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14. ABSTRACT

Epidermolysis bullosa 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, 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 ($IFN\gamma$). Also, $CD8^+$ cytotoxic T lymphocytes recognize *S. aureus*-infected RDEB keratinocytes and respond by producing interleukin-2 (IL-2) and $IFN\gamma$ 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.

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. During first two years of the project, we leveraged the ability of our clinical collaborator, Dr. J. Salas, to follow particular wounds on patients affected by different EB types while providing standard of care and our capacity to characterize healing capacities of EB-associated wounds on different genetic backgrounds. In addition, to define microbial biomarkers predictive of the wound outcome, we conducted comprehensive studies on the dynamics of bacterial and fungal communities during wound progression and healing. Ultimately, these studies will allow us to delineate molecular and cellular activities/mechanisms, which inhibit wound healing process.

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

In physiological conditions, skin microbiota establish symbiotic relations with the immune system and, as shown by a number of studies, play an important role in maintaining skin barrier function, regulating inflammation and promoting wound healing responses. However, in the case of skin injury, stability of microbial communities and their interaction with host immune system could be perturbed, leading to impaired healing as observed in 50% of chronic wounds of the diabetic foot ulcers (DFU). Bacterial infection is also one the major morbidities for patients with EB. Extensive areas of denuded skin, loss of the stratum corneum barrier and accumulation of body fluids represent a substantial challenge in management of the bacterial infection. The most common bacteria isolated from EB wounds include gram-positive *Staphylococcus aureus* and *Streptococci,* and gram-negative anaerobes such as *Pseudomonas aeruginosa* and *Proteus*. To date, the majority of microbiome studies employed cross-sectional designs, which did not allow correlating the relationship between wound healing and wound microbiome. This project is designed to test the hypothesis that dynamics of the microbiota community could be indicative of the EB wound outcomes and lead to novel approaches in management and treatment of EB wounds. With an estimated composition of 100 trillion cells, commensals outnumber host cells by at least a factor of 10 and encode 100-fold more antigens than host's genome. Despite such enormous antigenic load at the skin, our immune system establishes somewhat symbiotic relations with the commensals via development of the commensal-specific T-cell tolerance at the neonatal period and physical barriers such as the stratum corneum to limit physical contact between microorganisms and the immune system. This implies that the initial encounter of pathogens with the immune system occurs in an environment conditioned and regulated by microbiota. Commensals augment various skin responses including production of antimicrobial peptides, recruitment of leukocytes and T cell activation. 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 dermal-epidermal junction, as in junctional EB (JEB) and DEB. As adequate T cell responses are essential for immune-mediated protection against microbiota, within this study we also tested the hypothesis that a high number of activated T cells at wound sites can either exhaust or establish tolerance toward wound-colonizing bacteria.

4. KEY RESEARCH ACCOMPLISHEMNTS
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.

Progress: All samples were collected concurrently with bandages.

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.

Progress: DNA from swab specimens was isolated and used for bacterial microbiome analysis. Amplification of the 16S rRNA gene V1-V3 region was performed using the Illumina MiSeq platform with 300 bp paired-end 'V3' chemistry as described previously. This approach produces sufficient number of classifiable sequences ranging from 2,000 to 70,000 sequences per sample. DNA isolation, sequencing, and all standard bioinformatics analyses were done using the CHOP Microbiome Center (Philadelphia, PA). Briefly, operationale taxonomic unit (OTU) clustering was done via UCLUST algorithm. Sequences corresponding to taxa identified as contaminants in the negative controls were removed. Microbial diversity was calculated using Shannon diversity index, Faith's phylogenetic distance, and number of observed OTUs. Data analysis was done using R Statistical Package (R Core Team 2016) for all computations. Non-parametric Wilcoxon rank-sum tests were used to compare differences between groups. Spearman correlations was used to correlate continuous variables.

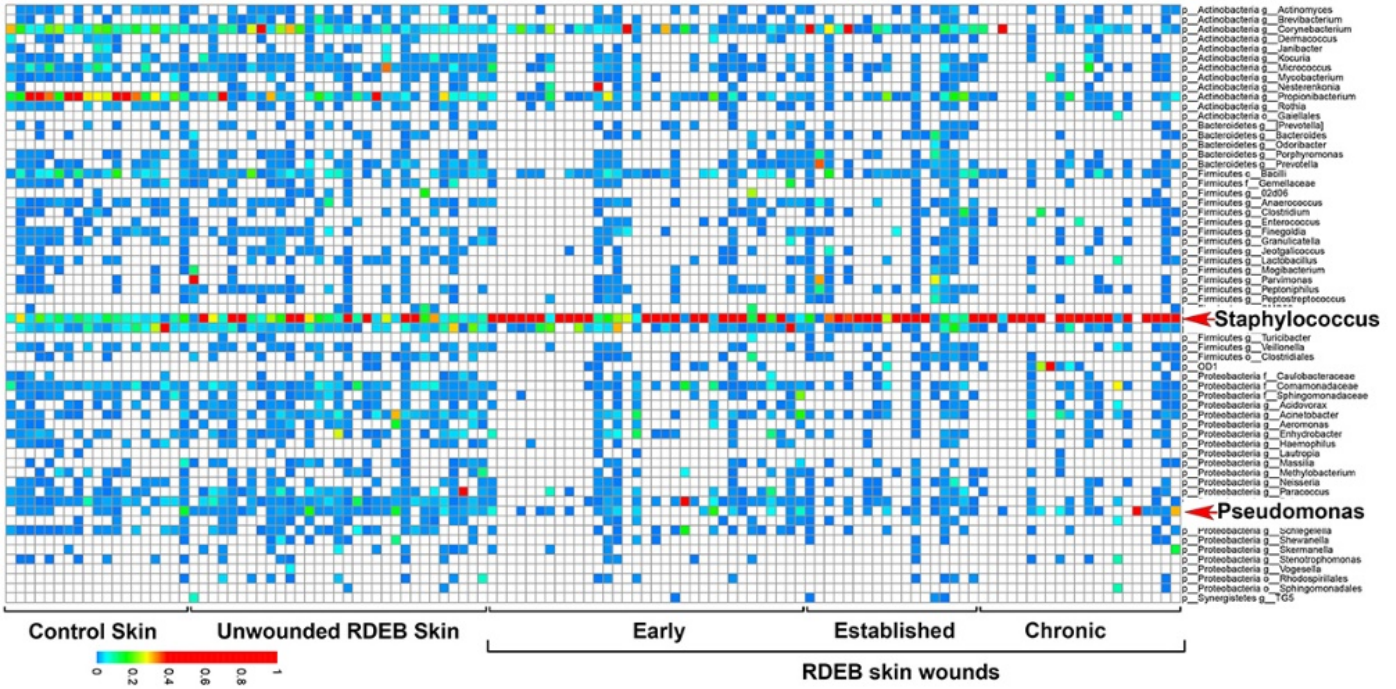

Fig. 1. A heat map illustrating RDEB wound-associated microbiome analysis and changes in bacterial communities in normal, RDEB unwounded, RDEB lesional skin (as indicated). Arrows point to most abundant species detected in RDEB wounds.

Kruskal-Wallis tests, followed by Wilcoxon rank sum post-hoc tests, were used for categorical variables. Linear models were calculated in base R; mixed-effect regressions were generated using the NLME package.

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 RDEB clinical outcomes. RDEB microbiomes were determined by sequencing of hypervariable regions VI through V3 of the 16S ribosomal RNA (rRNA) gene. 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 RDEB wounds (Fig. 1). PCR-based analysis of the most common RDEB wound contaminants showed that *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Pseudomonas aeruginosa* are the most common wound contaminants in RDEB lesions. Respectively, these species and, particularly *Staphylococcus sp.,* could be the primary targets for therapeutic intervention.

Milestone(s) Achieved: Timeline - 24 month. A well characterized analysis of bacterial communities of EB-associated wounds. Acquisition of statistically significant data. Manuscript in preparation.

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.

Progress: All samples were collected concurrently with bandages.

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.

Progress: DNA isolation, sequencing, and all standard bioinformatics analyses were done using the CHOP Microbiome Center (Philadelphia, PA). The fungal component of the wound microbiomes was analyzed by sequencing of the hypervariable internal transcribed spacer 1 (ITS1) region using the Illumina MiSeq platform (two 300-bp paired-end chemistry) (Fig. 2). As the ITS region has been formally recognized as the universal barcode for fungal identification (40), it was used for the assignment of the OUT using a curated fungal bar code reference database UNITE. ITS1 regions were clustered into OTUs with VSEARCH. Contaminants found in negative controls were removed at the OUT level. Reference-independent analyses were used to characterize diversity and variation of fungal communities. The Shannon diversity index, Simpson diversity index (1-Dominance), Faith's phylogenetic distance (PD), and number of observed species (richness) were calculated using the QIIME 1.8.0 alpha_diversity.py script.

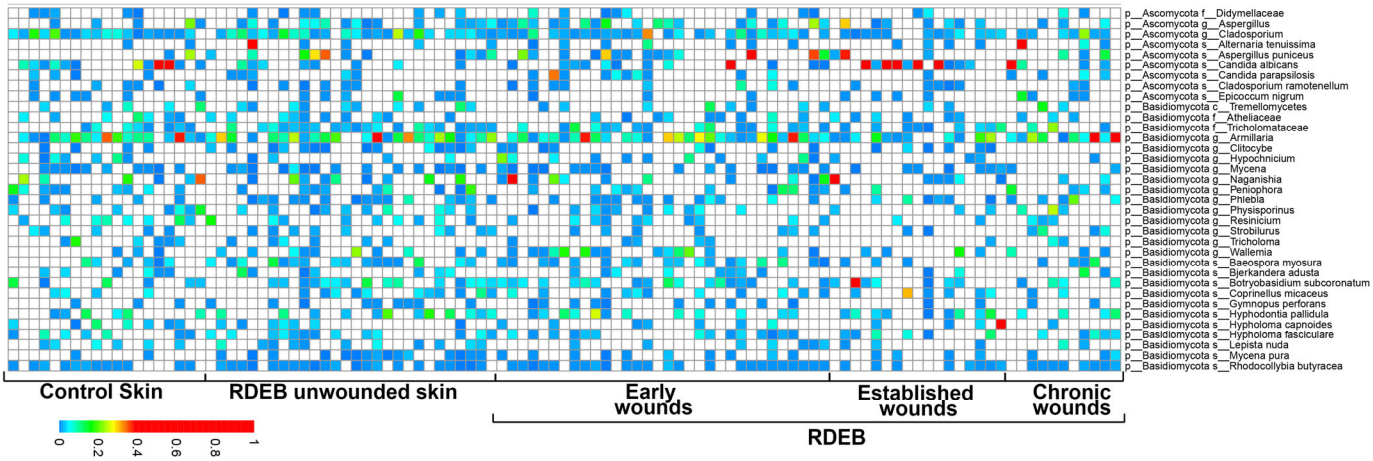

Fig. 2. A heat map illustrating RDEB wound-associated fungal community structure and changes in fungal communities in normal, RDEB unwounded, RDEB lesional skin (as indicated).

Milestone(s) Achieved: Timeline - 24 month. A well characterized analysis of fungi communities of EB-associated wounds. Acquisition of statistically significant data. Manuscript in preparation.

Together, completion of major tasks 3 and 4 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. 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.

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.

Progress for Subtasks 1 and 2: **Wound-associated *Staphylococcus aureus* could serve as a common target for T cell- mediated immunity in RDEB skin.** To define microbial species whose antigens could be recognized by the adaptive T cell-mediated immunity, we evaluated extracellular and intracellular microbial contaminants in cellular fraction and exudates obtained from RDED wounds with no documented active infection, as described previously. The samples were subjected to PCR-based analysis of the most common microbial contaminants identified in RDEB wounds with species-specific primers. This evaluation showed that *Staphylococcus aureus* is detected in more than 50% of the exudates (n=57) with high, medium, and low levels of contamination as defined by semi-quantitative PCR, whereas *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* were detected in a substantially lower percent of samples with mostly high and medium levels of contamination (Fig. 3a, b). Other examined bacterial and fungal contaminants were not detected. When bandage-derived cells were placed in culture conditions, one-third of samples showed the presence of *S. aureus* either in conditioned media or intracellularly (Fig. 3c, d). These findings demonstrated that *S. aureus* can infect and survive in keratinocytes at RDEB wounds.

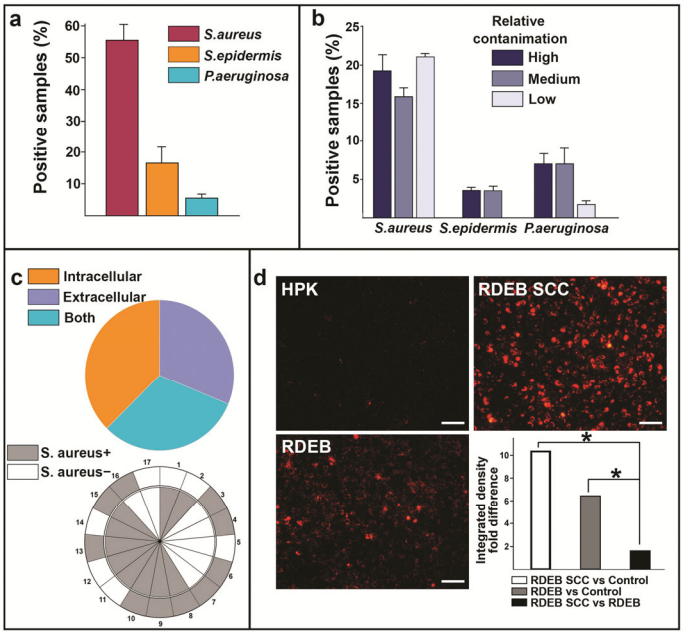

Fig. 3. Analysis of bacterial infection in RDEB wounds and keratinocytes.

Unlike viruses which most often utilize cell surface receptors to infect the host, bacteria are engulfed by the host cells.

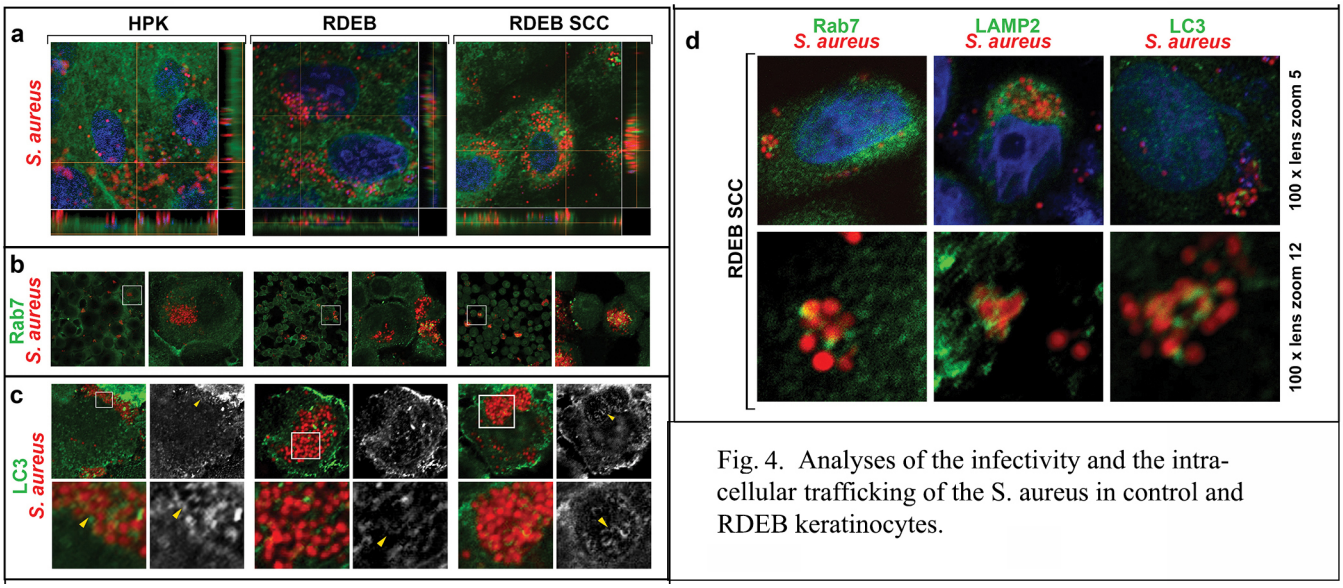

Fig. 4. Analyses of the infectivity and the intra-cellular trafficking of the S. aureus in control and RDEB keratinocytes.

Macrophages and neutrophils are well-equipped for bacterial phagocytosis. However, studies have shown that other cell types, particularly keratinocytes, could engulf the bacteria. Using AlexaFluor^{594}-labeled *S. aureus*, we evaluated its ability to infect control and RDEB-derived keratinocytes. Our assessment showed that overnight exposure of the cells to bacteria leads to a more robust infection of RDEB- (6-times) and RDEB-associated squamous cell carcinoma (RDEB SCC)-derived (10-times) keratinocytes with *S. aureus* when compared to keratinocytes from healthy donors (Fig. 3d). Using confocal microscopy to scan infected cells at 5 µm Z-position, we observed an overwhelming intracellular presence of bacterial particles in the RDEB cells. In control keratinocytes, bacterial particles were mostly detected on cell surface (Fig 4a). *S. aureus* co-localization with Rab7^{+} endosomes was very prominent in RDEB SCC (Fig.4b). Further analysis of the intracellular bacterial trafficking showed that *S. aureus* could be detected in association with LC3^{+} autophagosomes (Fig. 4c) and routed toward LAMP2^{+} phagolysosomes (Fig. 4d).

Microbial antigen-specific CD4^{+} and CD8^{+} T cells are present in RDEB skin wounds. To evaluate whether microbial antigen-specific CD4^{+} and CD8^{+} T cells are present in RDEB skin wounds, we isolated a pool of CD3^{+} T cells by positive selection from bandage-derived leukocytes and propagated them *in vitro*. FACS-based immuno-phenotyping showed that CD4^{+} and CD8^{+} T cells are present in the CD3^{+} population at an average ratio of 6 to 1, respectively (Fig. 5a). Exposure of RDEB-derived T cells to wound-associated pooled microbial Ag led to clonal expansion that was most prominent after 48 h of exposure (Fig. 5b). CFSE dilution assay confirmed these observations by showing that 2-day exposure to microbial Ag induced vigorous proliferation in about 30% of RDEB wound-derived T cells and did not substantially affect proliferation of control T cells in which anti-CD3/CD28 stimulation triggered robust proliferation (Fig. 5c). Intracellular staining for IFNγ combined with detection of CD107a degranulation marker confirmed microbial Ag-specific T activation in RDEB T cells (n=5). Two days of exposure induced IFNγ expression in about 50% of RDEB T cells, whereas induction of IL-2 was detected in only 10% of cells (Fig 5d, f). Quantitative ELISA showed a significant increase in INFγ secretion from RDEB T cells detected at 100 pg/ml after 1 day of exposure and 275 pg/ml after 7 days of exposure (Fig. 5e). However, analysis of IL-2 and IL-17 in culture media did not display any appreciable secretion of these cytokines. Population profiles showed the presence of a small CD107a^{high}IFNγ^{high} population of T cells that was identified as CD8^{+} T cells (Fig. 5e). Collectively, these data demonstrated that RDEB wounds contain a pool of primed T cells that react to microbial antigens by proliferation, degranulation, and secretion of IFNγ.

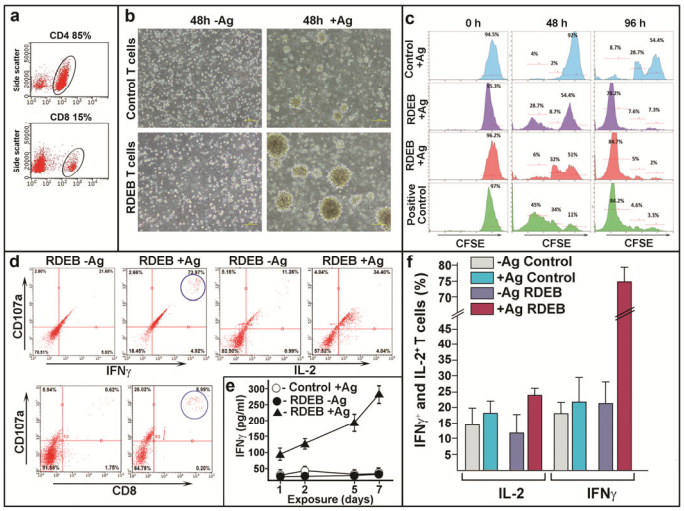

Fig. 5. Analysis of T cell responses to pooled RDEB wound-derived microbial antigens.

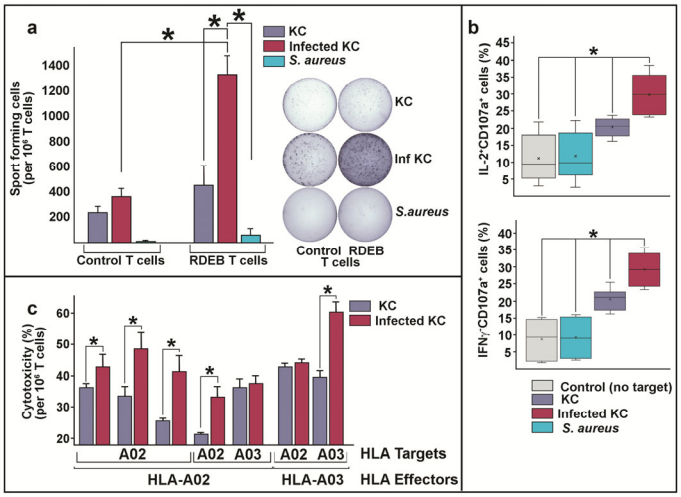

Fig. 6. Analysis of T cell activity against *S. aureus*-infected keratinocytes.

Wound-derived T cells can recognize *S. aureus* antigens and target infected keratinocytes in HLA-dependent manner. Considering our findings that *S. aureus* infects RDEB cells and that the presence of *S. aureus* super antigens could activate adaptive immunity, we tested the capacity of RDEB T cells to recognize and target *S. aureus*-infected keratinocytes. To set-up an IFNγ ELISpot assay, we screened available RDEB

keratinocytes (n=5) and patient-derived T cells (n=5) for HLA-A02 and HLA-A03 expression for HLA matching (data not shown). *S. aureus*-infected and uninfected keratinocytes were used as targets and control and RDEB T cells were used as effectors. Exposure of control or patient-derived T cells to soluble *S. aureus* Ag slightly increased a number IFNγ spot-forming cells (SFC) (Fig. 6a). Minor activation of control and RDEB T cells in response to uninfected keratinocytes was attributed to allogeneic response. Yet, a significantly greater number of SFC were detected when RDEB T cells were exposed to *S. aureus*-infected RDEB keratinocytes as compared to control (Fig. 6a). IL-2 and IFN-γ intracellular staining combined with detection of the CD107a degranulation marker confirmed a significantly higher percentage of cytokine-expressing RDEB T cells responded to *S. aureus* (Fig. 6b). CTL capable of targeting and killing infected keratinocytes were also identified among RDEB T cells (Fig. 6c). Moreover, CTL response to infected cells appeared to be HLA-restricted. Four independent patient-derived HLA-A02$^+$ T cells showed higher CTL activity toward HLA-A02$^+$ *S. aureus* infected targets. One of these T cell pools, while having specific CTL activity against HLA-A02$^+$ targets, showed unspecific allogeneic response toward HLA-A3$^+$ targets. Conversely, HLA-A03$^+$ patient-derived T cells were more specific against HLA-A03$^+$ than toward HLA-A2$^+$ infected targets (Fig. 6c). Collectively, these data demonstrated that *S. aureus*-specific memory/effector T cells capable of producing type 1 cytokines (IL-2 and IFN-γ) and killing infected keratinocytes are present in RDEB wounds.

PD-1 and Treg-dependent mechanisms may contribute to inhibition of bacteria-specific T cells in chronic RDEB wounds. In chronic infections, T cells exposure to persistent antigenic load could result in down-modulation of robust T cell effector function via activation of programmed cell death protein 1 (PD-1) and T cell exhaustion or through induction of regulatory T cells (Treg). One-day exposure of RDEB T cells to the pooled microbial Ag led to induction of PD-1 expression on about 20% of the cells with no substantial differences in the CD8$^+$ T cell population (Fig. 7a). By day 2, the CD69 T cell activation marker was induced along with PD-1 on about 30% of CD4$^+$ T cells. By day 3, about 60% of CD4$^+$ T cells were identified as CD69$^+$PD-1$^+$ cells. However by day 6, CD69 expression was diminished, yet, about 60% of CD4$^+$ T cells continue to express PD-1. During the observed period, no induction of CD57, a marker of PD-1-mediated T cell exhaustion, was detected in control (not shown) and RDEB T cells (Fig. 7b). Exposure to pooled *S. aureus*-derived Ag for 2 days also triggered prominent PD-1 expression in about 40% of RDEB T cells, a level of expression that was significantly higher than in control T cells (Fig. 7c). However, 6 days of exposure of both T cell types led to PD-1 expression in more than 60% of T cells. Unlike exposure to microbial Ag, exposure to *S. aureus* Ag led to a separation resulting in two distinct populations with lower and higher levels of CD4. The entire CD4^{high} population was PD-1 positive (Fig. 7c). CD57 was expressed on only 2% of RDEB T cells.

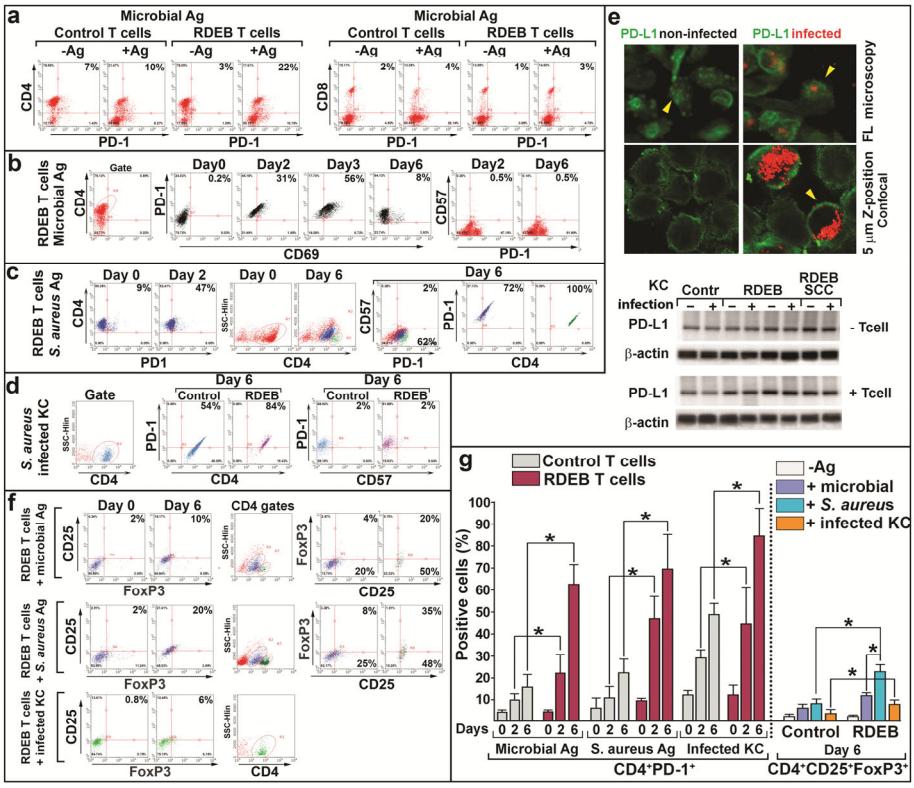

Fig. 7. Analysis of RDEB-associated putative microbial infection-induced T cell impairment mechanisms.

To test whether the interaction of T cells with MHC-presented Ag triggers a similar response, we infected RDEB SCC keratinocytes with inactivated *S. aureus* and exposed them to T cells. After 6 days of exposure, PD-1 expression was induced in control and RDEB-derived T cells up to 50% and 80%, respectively.

CD57 expression was detected in about 2% of PD-1^+ T cells (Fig. 7d). No separation of $CD4^+$ T cells into 2 district populations was detected. Suggesting that PD-1 could be activated by PD-1 ligand (PD-L1) expressed by infected keratinocytes, we evaluated its expression in infected and non-infected RDEB SCC. Indirect immuno-fluorescence and western blot analyses showed no significant induction of PD-L1 in the cells after infection. However, in uninfected RDEB cells, PD-L1 was detected mostly intracellularly, whereas in infected cells it was localized to the membrane as defined by light and confocal microscopy (Fig. 7e).

Since immune-inhibitory activity of regulatory T cells (Treg) may also play a role in diminishing T cell-mediated response, we evaluated the presence of Treg in control and RDEB T cells in similar settings. FACS-based analysis showed that both control (not shown) and RDEB T cells contain a small (~2%) population of $CD4^+CD25^+FOXP3^+$ Treg among CD4-gated T cells and that this percentage was increased after 6 days of exposure to microbial and *S. aureus* Ag up to 10% in the control and 20% in the RDEB T cells (Fig. 7f). The majority of $CD25^+FoxP3^+$ Treg were identified in the $CD4^{high}$ population (Fig. 7f). When T cells were exposed to *S. aureus*-infected keratinocytes for 6 days, much lower (i.e., up to 7%) induction of Treg was detected in the RDEB T cells (Fig. 7f). No substantial Treg induction was detected in control T cells (not shown). All detected differences between control and RDEB T cells to microbial, *S. aureus*, and *S. aureus*-infected keratinocytes were significant (Fig. 7g).

Milestone(s) Achieved: Timeline - 24 month. A well characterized role of T cell immunity in controlling microbiota in RDEB-associated wounds. All tasks are accomplished, manuscript submitted.

Overall Progress: Major tasks 3, 4, and 5 are fully accomplished.

5. OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

Nothing to report

6. IMPACT

It is anticipated that the proposed analyses will allow us to define precipitating factors, which predefine abnormal wound healing in EB skin lesions. In the short term, these investigations will allow us to develop clinically relevant tests and algorithms to assess wound healing capacities of the EB-associated wounds. 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. 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 fibrosis.

7. CHANGES/PROBLEMS

Nothing to report.

8. 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; 100:209-216. https://pubmed.ncbi.nlm.nih.gov/33143962

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9. PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

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Contribution to Project:	Dr. Igoucheva supervised all aspects of the study and performed microbiome studies as well analyses of patient-derived samples related to T cell-mediated immunity	Dr. Alexeev was involved in all aspects of the Aim 2, including microbiome analysis of bandage-derived organisms and T cell-mediated immunity	Dr. Huitema was involved in all aspects of the Aim, including analysis of bandage-derived microbiomes and T cell-mediated immunity	Dr. Uitto has provided clinical expertise of collected patient material and patient data assessment	Ms. Phillips was involved in studies related to T cell- mediated immunity, and maintenance of mouse colony for studies outlined in Aim 3
Funding Support:	No change	Internal support from Dr. Hatomi Sato Dr. Misue Terai Hasumi contract	No change	NIH R01 grant NIH R01 subcontract	No change

No other organizations were involved as partners.

10. SPECIAL REPORTING REQUIREMENTS

Nothing to report

11. APPENDICIES

Nothing to report