: Dr. Chervoneva provided biostatistical data analysis by applying univariate and multivariate methods and biostatistical modeling.
Funding Support: DOD W81XWH2110003, NIH P30 5P30CA056036-23, NIH R01 5R01CA253977-03, NIH R01 5R01CA222847-04, NIH R01 5R01CA160495-10, NIH R03 1R03CA259594-01, DOD W81XWH2010554, R01 5R01CA255792-02, NIH R01 5R01CA182635-08

Name: Leonie Huitema
Project Role: Research Associate
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0003-2947-021X
Nearest person month worked: 12
Contribution to Project: Dr. Huitema was involved in all aspects of the Aims 1 and 2, including analysis of bandage-derived cells and T cell-mediated immunity.
Funding Support: No change

Name: Taylor Phillips
Project Role: Research Technician
Researcher Identifier (e.g. ORCID ID): https://orcid.org/0000-0001-9843-4736
Nearest person month worked: 12
Contribution to Project: Ms. Phillips was involved in studies related to leukocytic infiltrates, T cell-mediated immunity as well as maintenance of mouse colony and general maintenance of the laboratory.
Funding Support: No change

What other organizations were involved as partners?
No other organizations were involved as partners

8. SPECIAL REPORTING REQUIREMENTS
Nothing to report

9. APPENDICIES
Nothing to report