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Platelet Hyperactivity in *TNFSF14/LIGHT* Knockout Mouse Model of Impaired Healing

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Objective: Impaired and chronic wounds occur due to defects in one or more of the overlapping stages of healing. However, problems related to the vascular system are critical for nonhealing, and chronic wounds in humans often show the presence of fibrin cuffs/clots. We hypothesized that these clots are due to alterations in platelet function; hence, we have investigated whether alterations in platelet function are present during impaired healing.

Approach: Platelets were subjected to different agonists to determine the rate of aggregation and evaluate the molecules involved in adhesion and aggregation that could lead to faster thrombosis and potentially contribute to impaired wound healing.

Results: We show that wounding of *TNFSF14/LIGHT*^{-/-} mice, which have impaired healing, leads to an enhanced response in platelet aggregation and a faster time to blood vessel occlusion (thrombosis). In addition, after wounding, platelets from these mice have increased levels of P-selectin, integrin $\alpha_{IIb}\beta_3$, and phosphatidylserine, molecules that contribute to platelet adhesion. They also have more extensive open canalicular system than platelets of control mice, suggesting increased surface area for interactions upon activation.

Innovation: These results show a novel function for *TNFSF14/LIGHT* during wound healing.

Conclusion: The absence of *TNFSF14/LIGHT* from the cell surface of platelets causes rapid platelet aggregation and thrombus formation that may contribute to impaired healing by reducing the ability of the blood vessels to transport nutrients and oxygen and other molecules needed for proper healing.

Keywords: platelet aggregation, phosphatidylserine, thrombosis and occlusion, P-selectin, integrin $\alpha_{IIb}\beta_3$, fibrin cuffs

INTRODUCTION

THE OCCURRENCE OF chronic wounds in humans has risen significantly in the last decade. In the United States alone, chronic wounds affect 6.5 million people and cost ~\$25B/year.¹ Abnormalities associated with impaired and chronic wounds include a prolonged inflammatory phase, abnor-

mal angiogenesis, formation of fibrin cuffs, abnormal extracellular matrix (ECM) deposition, decreased rate of re-epithelialization, and presence of biofilm-forming bacteria.²⁻⁴ Prediction of healing outcomes remains elusive due to the lack of understanding of the mechanisms involved in the etiology of impaired and



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chronic wounds, and often the coexisting morbidities. Furthermore, although a murine model for chronic wounds has been developed recently,^{5,6} mechanistic studies are still lacking, thereby hindering the advancement in the development of diagnostics and therapeutics to treat these wounds.⁷⁻⁹

Tissue injury causes the disruption of blood vessels and extravasation of blood constituents. Blood clotting reestablishes hemostasis and provides a provisional ECM for cell migration and proliferation. A variety of vasoactive mediators and chemotactic factors are generated by coagulation, the activated complement pathways, and by injured or activated parenchymal cells.¹⁰ This vascular injury leads to the activation of platelets as first responders to help stop hemorrhage. Platelets are major players in hemostasis and they are crucial in the pathophysiology of vascular clots and thrombosis. Moreover, platelets release molecules that interact with various factors related to inflammation, proliferation, adhesion, aggregation, and vasoconstriction.¹¹ The α -granules of platelets contain clotting proteins such as von Willebrand factor (vWF), fibrinogen, fibronectin, and factor V. The dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), and ionized calcium. Contents present in both α - and dense granules are involved in activation, aggregation, and recruitment of resting platelets to sites of injury.

The activation and aggregation of platelets occur due to their interactions with ligands on endothelial cells, leukocytes, and various ECM proteins.¹¹ P-selectin is an adhesion molecule present in α -granules that becomes exposed on the platelet membrane upon activation. Activation of platelets is followed by adhesion and aggregation, which involves various factors, including the fibrinogen receptor integrin $\alpha_{IIb}\beta_3$.¹¹ Activation-dependent conformational changes occur in integrin $\alpha_{IIb}\beta_3$, making it competent to bind soluble fibrinogen.¹² Platelet activation also leads to the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the platelet membrane bilayer. PS has been shown to be involved in thrombin generation and hence blood coagulation.^{13,14} Assembly of coagulation factor complexes such as tenase and prothrombinase (which are necessary for thrombin generation), on the membrane bilayer is also dependent on PS exposure on activated platelets.¹⁵ Furthermore, procoagulant platelets display features of apoptosis, including blebbing, microvesiculation, caspase activation, and membrane contraction.¹⁴

We have previously shown that *TNFSF14/LIGHT*^{-/-} mice display marked abnormalities in

wound healing processes, including increased inflammation, oxidative and nitrosative stress, excessive coagulation and fibrin cuffs, vascular defects, and abnormal ECM deposition.^{5,16} Furthermore, we also showed that the basement membrane of the blood vessels was not continuous, the microvessels had very few associated α -smooth muscle actin-expressing periendothelial cells, and contained significant intravascular coagulation,¹⁶ resulting in immature/defective vessels. Moreover, we have recently shown that in these mice with impaired healing, platelets have enhanced aggregation as well as shorter bleeding time.⁶ We also found that after injury there was a significant increase in the eicosanoids 11-, 12- and 15-HETE, and the proinflammatory leukotrienes (LTD₄ and LTE₄) and prostaglandins (PGE₂ and PGF_{2 α})⁶ that are important in platelet function.

The goal of this study is to further determine whether the ability of the platelets of this knockout (KO) mouse to aggregate faster than control platelets is due to the presence of higher levels of molecules involved in adhesion and aggregation, whether this leads to faster thrombosis, and do they behave differently than control mice in the absence of injury/wounding. We show that, upon injury, *TNFSF14/LIGHT*^{-/-} mice have enhanced aggregation of platelets in the presence of the ADP agonist and an agonist for the thromboxane A₂ (TXA₂) receptor (U46619), and that thrombosis is faster than the control 3 days after injury. Moreover, we show significant differences in the levels of several molecules involved in adhesion, activation, and aggregation of the *TNFSF14/LIGHT*^{-/-} platelets as well as a more extensive canalicular system. Because it has been demonstrated that mouse and human platelets share similar structural features,¹⁷ our findings in this mouse model of impaired healing may provide insight into the mechanisms involved in blood vessel clotting that could be instrumental in understanding similar processes in human problematic wounds.

CLINICAL PROBLEM ADDRESSED

Impaired and chronic wounds are affecting an ever-increasing population and results in the rising cost of healthcare. The confounding number of factors that lead to the development of chronic wounds requires in-depth understanding of the mechanisms. Our data presented in this study provide evidence for the role of platelets in the development of impaired and chronic wounds. Furthermore, the results suggest a possible role of *TNFSF14/LIGHT* on platelets that could lead to their impaired function. Therefore, these find-

ings could be influential in deciphering processes underlying the etiology of chronic wound development and advancement of wound therapeutics.

MATERIALS AND METHODS

Dermal excision wound model

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME), and *TNFSF14/LIGHT*^{-/-} mice were a gift from Carl Ware (La Jolla Institute for Allergy and Immunology, San Diego, CA). Animals were housed at the University of California, Riverside (UCR) vivarium. All experimental protocols were approved by the UCR Institutional Animal Care and Use Committee (IACUC) and all methods were carried out according to the approved guidelines. Experiments were performed using 16-week-old mice. The procedure used was performed as previously described.¹⁸ Briefly, mice were anesthetized with a single intraperitoneal injection of ketamine (80 mg/kg body weight)/xylazine (16 mg/kg body weight). Full-thickness 7 mm punch wounds (excision of the skin and the underlying panniculus carnosus) were made on the back of the mice 24 h after nairing and shaving the hair.

Murine platelet preparation

C57BL/6 (control) or *TNFSF14/LIGHT*^{-/-} mice were anesthetized and blood was collected from the ventricle in 3.8% w/v sodium citrate solution (one part sodium citrate to nine parts blood) to prevent coagulation. Platelet-rich plasma was obtained by centrifugation at room temperature. Platelets were counted with an automated hematology analyzer (Drew Scientific, Dallas, TX) and their count adjusted to 7×10^7 platelets/mL, before each experiment.

In vitro platelet aggregation assay

C57BL/6 or *TNFSF14/LIGHT*^{-/-} platelets were activated with different concentrations of ADP and U46619. Platelet aggregation was measured by the turbidometric method using the model 490 aggregometer (Chrono-Log Corporation, Havertown, PA). There are no standard deviations because, to measure % aggregation increase, we needed to pool the blood of five samples given that the amount of blood we obtain from each mouse is very small.

In vivo thrombosis model

These studies were performed as described previously.¹⁹ Briefly, C57BL/6 or *TNFSF14/LIGHT*^{-/-} mice were anesthetized with ketamine (200 mg/kg). Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with the Transonic micro-flow probe

(0.5 mm; Transonic Systems, Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride (FeCl₃) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 min. Blood flow was continuously monitored for 30 min, or until blood flow reached stable occlusion (zero blood flow for 2 min). Data were recorded and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the FeCl₃-containing filter paper. An occlusion time of 30 min was considered the cut-off time for the purpose of statistical analysis.

Flow cytometric analysis

Flow cytometric analysis was performed as described before.¹⁹ Briefly, platelets (2×10^8) were stimulated with ADP (0.0625 and 0.25 μ M) or U46619 (0.5 μ M) for 3 min and then fixed with 2% formaldehyde for 30 min at room temperature, followed by incubation for 30 min at room temperature with FITC-conjugated anti-P-selectin and Annexin V (Cell Signaling Technology, Inc., Danvers, MA), FITC-conjugated JON/A (Integrin $\alpha_{IIb}\beta_3$; EMFRET Analytics, Würzburg, Germany). Fluorescent intensities of the platelets diluted 2.5-fold with HEPES/Tyrode's buffer (pH 7.4) were measured using a BD Accuri C6 flow cytometer. Results were analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ). Each experiment was repeated at least three times with blood pooled from $n = 6$ mice.

Transmission electron microscopy

Mouse platelets were isolated and processed for transmission electron microscopy as described previously.²⁰ Briefly, equal volumes of 0.1% glutaraldehyde in White's saline were added to the C57BL/6 or *TNFSF14/LIGHT*^{-/-} platelet suspension for 15 min at 37°C. The platelets were centrifuged and incubated in ice-cold 3% glutaraldehyde in White's saline at 4°C for 1 h. After three washes, the platelets were incubated with 1% OsO₄. Osmicated platelets were washed twice with water and dehydrated with a series of ethanol solutions (50%, 70%, 80%, 90%, and 100%). The platelets were rinsed twice with propylene oxide and infiltrated overnight in a 1:1 mixture of propylene oxide and Spurr's resin (4.1 g ERL-4221 [3,4 epoxy cyclohexylmethyl 3,4 epoxy cyclohexyl carboxylate], 5.9 g nonenyl succinic anhydride, 1.43 g DER-736 epoxy resin, and with final addition of 0.1 g 2-dimethylaminoethanol). After several washes in pure Spurr's resin, samples were embedded in Spurr's resin and polymerized at 60°C for 24 h. Polymerized blocks were sectioned (70 nm) and mounted on copper grids. Following

counterstaining with uranyl acetate and lead citrate, samples were examined using FEI Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR) and images were obtained with Gatan Digital micrograph software (Pleasanton, CA).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A one-way ANOVA, followed by a pairwise comparison post-test, was conducted wherever appropriate. A 95% confidence interval was considered as statistically significant.

RESULTS

TNFSF14/LIGHT^{-/-} wounded mice have enhanced platelet aggregation

To examine platelet behavior in the *TNFSF14/LIGHT*^{-/-} mice after injury, we performed platelet aggregation assays using different concentrations of the platelet aggregation agonists, ADP and U46619. We observed that platelet aggregation in the presence of ADP is increased at 3 days postwounding in the *TNFSF14/LIGHT*^{-/-} mice when

compared with the control C57BL/6 mice and that aggregation increased in a dose-dependent manner (Fig. 1A). Similar data were obtained when using U46619 the TXA₂ mimetic (Fig. 1B). Furthermore, we examined whether the increase in platelet aggregation also occurred at a later time postwounding. Higher levels of platelet aggregation still occurred in the *TNFSF14/LIGHT*^{-/-} mice at day 7 postwounding, also in a dose-dependent manner (Fig. 1C, D). However, before wounding there are no differences between the platelets in the two types of mice (Fig. 2).

Thrombogenesis is enhanced in wounded *TNFSF14/LIGHT*^{-/-} mice

To determine whether thrombus formation, evaluated by occlusion time, was affected in the *TNFSF14/LIGHT*^{-/-} mice, we used a FeCl₃-induced carotid artery injury thrombosis model (Fig. 3). No significant differences in occlusion time were observed between unwounded *TNFSF14/LIGHT*^{-/-} and control (C57BL/6) mice. However, at day 3 postwounding, the time to occlusion in *TNFSF14/LIGHT*^{-/-} mice was significantly decreased in comparison to control C57BL/6 mice. Complete occlusion of the vessel wall after FeCl₃

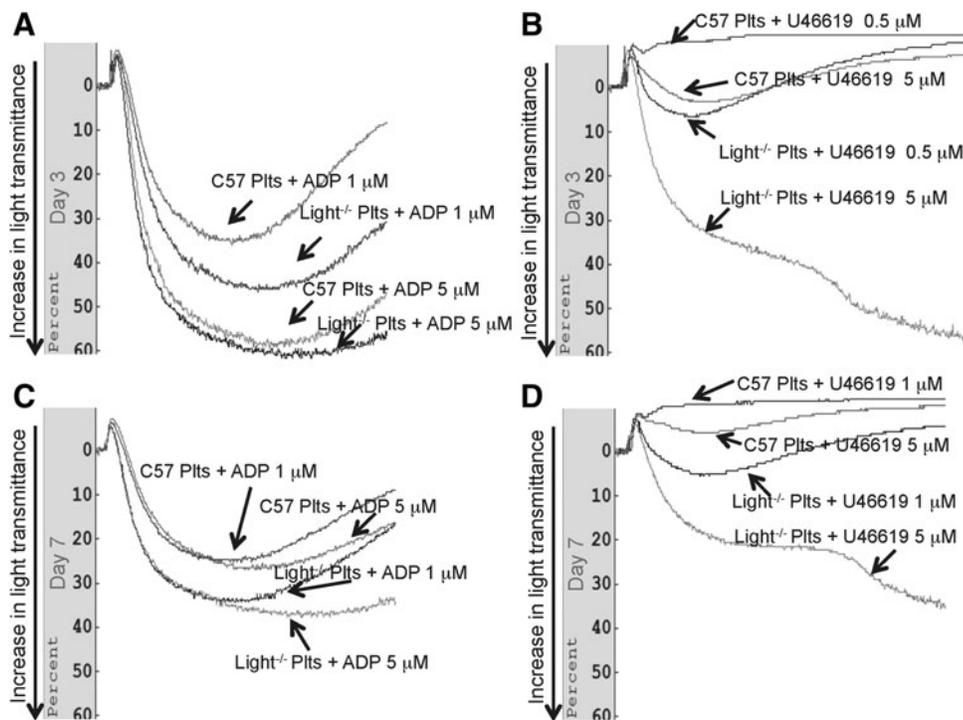


Figure 1. Platelet aggregation is increased during impaired healing. Platelet aggregation was determined using an aggregometer and the agonists ADP and U46619. (A) Platelet aggregation 3 days postwounding was significantly enhanced in the *TNFSF14/LIGHT*^{-/-} mice and the aggregation occurred in an ADP dose-dependent manner. (B) Similar effects were seen when the platelets were stimulated with the agonist U46619. (C, D) At day 7 postwounding, there was enhanced platelet aggregation in *TNFSF14/LIGHT*^{-/-} mice when both ADP and U46619 were used as the agonist for activation. We pooled the blood from five mice because this assay requires a significant amount of blood. Therefore, in total $n=5$ for each assay. *TNFSF14*, tumor necrosis factor superfamily 14. ADP, adenosine diphosphate.

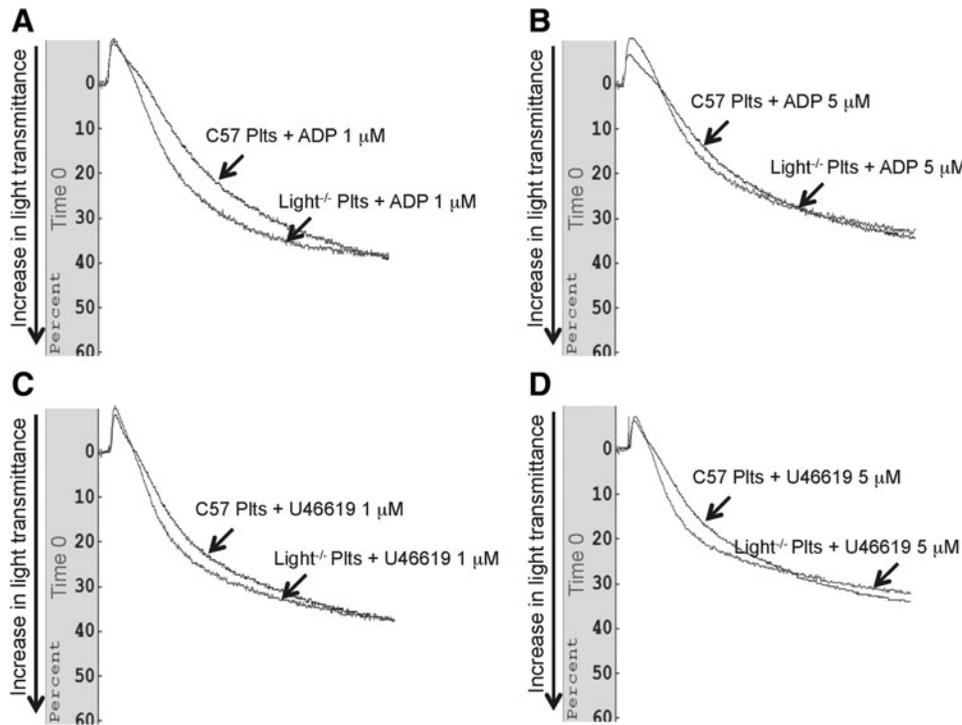


Figure 2. Platelets of unwounded mice show no difference in time to aggregation. Platelet aggregation was determined using an aggregometer and the agonists (A, B) ADP and (C, D) U46619. No difference in time to aggregation was seen. For each assay we pooled the blood from five mice.

treatment of the KO mouse occurred in about 4 min, whereas wounded control mice took more than 22 min. At day 7 postwounding, the occlusion time in *TNFSF14/LIGHT*^{-/-} wounded mice continued to be low, but because of variability in the occlusion

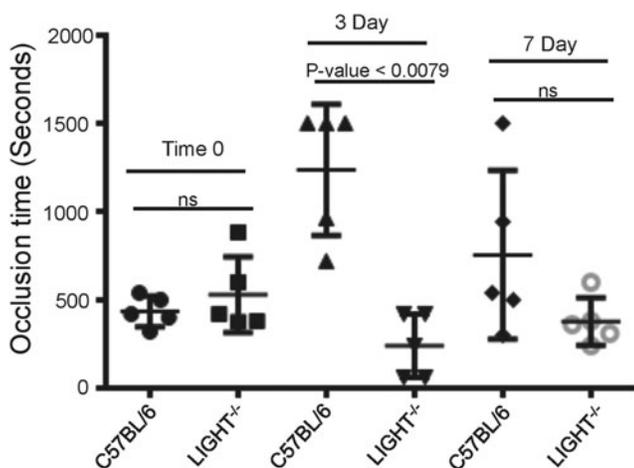


Figure 3. Occlusion time is shortened during impaired healing. C57BL/6 (control) and *TNFSF14/LIGHT*^{-/-} mice were anesthetized and the FeCl₃-induced carotid artery injury model was performed as discussed in Materials and Methods section. Each point represents the occlusion time of a single animal. Occlusion time postwounding in *TNFSF14/LIGHT*^{-/-} mice was significantly reduced compared with the C57BL/6 wounded mice at day 3 postwounding ($n=5$).

time for the control C67BL/6 mice, the difference in time to occlusion between the two strains of mice was no longer statistically different.

Activated *TNFSF14/LIGHT*^{-/-} platelets have enhanced P-selectin levels

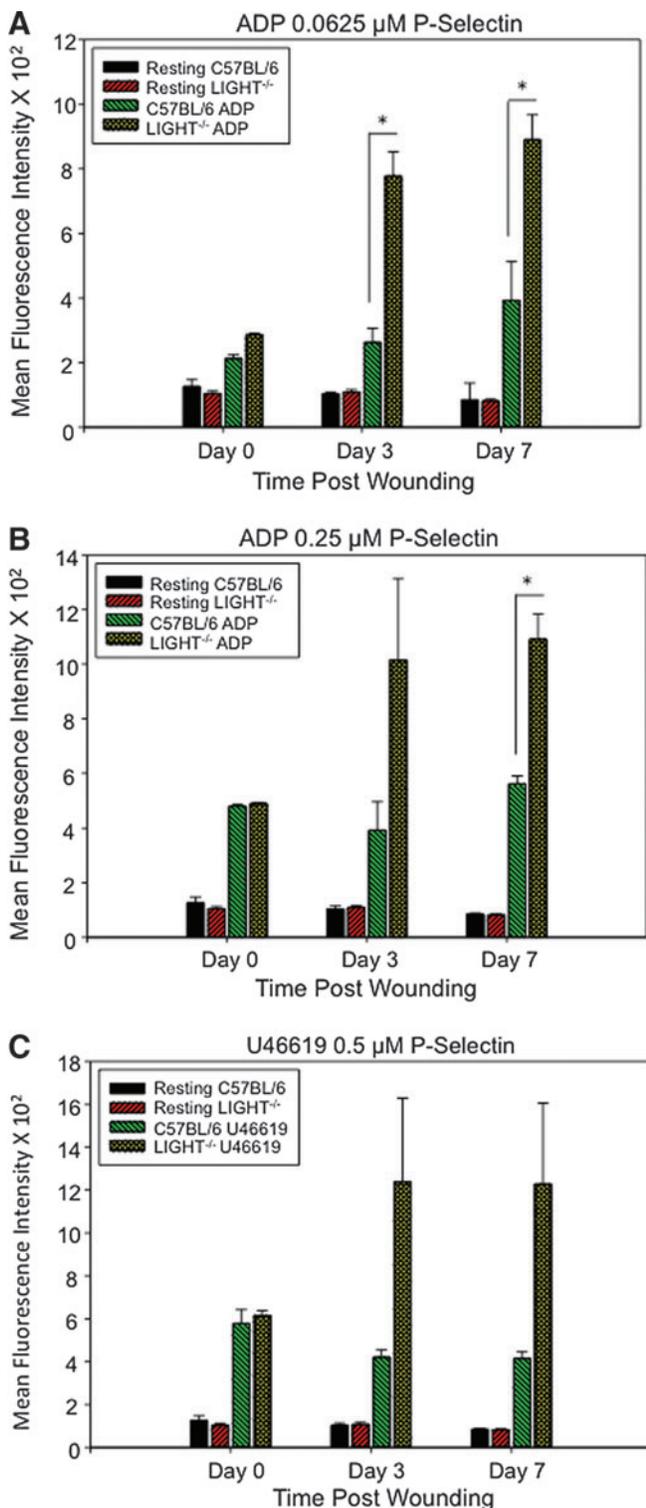
Release of granule content is the earliest event in the platelet activation process. The results described above show that the platelets in wounded *TNFSF14/LIGHT*^{-/-} are hyperactive. Therefore, we conducted platelet responsiveness studies to different agonists by measuring the levels of P-selectin using flow cytometry. P-selectin levels in activated platelets of unwounded mice were not significantly different in *TNFSF14/LIGHT*^{-/-} and C57BL/6 mice (Fig. 4A). However, by day 3 postwounding, *TNFSF14/LIGHT*^{-/-} platelets displayed significantly higher levels of P-selectin than those of C57BL/6 mice, a difference that continued for day 7 wounds. The ADP increased the P-selectin levels in a dose-dependent manner (Fig. 4B) and similar results were found using U46619 (0.5 μm), the mimetic for the TXA₂ receptor (Fig. 4C).

Integrin $\alpha_{IIb}\beta_3$ levels are higher in activated platelets from wounded *TNFSF14/LIGHT*^{-/-}

It is well documented that integrin $\alpha_{IIb}\beta_3$ plays a critical role in platelet aggregation in response to physiological agonists and that it mediates

thrombus formation.²¹ To determine whether the enhanced platelet aggregation from wounded *TNFSF14/LIGHT*^{-/-} mice is associated with an increase in the levels of integrin $\alpha_{IIb}\beta_3$, we performed fluorescence-activated cell sorting analysis with an antibody specific to this integrin. We show that, upon activation with ADP (0.062 μ M and

0.25 μ M), the platelets collected from unwounded *TNFSF14/LIGHT*^{-/-} and C57BL/6 mice showed equal levels of integrin $\alpha_{IIb}\beta_3$ on the plasma membrane. However, upon wounding, the levels of integrin $\alpha_{IIb}\beta_3$ on the platelet membrane in the *TNFSF14/LIGHT*^{-/-} mice at both days 3 and 7 were significantly higher than those of C57BL/6 platelets (Fig. 5A, B). We also found that the levels of integrin $\alpha_{IIb}\beta_3$ in the wounded *TNFSF14/LIGHT*^{-/-} mice platelets were significantly enhanced with the agonist for the TXA2 receptor U46619 (Fig. 5C). However, there was no significant difference on the levels of integrin $\alpha_{IIb}\beta_3$ between days 3 and 7 postwounding.



PS levels are higher in platelets from wounded *TNFSF14/LIGHT*^{-/-}

Exposure of negatively charged PS to the outer leaflet of the plasma membrane bilayer occurs in activated platelets and is followed by increased platelet procoagulant activity.²² Therefore, we determined whether the platelets from wounded *TNFSF14/LIGHT*^{-/-} mice had enhanced PS levels upon agonist activation. Platelets from unwounded *TNFSF14/LIGHT*^{-/-} and C57BL/6 mice, when activated using ADP, show no significant difference in flipping of PS to the outer leaflet of the membrane (Fig. 6A, B; day 0). However, platelets from wounded *TNFSF14/LIGHT*^{-/-} mice show an augmented presence of PS on the outer leaflet of the plasma membrane compared with platelets from C57BL/6 mice (Fig. 6A, B; days 3 and 7). Moreover, U46619 activation of platelets also caused significant increases in PS exposure on *TNFSF14/LIGHT*^{-/-} wounded mice platelets in comparison to controls (Fig. 6C; days 3 and 7).

Platelets from wounded *TNFSF14/LIGHT*^{-/-} mice have a more extensive open canalicular system

Because we observed a significant increase in the levels of aggregation and of prothrombotic

Figure 4. Surface P-selectin levels are enhanced during impaired wounding. Surface P-selectin levels on the platelet plasma membrane after platelet activation were measured using FACS. (A, B) Platelet activation using 0.0625 μ M ADP led to significant increases in P-selectin on the platelet membrane in *TNFSF14/LIGHT*^{-/-} mice when compared with C57BL/6 at both day 3 and 7 postwounding. An overall increase in P-selectin was observed at day 3 in *TNFSF14/LIGHT*^{-/-} mice and the levels were significantly higher at day 7 postwounding in *TNFSF14/LIGHT*^{-/-} mice when compared with C57BL/6 mice. (C) Agonist U46619 0.5 μ M caused similar effects on P-selectin levels in *TNFSF14/LIGHT*^{-/-} wounds after wounding when compared with C57BL/6 wounded mice ($n=5$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

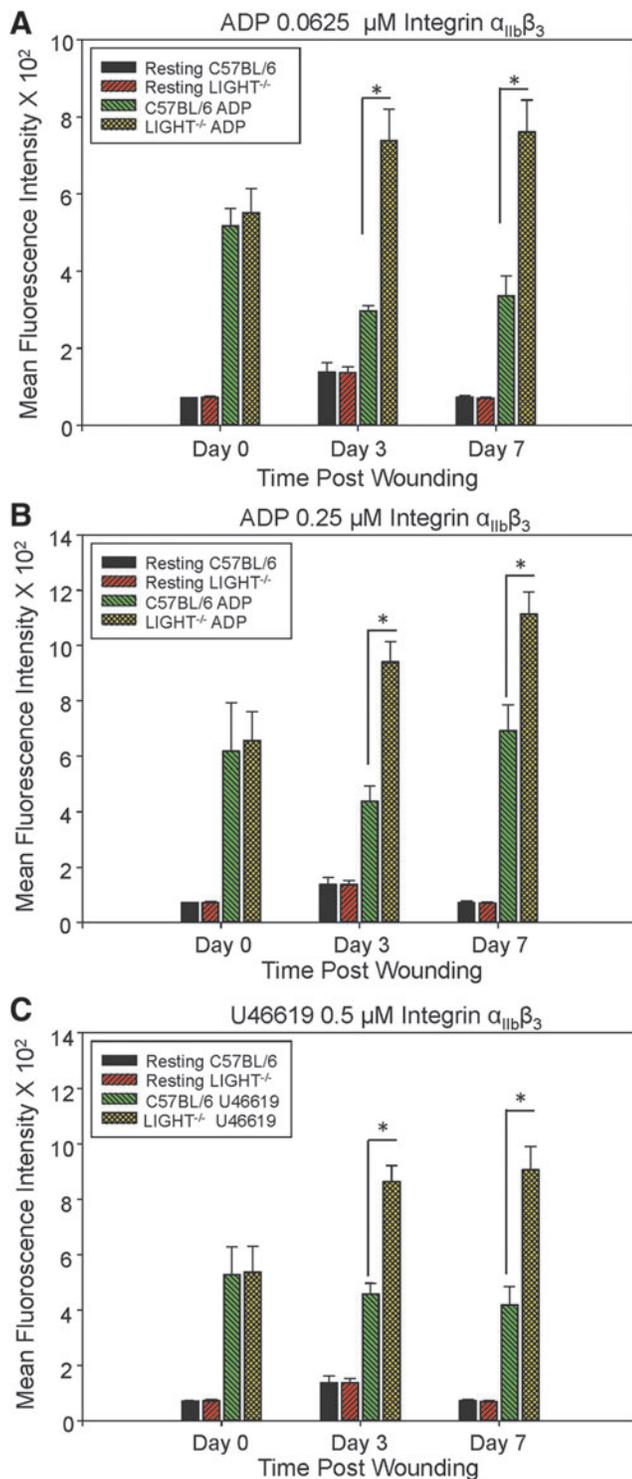


Figure 5. Surface platelet integrin $\alpha_{\text{IIb}}\beta_3$ levels are increased in impaired healing. Integrin $\alpha_{\text{IIb}}\beta_3$ levels resulting from platelet activation were determined by flow cytometry. Integrin $\alpha_{\text{IIb}}\beta_3$ levels were significantly increased in *TNFSF14/LIGHT*^{-/-} mice by (A) 0.0625 μM ADP, (B) 0.25 μM ADP, and (C) 0.5 μM U46619. Significant increases in receptor levels were observed in *TNFSF14/LIGHT*^{-/-} mice at day 3 and 7 postwounding. No difference was seen in $\alpha_{\text{IIb}}\beta_3$ levels in activated platelets from unwounded mice of both strains (day 0) ($n=5$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

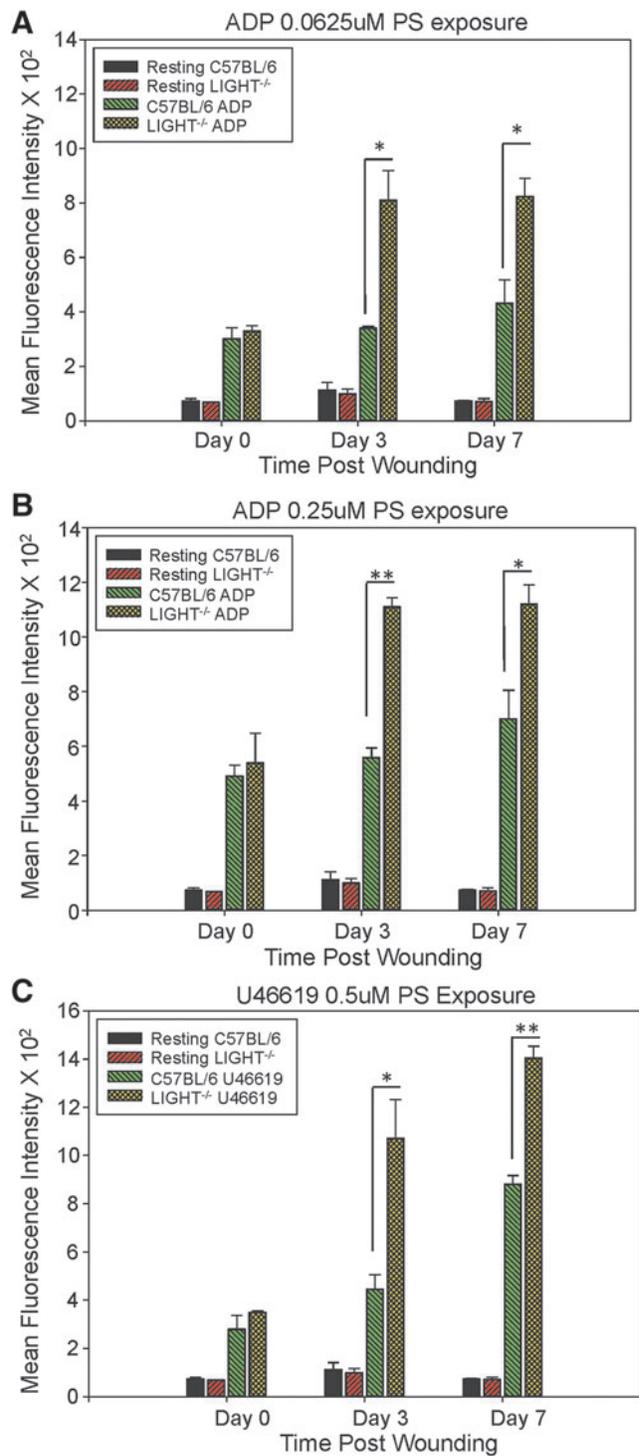


Figure 6. Wounded *TNFSF14/LIGHT*^{-/-} mice platelets exhibit increased PS on the outer leaflet of the membrane. Exposure of PS on the surface of platelets in the resting and activated state was estimated using FACS. The translocation of PS onto the outer leaflet of the platelet plasma membrane was significantly increased in *TNFSF14/LIGHT*^{-/-} mice by (A) 0.0625 μM ADP, (B) 0.25 μM ADP, and (C) 0.5 μM U46619 agonist activation of platelets. The significant increase in translocation of PS onto the outer leaflet of the platelet plasma membrane from wounded *TNFSF14/LIGHT*^{-/-} when compared with wounded *C57BL/6* mice, supports our results of increase in aggregation of platelets in *TNFSF14/LIGHT*^{-/-} mice ($n=5$). PS, phosphatidylserine. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/wound

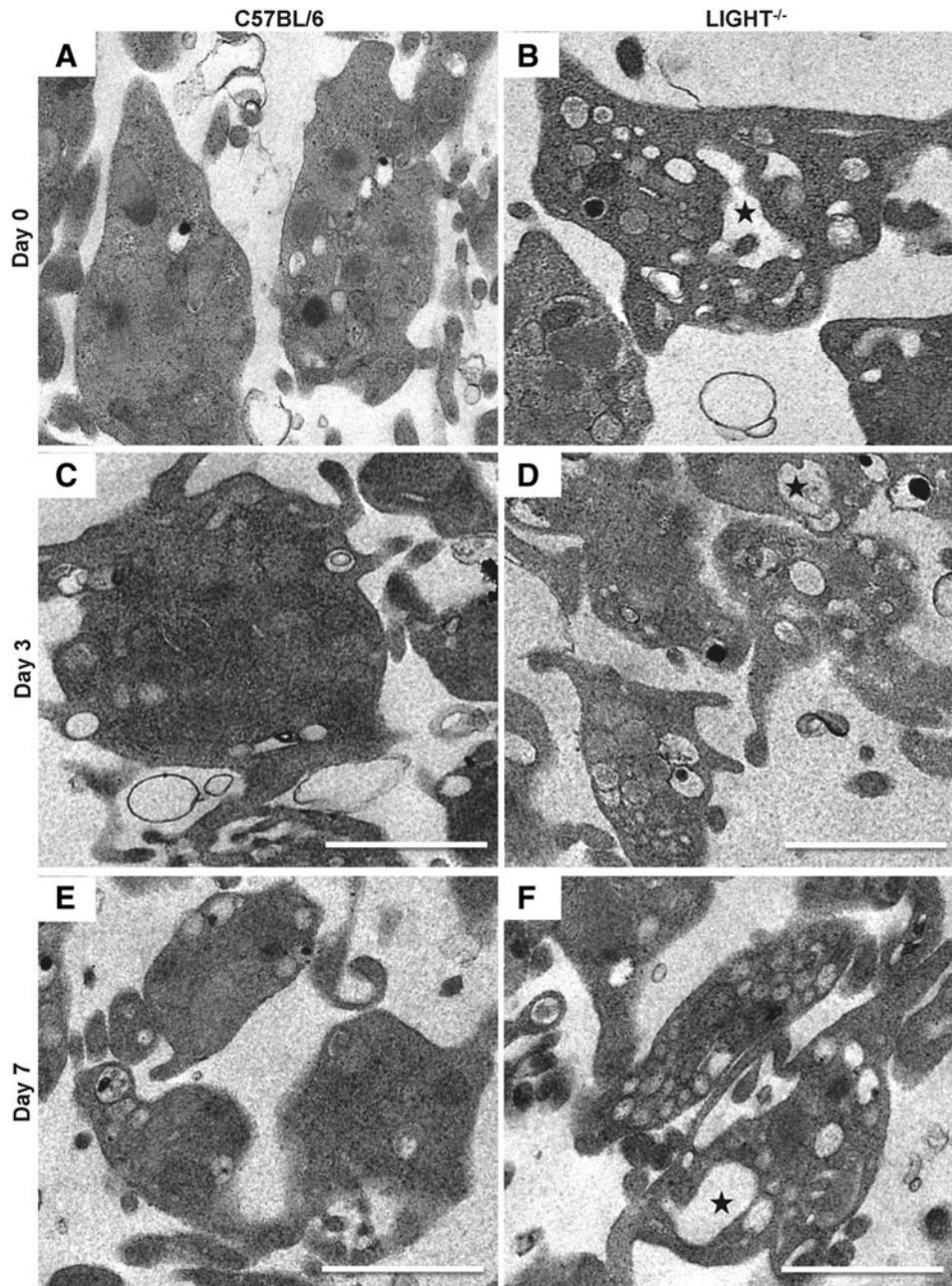


Figure 7. Resting *TNFSF14/LIGHT*^{-/-} mice platelets show increased OCS. Resting C57BL/6 (A, C, E) and *TNFSF14/LIGHT*^{-/-} (B, D, F) platelet structure was assessed by using transmission electron microscopy. Increased presence of a dilated OCS was observed only in resting platelets from *TNFSF14/LIGHT*^{-/-} mice (stars in B, D, F), suggesting increased surface area for interaction upon activation and granule release. OCS, open canalicular system.

markers after activation, we used transmission electron microscopy to determine whether the resting platelets showed differences in their structural integrity. We found that while the overall structure of the platelets was intact in both the C57BL/6 and *TNFSF14/LIGHT*^{-/-} mice, the presence of a dilated open canalicular system (OCS) was more extensive in *TNFSF14/LIGHT*^{-/-} platelets (Fig. 7A–F).

DISCUSSION

The role of platelet function in impaired wound healing has not been extensively studied. In the present study, we provide the first evidence of the presence of hyperactive platelets and problems in thrombosis in a model of impaired healing, the *TNFSF14/LIGHT*^{-/-} mouse. Platelets from these wounded KO mice have higher aggregation re-

sponse when treated with agonist than do the wounded control mice. They also have higher levels of P-selectin, integrin $\alpha_{IIb}\beta_3$, and PS on the plasma membrane after activation than do the wounded control C57BL/6 mice. Resting platelets obtained from *TNFSF14/LIGHT*^{-/-} mice have a large OCS when compared with control resting platelets. The enlarged presence of OCS in resting platelets in the *TNFSF14/LIGHT*^{-/-} mice suggests availability of extensive surface for release of α -granules and other platelet components, including provision for increased area for surface interaction after activation.

The significantly higher levels of P-selectin on activated platelets from wounded *TNFSF14/LIGHT*^{-/-} mice suggest an increase in adhesion and formation of platelet-leukocyte complexes. Furthermore, high levels of P-selectin have been associated with increasing size and stability of platelet aggregates.²³ This suggests that the formation of clots in blood vessels in various tissues of wounded *TNFSF14/LIGHT*^{-/-} mice¹⁶ is a result of abnormal platelet behavior. Moreover, these aggregates could play a critical role in creating a hypoxic environment that can further contribute to impaired wounds.

Elevated levels of integrin $\alpha_{IIb}\beta_3$ detected on activated platelets of wounded *TNFSF14/LIGHT*^{-/-} mice indicate that they have a significantly high risk of formation of atherosclerotic plaques.²⁴ Indeed, we observed that some of these mice died and, when analyzed, had very large numbers of clots in vessels of the heart (unpublished results). The increased levels of integrin $\alpha_{IIb}\beta_3$ could also explain its increased availability for binding to fibrinogen, where fibrinogen and vWF compete (fibrinogen outcompetes vWF) to bind to integrin $\alpha_{IIb}\beta_3$.²⁵ Furthermore, integrin $\alpha_{IIb}\beta_3$ -fibrinogen interactions help in the firm bridging of platelets with the endothelium causing thrombus formation, initially stabilized by the presence of P-selectin, which was also highly present in *TNFSF14/LIGHT*^{-/-} mice platelets. These observations suggest critical interactions facilitating the development of larger platelet aggregates.^{23,26} Moreover, increase in PS in wounded *TNFSF14/LIGHT*^{-/-} mice indicates a higher degree of thrombin generation and coagulation observed in the blood of these mice. In addition, increase in platelet integrin $\alpha_{IIb}\beta_3$ that supports the binding of activated platelets to the fibrin network could also increase PS exposure on the platelet surface.²⁷

An important structure of the platelet membrane is the presence of invaginations known as OCS that provide the capability to increase the

surface to volume ratio of the membrane upon activation of platelets. The OCS provides the vehicle for external microenvironmental components to interact with the interior of the platelet, while the platelet contents, including the granules, exit through the OCS upon activation.²⁸ The presence of bigger and more dilated OCS suggests the availability of more surface-to-volume interaction of the *TNFSF14/LIGHT*^{-/-} platelets with the surrounding microenvironment. Furthermore, the presence of integrin $\alpha_{IIb}\beta_3$ on the surface of OCS before activation of the platelets, may explain the significant increase in the exposure of this integrin in activated *TNFSF14/LIGHT*^{-/-} platelets.²⁹

Although there have been a few studies showing that *TNFSF14/LIGHT* plays a role in platelet biology, they have been primarily focused on the release of this protein from the platelets. Indeed, these studies have shown that when *TNFSF14/LIGHT* is released in high levels from the platelets, it goes into circulation and induces a vigorous inflammatory response that affects both endothelial cells and inflammatory cells alike, resulting in a variety of diseases/disorders.³⁰⁻³⁴ It has also been proposed that, upon activation, platelets release superoxide anions and that the availability of superoxide anions leads to greater metabolism of ADP, enhancing further recruitment and activation of platelets.³⁵ Furthermore, collagen-induced platelet activation in wounds can produce high amounts of H₂O₂ that could stimulate platelet aggregation pathways.³⁶ We recently showed that *TNFSF14/LIGHT*^{-/-} mice have high levels of oxidative stress early in wound healing with increased presence of H₂O₂ and isoprostanes,¹⁸ suggesting the enhancement of the platelet activation we now report here. Moreover, reactive oxygen species frequently observed in impaired and chronic wounds have increasingly been shown to be major players in the activation of platelets and to cause thrombus formation.^{37,38}

In conclusion, this work shows a novel function for *TNFSF14/LIGHT* during wound healing; in the absence of this molecule from the cell surface, platelets show higher levels of aggregation, potentially contributing to rapid thrombosis during the early stages of healing, which might contribute to impaired healing by reducing the ability of the blood vessels to transport nutrients and oxygen as well as other serum molecules needed for proper healing of the wound tissue. Furthermore, this environment can lead to cell death, contributing to impaired healing. These findings also suggest a role for *TNFSF14/LIGHT* molecule in controlling platelet function during human wound healing.

Because chronic venous stasis ulcers in humans have an underlying state of platelet activation similar to those seen in *TNFSF14/LIGHT*^{-/-} mice, we speculate that dysregulation of platelet function in these chronic wounds could be due to decreased levels or absence of *TNFSF14/LIGHT* on the surface of platelets.

INNOVATION

Advancement in therapeutics to treat impaired wounds has been hindered due to lack of understanding of mechanisms behind chronic wound etiology. Upon injury, reestablishment of hemostasis occurs through activation of platelets, the first responders that are also crucial in the pathophysiology of vascular clots and thrombosis. However, their role in impaired wounds is not well understood. Higher levels of aggregation observed in platelets of injured *LIGHT*^{-/-} mice might explain their part in establishing an environment conducive to improper healing. Our results, for the first time, suggest that dysregulated platelet function could be due to abnormal expression of *TNFSF14/LIGHT* on the surface of platelets.

LIMITATIONS OF THE SYSTEM

Because the *TNFSF14/LIGHT KO* mouse has a global and not platelet-specific deletion of the gene, our FeCl₃ thrombosis model could also have contributions from the endothelial cells in the vasculature and other blood components. Furthermore, the aggregation response observed in the platelets requires pooling of the blood from several mice making it difficult to analyze platelets from individual animals to better understand the variability.

AUTHORS' CONTRIBUTIONS

S.D., Z.A.K., F.K., and M.M.G. conceived and designed the study. S.D. and Z.A.K. performed the experiments. S.D., Z.A.K., F.K., and M.M.G. discussed the results and contributed to the data interpretation. S.D. and M.M.G. wrote the article and Z.A.K. and F.K. edited the article. All authors reviewed and revised the semi-final version of the article.

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KEY FINDINGS

- Enhanced response in platelet aggregation and faster time to blood vessel occlusion (thrombosis) was observed upon wounding in the *TNFSF14/LIGHT*^{-/-} mouse model of impaired healing.
- Platelets from wounded *TNFSF14/LIGHT*^{-/-} mice have increased levels of P-selectin, integrin $\alpha_{IIb}\beta_3$, and PS.
- *TNFSF14/LIGHT*^{-/-} platelets have more extensive OCS than platelets of control mice.

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AUTHOR DISCLOSURE AND GHOSTWRITING

The authors have no conflicts of interest to declare. There are no ghostwriter contributions in this work.

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Abbreviations and Acronyms

ADP	=	adenosine diphosphate
ATP	=	adenosine triphosphate
ECM	=	extracellular matrix
FeCl ₃	=	ferric chloride
HETE	=	hydroxyeicosatetraenoic acid
KO	=	knockout
LIGHT	=	lymphotoxin-like inducible protein that competes with Glycoprotein D for binding herpes virus entry mediator on T cells
LT	=	leukotrienes
OCS	=	open canalicular system
OsO ₄	=	osmium tetroxide
PG	=	prostaglandins
PS	=	phosphatidylserine
TNFSF14	=	tumor necrosis factor superfamily 14
TX	=	thromboxane
UCR	=	University of California, Riverside
vWF	=	von Willebrand factor