Investigation of a Thromboxane A$_2$ Receptor–Based Vaccine for Managing Thrombogenesis

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Background—Despite the well-established role for the thromboxane A$_2$ receptor (TPR) in the development of thrombotic disorders, none of the antagonists developed to date has been approved for clinical use. To this end, we have previously shown that an antibody targeted against TPR's ligand-binding domain inhibits platelet activation and thrombus formation, without exerting any effects on hemostasis. Thus, the goal of the present studies is to design a novel TPR-based vaccine, demonstrate its ability to trigger an immune response, and characterize its antiplatelet and antithrombotic activity.

Methods and Results—We used a mouse keyhole limpet hemocyanin/peptide-based vaccination approach rationalized over the TPR ligand-binding domain (ie, the C-terminus of the second extracellular loop). The biological activity of this vaccine was assessed in the context of platelets and thrombotic diseases, and using a host of in vitro and in vivo platelet function experiments. Our results revealed that the TPR C-terminus of the second extracellular loop vaccine, in mice: (1) triggered an immune response, which resulted in the development of a C-terminus of the second extracellular loop antibody; (2) did not affect expression of major platelet integrins (eg, glycoprotein IIb-IIIa); (3) selectively inhibited TPR-mediated platelet aggregation, platelet-leukocyte aggregation, integrin glycoprotein IIb-IIIa activation, as well as dense and α granule release; (4) significantly prolonged thrombus formation; and (5) did so without impairing physiological hemostasis.

Conclusions—Collectively, our findings shed light on TPR's structural biological features, and demonstrate that the C-terminus of the second extracellular loop domain may define a new therapeutic target and a TPR vaccine-based approach that should have therapeutic applications. (*J Am Heart Assoc*. 2018;7:e009139. DOI: 10.1161/JAHA.118.009139.)

Key Words: antiplatelet agent • platelets • thrombosis • thromboxane A$_2$ receptor • vaccine

On injury to a blood vessel, the subendothelial matrix that is normally shielded from platelets gets exposed, revealing many agonists and factors that are critical for platelet adherence and subsequent activation.[1–4] Consequently, platelets will interact with the exposed subendothelial matrix,[4,5] leading to intraplatelet signaling and activation, which is associated with several events, including release of arachidonic acid from the membrane phospholipids.[6,7] The liberated arachidonic acid is metabolized by the cyclooxygenase enzyme and thromboxane A$_2$ (TXA$_2$) synthase, leading to the formation of TXA$_2$.[8–10] TXA$_2$, a well-studied platelet agonist, is a labile lipid mediator that acts through binding to its G-protein–coupled receptor[11–13] (namely, the TXA$_2$ receptor [TPR]). This interaction causes a wide variety of biological effects, including platelet aggregation. To this end, TPR's clear role in normal hemostasis is supported by the finding that "patients" have a bleeding disorder as a result of a point mutation in the receptor protein.[14–16] On the other hand, upregulated signaling through TPR has been implicated in the pathogenesis of multiple cardiovascular and thrombosis-based diseases.[17–20] Consistent with this concept are clinical findings indicating that inhibition of platelet TXA$_2$ production provides therapy for thromboembolic diseases,[21–23] which is the underlying rationale for the use of aspirin in such diseases.[24,25]

Consequently, the TXA$_2$ pathway has been targeted for pharmacological intervention to either inhibit its formation or modulate binding to its receptor. In light of this fact, several possibilities have emerged to achieve this goal, with cyclooxygenase enzyme inhibitors and thromboxane synthase inhibitors being the initial lead candidates. However, although the

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Clinical Perspective

What Is New?

- This is the first study to demonstrate that a vaccine modeled over the ligand-binding domain of a platelet G-protein–coupled receptor (namely, the thromboxane A_2 receptor) inhibits platelet activation and exhibits protective effects against thrombogenesis.

What Are the Clinical Implications?

- On the basis of our findings, as well as future human studies, and approval by the Food and Drug Administration, clinicians may elect to recommend/prescribe this vaccine as a therapeutic approach for managing patients under the risk of thrombotic disease.

cyclooxygenase enzyme inhibitor aspirin is in clinical use, it is associated with inherent limitations.[26–28] Thus, aspirin: was found to (1) lack selectivity to TXA_2 because it also inhibits prostaglandin I_2 synthesis[25]; (2) cause bleeding[29] and gastric ulcers,[28] adverse effects that in some instances mandate its discontinuation; and (3) redirect arachidonic acid metabolism to isoprostanes, which themselves modulate platelet function[30]; and is (4) associated with sensitivity; and (5) an increasing rate of resistance worldwide.[26–28]

The thromboxane synthase inhibitors, on the other hand, exhibited minimal activity[31,32] because the immediate precursor of TXA_2, prostaglandin H_2, binds to the same receptor[7,33,34] and can, therefore, induce platelet aggregation.[35] On this basis, it became clear that more selective means of blocking TXA_2-mediated platelet aggregation needed to be developed, and receptor blockade seemed to be the most logical and promising approach. Thus, several TPR antagonists were designed throughout the years and tested for biological activity.[35–38] Although in vitro results were encouraging, the in vivo effectiveness of these molecules was limited by short biological half-life, toxicity, or limited tissue distribution. One apparent reason for this failure is because these agents were empirically designed on the basis of the complex structures of prostaglandin H_2 and/or TXA_2, with little information on the actual TPR binding domains. In this connection, research efforts[11,30,39–41] by us and others to map the TPR ligand-binding domain revealed the following: (1) the ligand-binding domain resides in the C-terminus of the second extracellular loop (C-EL2; C^{183}-D^{193}) of the receptor protein; (2) this extracellular segment contains ligand–amino acid coordination sites; and (3) an antibody raised against this sequence (ie, abbreviated C-EL2Ab) inhibits TPR ligand binding and platelet aggregation and protects from thrombogenesis without any apparent bleeding diathesis, making it the first functional antibody against platelet TPRs. Based on these considerations, we sought to further assess the

contributions of the C-EL2 domain to in vivo TPR-dependent platelet activation (eg, hemostasis/bleeding time) and the genesis of thrombosis, by using a vaccine-based approach using the cognate TPR C-EL2 peptide as an immunogen. Our results revealed that immunizing mice with the C-EL2 peptide TPR vaccine did lead to the production of a C-EL2 TPR antibody, and consequently inhibition of aggregation induced by the TPR agonist U46619. However, it produced no effects on aggregation stimulated by separate agonists (namely, ADP and thrombin receptor-activating peptide 4 [TRAP4]). Moreover, platelets from the immunized mice also exhibited selective defects in TPR-dependent dense and α granule release, platelet-leukocyte aggregation, and glycoprotein IIb-IIIa. In terms of its in vivo activity, the TPR C-EL2 vaccinated mice exhibited a prolonged time for occlusion, but their bleeding time was no different from the controls. On the other hand, vaccinating mice with the random version of the C-EL2 peptide (ie, C-EL2r vaccine) or keyhole limpet hemocyanin (KLH) exerted no effects on platelet function, in vitro and in vivo. Together, these findings indicate that the TPR C-EL2–based vaccine protects against thrombogenesis, without impairing hemostasis. Among other advantages, this active vaccination approach would not be expected to face some of the functional limitations an antagonist does, including frequent administration and high costs.

Methods

Reagents and Materials

Stir bars and other disposables were from Chrono-Log (Havertown, PA). U46619, the thromboxane receptor (TPR) agonist, and SQ29,548, the TPR antagonist, were obtained from Cayman Chemical (Ann Arbor, MI); and the protease activated receptor 4 agonist peptide (TRAP4) was from Peptides International (Louisville, KY). ADP, KLH, and complete and incomplete Freund's adjuvants were obtained from Sigma Aldrich (St Louis, MO). The anti–P-selectin antibody was purchased from Cell Signaling Technology, Inc (Danvers, MA), whereas anti–glycoprotein IIb-IIIa, anti–glycoprotein Ib, and JON/A antibodies were purchased from Emfret Analytics (Germany). The anti–glycoprotein VI antibody was from R&D Systems (Minneapolis, MN). The anti-CD11b antibody was from Thermo Fisher Scientific (Waltham, MA). The BD FACS™ lysing solution was from BD Biosciences (Franklin Lakes, NJ).

Animals

C57BL/6J mice (aged 8–10 weeks, mixed sex) were from Jackson Laboratories (Bar Harbor, ME) and were housed in groups of 1 to 4 at 24°C, under 12/12-hour light/dark cycles, with access to water and food ad libitum. All experiments involving animals were performed in compliance with the

institutional guidelines and were approved by the Institutional Animal Care and Use Committee.

Methods

The data, analytic methods, and study materials will not be made available to other researchers for purposes of reproducing the results or replicating the procedure at this time, because of patent considerations. However, once the US Patent and Trademark Office makes a final decision on the patent application pertaining to the present "vaccine," requests will be honored on a case–by–case basis, and will be provided by the corresponding author.

Vaccination Protocol

Mice were immunized with KLH-coupled peptide corresponding to the human C-EL2 sequence CFLTLGAESGD (C[183]-D[193]), and control animals were immunized with random C-EL2 (abbreviated C-EL2r; STLACGFDGEL), with an additional cysteine synthesized at the end of this peptide to allow coupling with KLH (CSTLACGFDGEL) or with KLH alone. Mice received intraperitoneal injections of peptides (35 μg) or KLH dissolved in Freund's complete adjuvant. The animals were boosted 3 times with peptides (and Freund's incomplete adjuvant) at days 14, 28, and 42. Described were performed 2 weeks after the conclusion of the immunizations protocol (day 56), unless otherwise indicated. The mouse and the human platelet TPRs are identical in 17 of the 21 amino acids that are located in the second extracellular loop region of the receptor protein.

Measurement of Antibody Production in the Vaccinated Mice Using ELISA

ELISAs were performed to test the antibody development in the immunized mice (C-EL2 vaccine, C-EL2r vaccine, and KLH). Nunc-Immuno MicroWell 96-well plates were coated with 12.5 μg/well C-EL2 peptide for 18 to 24 hours at room temperature. After the incubation, the plates were washed 3 times with 200 μL/well modified Tyrode's buffer (0.1% BSA; 20 mmol/L HEPES/KOH, pH 7.4; 128 mmol/L NaCl; 2.8 mmol/L KCl; 1 mmol/L MgCl$_2$; 0.4 mmol/L NaH$_2$PO$_4$; 12 mmol/L NaHCO$_3$; and 5 mmol/L D-glucose). Then, non-specific sites were blocked by incubation for 1 hour with 5% BSA (200 μL/well) in the same buffer. Plates were again washed 3 times with the modified Tyrode's buffer before applying serial dilutions of C-EL2 peptide to the wells in triplicate. Antibodies were allowed to incubate for 1 hour, followed by 3 more washes. Antibodies bound to the immobilized peptide were detected by incubation (1 hour) with goat anti-mouse IgG (heavy+light) conjugated to horseradish peroxidase. The wells were then washed a final time before the addition of the horseradish

peroxidase substrate solution. After 10-minute incubation in the dark, the reaction was quenched with 2 N H$_2$SO$_4$ (200 μL/well). The presence of specific antibodies was measured by the absorbance at 490 nm.

Preparation of Murine Platelets

Mouse platelets were prepared, as discussed previously.[42,43] Briefly, mouse blood was collected from a ventricle, and the citrated (0.38%) blood was mixed with PBS, pH 7.4, and was incubated with prostaglandin I$_2$ (10 ng/mL; 5 minutes), followed by centrifugation at 237g for 10 minutes at room temperature. Platelet-rich plasma was recovered, and platelets were pelleted at 483g for 10 minutes at room temperature. The pellets were resuspended in HEPES/Tyrode's buffer (20 mmol/ L HEPES/KOH, pH 6.5; 128 mmol/L NaCl; 2.8 mmol/L KCl; 1 mmol/L MgCl$_2$; 0.4 mmol/L NaH$_2$PO$_4$; 12 mmol/L NaHCO$_3$; and 5 mmol/L D-glucose) supplemented with 1 mmol/L EGTA, 0.37 U/mL apyrase, and 10 ng/mL prostaglandin I$_2$. Platelets were washed and resuspended in HEPES/Tyrode's buffer (pH 7.4) without EGTA, apyrase, or prostaglandin I$_2$. Platelets were counted with an automated hematology analyzer (Drew Scientific, Dallas, TX) and adjusted to the indicated concentrations.

Platelet Aggregation

Blood was collected from mice that were vaccinated with C-EL2, C-EL2r, and KLH, and the platelets were harvested by centrifugation. Platelets (with counts adjusted, as previously described[42–44]) were stimulated with 1 μmol/L U46619, 5 μmol/L ADP, or 80 μmol/L TRAP4, and aggregation was measured. Each experiment was repeated at least 3 times, with blood pooled from at least 3 separate groups of 6 to 8 immunized mice.

ATP Release

These experiments were performed, as we previously described.[42–44] Platelets from vaccinated mice (C-EL2, C-EL2r, and KLH) were prepared, as previously described (250 μL; 2.5×10^8/mL), before being placed into siliconized cuvettes and stirred for 5 minutes at 37°C at 237 × g . The luciferase substrate/luciferase mixture (12.5 μL; Chrono-Log) was then added, followed by the addition of the agonist U46619 (1 μmol/ L), ADP (5 μmol/L), or TRAP4 (80 μmol/L). Each experiment was repeated at least 3 times, with blood pooled from at least 3 separate groups of 6 to 8 immunized mice.

Surface Expression of Integrin Glycoproteins IIb-IIIa, Ib, and VI

Flow cytometric analysis was performed on platelets from vaccinated mice (C-EL2, C-EL2r, and KLH), as previously

discussed.[42–44] Briefly, platelets (2×10^8) were fixed with 2% formaldehyde for 30 minutes at room temperature, before incubation with anti-mouse glycoprotein IIb-IIIa, anti–glycoprotein Ib, or anti–glycoprotein VI antibodies at room temperature for 30 minutes in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/Tyrode's buffer (pH 7.4). The samples were transferred to fluorescence-activated cell sorting tubes, and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences).

Platelet-Leukocyte Aggregates

These experiments were performed, as previously described.[45,46] Briefly, blood from vaccinated mice (C-EL2, C-EL2r, and KLH) was incubated with anti-CD11b and anti–P-selectin antibodies before stimulation with 1 µmol/L U46619 or 80 µmol/L TRAP4. The reactions were stopped by adding BD FACS™ lysing solution. Events double positive for CD11b and P-selectin identified platelet-leukocyte aggregates and were recorded as a percentage of a total of 10 000 gated leukocytes. Measurements were performed using a BD Accuri C6 flow cytometer, and data were analyzed using CFlow Plus.

Integrin Glycoprotein IIb-IIIa Activation and P-Selectin Expression

Flow cytometric analysis was performed on platelets from vaccinated mice (C-EL2, C-EL2r, and KLH), as previously discussed.[42–44] Briefly, platelets (2×10^8) were stimulated with 1 µmol/L U46619, 5 µmol/L ADP, or 80 µmol/L TRAP4 for 3 minutes. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 minutes at room temperature. Finally, platelets were incubated with fluorescein isothiocyanate–conjugated JON/A or anti–P-selectin antibodies at room temperature for 30 minutes in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/Tyrode's buffer (pH 7.4). The samples were transferred to fluorescence-activated cell sorting tubes, and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus. We also measured glycoprotein IIb-IIIa activation with fluorescein isothiocyanate–conjugated JON/A in the presence or absence of 150 nmol/L of SQ29,548 (TPR antagonist).

In Vivo Thrombosis Model

These studies were performed, as described previously.[19,42,44] Briefly, vaccinated mice (C-EL2, C-EL2r, and KLH) were anesthetized with isoflurane. Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic micro–flow probe (0.5 mm; Transonic Systems Inc, Ithaca, NY). After stabilization of blood flow, 7.5%

ferric chloride was applied to a filter paper disc (1-millimeter in diameter) that was immediately placed on top of the artery for 3 minutes. Blood flow was continuously monitored for 45 minutes, or until blood flow reached stable occlusion (zero blood flow for 2 minutes). Data were recorded, and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with ferric chloride). An occlusion time of 45 minutes was considered as the cutoff time for the purpose of statistical analysis.

Tail Bleeding Time

Hemostasis in the vaccinated mice (C-EL2, C-EL2r, and KLH) was examined using the tail transection technique.[19,42,44] Briefly, mice were anesthetized with isoflurane and placed on a 37°C homeothermic blanket, and their tails were transected 5 mm from the tip. The tail was placed in 37°C saline, and the time to blood flow cessation was measured. Clotting was not considered complete until bleeding had stopped for 1 minute. When required, measurements were terminated at 15 minutes, to avoid excessive blood loss.

Statistical Analysis

All experiments were performed at least 3 times. Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) by using 1-way ANOVA and with Tukey's multiple comparisons test as post hoc. Data are presented as mean ±SEM or mean±SD, as specified. The Mann-Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using *t*-test, and similar results were obtained. Significance was accepted at $P<0.05$ (2-tailed *P* value), unless stated otherwise.

Results

C-EL2 TPR Vaccine Elicits an Immune Response, Without Altering Peripheral Blood Cell Count

We first sought to confirm that vaccination with the TPR C-EL2 peptide does elicit an immune response. Thus, serum was prepared from blood from mice that were vaccinated with C-EL2, C-EL2r, or KLH, and the antibody titers were determined by ELISA, using the C-EL2 cognate peptide. It was found that when C-EL2 was used as an immunogen, these mice had significant levels of the "C-EL2Ab" in their serum (Figure 1), unlike C-EL2r and KLH (Figure 1). This finding indicates that our immunization approach is successful in generating an antibody targeting the C-EL2, which presumably would interact with that region, and exert biological activity. Given that some vaccines have been previously found to modulate platelet and other blood cell counts, we determined whether the C-EL2 vaccine would exhibit such effects. Thus, after immunization of the mice
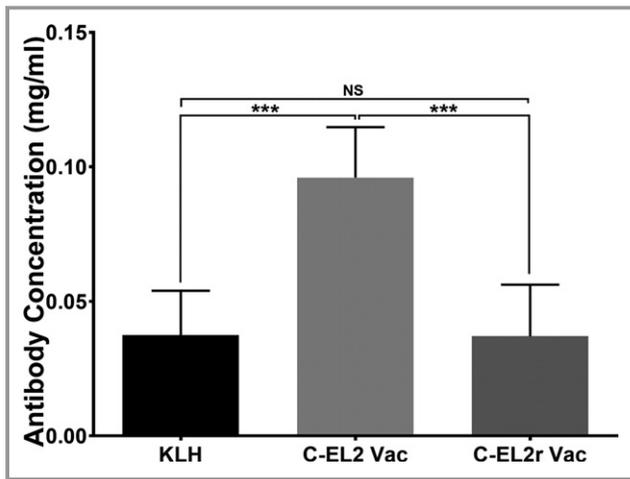
Figure 1. The C-terminus of the second extracellular loop vaccination (C-EL2 Vac) elicits an immune response. Mice were vaccinated with C-EL2, the random C-EL2 (C-EL2r), or keyhole limpet hemocyanin (KLH), and the presence of the C-EL2 antibody/immunoglobulin G was detected using ELISA and the C-EL2 cognate peptide, as described in the Methods section. The error bars in this figure represent SEM. The titers were measured in 5 different groups per vaccination, with each group consisting of 5 mice. NS indicates not significant, whereas ***$P < 0.0001$.

with TPR C-EL2 antigen, peripheral blood samples were analyzed, and the results showed that these mice have a normal platelet count, and other blood parameters (eg, white blood cells; Table 1), relative to control mice. We also measured platelet count 1, 2, and 3 months after the final boost of C-EL2 vaccine and observed no differences; yet, the antibody titers remained relatively "high" (Table 2). These data suggest that vaccinations with the C-EL2 antigen do not affect the "hematology" profile in mice.

C-EL2 TPR Vaccine Inhibits Platelet Aggregation

In light of our previous findings that treatment with the C-EL2Ab inhibits platelet aggregation,[19,47] we decided to

examine whether immunizations of live animals with the C-EL2 peptide would also translate into inhibition of platelet aggregation. Indeed, it was found that vaccination with C-EL2 (35 μg), unlike C-EL2r or KLH, resulted in inhibition of 1 μmol/L U46619-stimulated platelet aggregation (Figure 2A), whereas it had no effects on that induced by either ADP (5 μmol/L; Figure 2B) or TRAP4 (80 μmol/L; Figure 2C). Hence, these data suggest that C-EL2 TPR vaccine has the capacity to selectively inhibit platelet aggregation.

C-EL2 TPR Vaccine Inhibits Platelet-Dense Granule and α Granule Secretion

Platelet secretion is an important and early event in platelet activation,[48] and is known to be triggered by TXA_2. Thus, we next determined whether the TPR vaccine would exert inhibitory effects on platelet secretion. It was found that the C-EL2 (35 μg) vaccine inhibited platelet-dense granule secretion (ATP release) and α granule secretion (P-selectin expression), in response to the TPR agonist U46619 (1 μmol/L; Figures 3A and 4A), but not that by ADP (5 μmol/L; Figures 3B and 4B) or TRAP4 (80 μmol/L; Figures 3C and 4C), when compared with C-EL2r vaccine or KLH. These data show that immunizing mice with C-EL2 antigen does exert inhibitory effects on platelet granule secretion.

C-EL2 TPR Vaccine Inhibits Platelet Glycoprotein IIb-IIIa Activation

It is well documented that integrin glycoprotein IIb-IIIa (αIIbβ3) plays a crucial role in platelet aggregation in response to physiological agonists, and that it mediates thrombus formation.[49–51] Having established that the TPR C-EL2 vaccine has the capacity to inhibit platelet aggregation, we next investigated whether it would be associated with a commensurate inhibition of integrin αIIbβ3 activation. Our results revealed that immunization with C-EL2

Table 1. Peripheral Blood Cell Counts in KLH, C-EL2, and C-EL2r Vaccinated Mice

Type of Blood Cell	KLH	C-EL2	C-EL2r	P Values*
Platelets	1087.48±41.2	1154.13±38.7	1121.84±55.4	NS
MPV	4.63±2.44	4.81±2.78	4.94±3.12	NS
Red blood cells	8.13±1.39	8.76±1.44	8.35±1.21	NS
Lymphocytes	6.33±1.72	6.91±1.38	6.65±1.41	NS
Monocytes	0.039±0.024	0.042±0.031	0.044±0.021	NS
Granulocytes	2.51±2.22	2.36±2.61	2.43±2.09	NS

Data are represented as mean±SD. The counts were performed on 3 different groups, with each group consisting of 5 mice. Blood was collected from the heart and counted, as mentioned in the Methods section. All counts are thousands per microliter, except for red blood cells, which are millions per microliter. C-EL2 indicates C-terminus of the second extracellular loop; C-EL2r, random C-EL2; KLH, keyhole limpet hemocyanin; MPV, mean platelet volume; NS, not significant.
*Comparisons were made between KLH and C-EL2 vaccine, as well as C-EL2 and C-EL2r vaccine.

Table 2. Time Course of Platelet Count and Antibody Titer in C-EL2 Vaccinated Mice

Time, mo	Antibody Titer, mg/mL	Platelet Count	P Values*
1	0.91±0.018	1102.26±29.8	NS
2	0.84±0.022	1135.18±41.1	NS
3	0.79±0.027	1116.73±48.9	NS

Data are represented as mean±SD. The counts were performed on 3 different groups, with each group consisting of 5 mice. Blood was collected from the heart and was counted as mentioned in the Methods section. All counts are thousands per microliter, and were performed on different mice. C-EL2 indicates C-terminus of the second extracellular loop; NS, not significant.

*Comparisons were made between platelet counts at various time intervals (1–3 months after the final boost of the C-EL2 vaccine immunization) with the platelet keyhole limpet hemocyanin counts in Table 1.

antigen (35 µg), unlike C-EL2r or KLH, results in significant inhibition of U46619-triggered JON/A binding (1 µmol/L; Figure 5A), but not that induced by ADP (5 µmol/L; Figure 5B) or TRAP4 (80 µmol/L; 5C), which indicates abrogation of αIIbβ3 activation. Moreover, the TPR antagonist SQ29,548 inhibited U46619 (1 µmol/L)–mediated glycoprotein IIb-IIIa activation in the KLH and C-EL2r vaccinations, but had no effect on TRAP4 (80 µmol/L)–triggered integrin activation, in any of the immunizations (Figure 5D).

C-EL2 TPR Vaccine Delays the Time for Thrombotic Occlusion But Not the Tail Bleeding Time

In our previous studies, we have shown that C-EL2Ab (passive immunization) protects against thrombus formation, and that it does so without impairing physiological hemostasis.[19] Therefore, we sought to investigate whether the active immunizations (with the TPR C-EL2 vaccine) would produce similar effects. Indeed, it was found that mice that were immunized with C-EL2 peptide (35 µg), and subjected to the ferric chloride carotid artery thrombosis model, exhibited a prolonged time for arterial thrombosis (Figure 6A), relative to the controls C-EL2r and KLH. In fact, complete occlusion occurred by 2.5 minutes in the control animals, compared with >12 minutes in the C-EL2–vaccinated animals.

We next investigated whether the C-EL2 vaccine would exert negative consequences on hemostasis by measuring the tail bleeding time. Interestingly, it was found that C-EL2–vaccinated mice had tail bleeding times that were not different from those that were vaccinated with C-EL2r or KLH (Figure 6B). Taken together, these results provide evidence that a C-EL2 TPR vaccine exerts antithrombotic activity, without increasing the risk of bleeding.
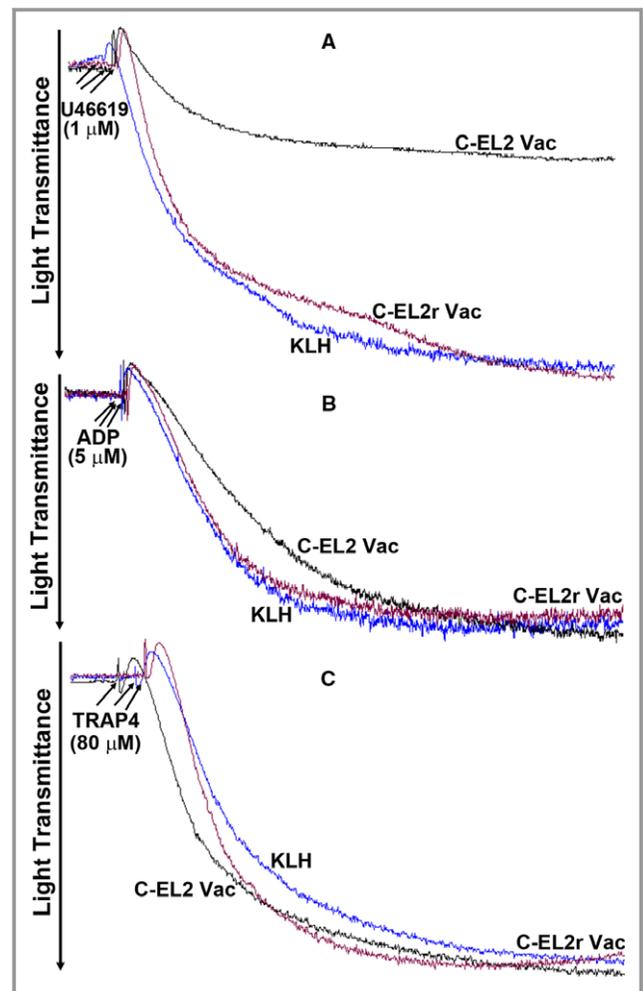

Figure 2. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac), but not the random C-EL2 (C-EL2r) or keyhole limpet hemocyanin (KLH), inhibits platelet aggregation. Platelets from vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were stimulated with 1 µmol/L U46619 (A), 5 µmol/L ADP (B), or 80 µmol/L thrombin receptor-activating peptide 4 (TRAP4; C), and their aggregation response was monitored using an aggregometer. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 6 mice per group.

C-EL2 TPR Inhibits Platelet-Leukocyte Aggregate Formation

Platelet-leukocyte aggregates are known to contribute to the pathogenesis of thrombotic disorders,[52,53] and TPR antagonists were found to reduce their formation.[54] Thus, we investigated whether the C-EL2 TPR vaccine would exert similar effects. Indeed, it was found that U46619 (1 µmol/L)–induced platelet-leukocyte complexes are reduced with the C-EL2 peptide, but not with the KLH or the C-EL2r controls (Figure 7). On the other hand, neither the C-EL2 vaccine nor the controls exert any effects on TRAP4 (80 µmol/L)–triggered platelet-leukocyte aggregation (Figure 7).
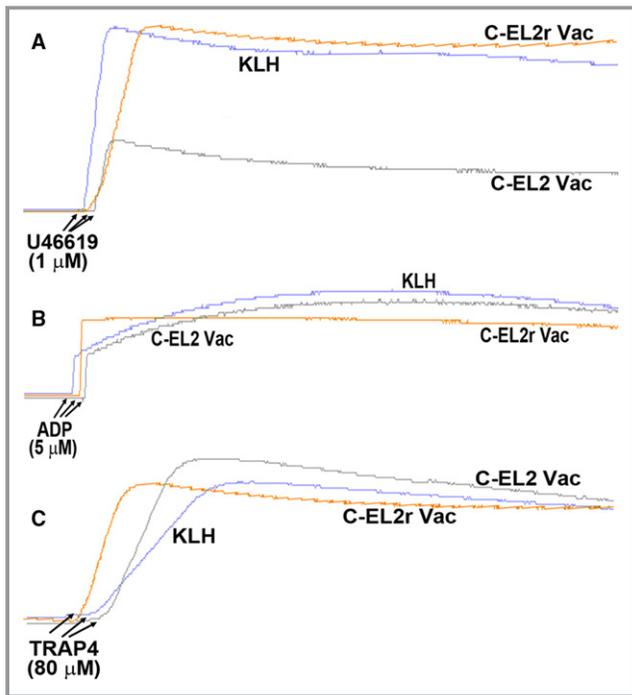
Figure 3. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac), but not the random C-EL2 (C-EL2r) or keyhole limpet hemocyanin (KLH), inhibits dense granule secretion. A through C, Platelets from vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were incubated with luciferase luciferin (12.5 µL), before being stimulated with 1 µmol/L U46619 (A), 5 µmol/L ADP (B), or 80 µmol/L thrombin receptor-activating peptide 4 (TRAP4; C). ATP release (for dense granules) was detected as luminescence, and measured by a lumiaggregometer. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 6 mice per group.

C-EL2 TPR Vaccine Does Not Affect Platelet Glycoprotein IIb-IIIa, Ib, and VI Surface Expression

To exclude the possibility that the phenotype observed with the C-EL2 vaccine may derive, in part, from unintended effects on major platelet integrin receptors, their expression levels were measured. As can be seen in Figure 8, immunizations with C-EL2, KLH, or C-EL2r produced no effects on the surface expression of glycoproteins IIb-IIIa, Ib, and VI. These findings further support the "specificity" of the effects produced by the C-EL2 vaccine.

Discussion

The TXA_2-TPR signaling pathway is known to play an important role in platelet function in vivo, and has been implicated in the genesis of various forms of cardiovascular disorders.[55–57] However, despite its clear involvement in such diseases, considerable gaps remain in our understanding of TPR's structural biological features, which we believe have
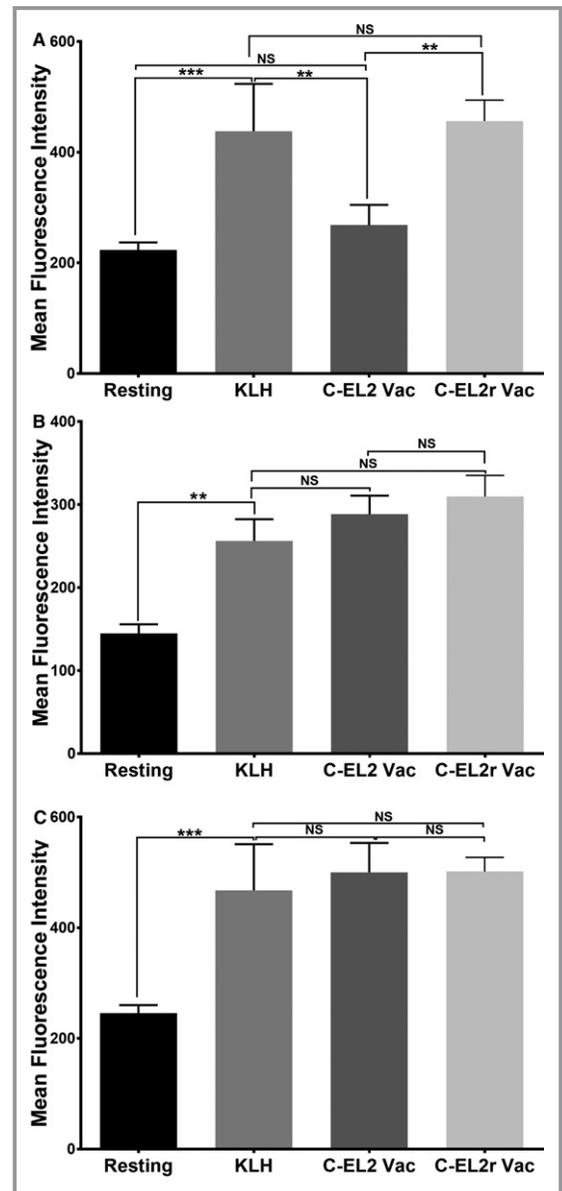

Figure 4. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac), but not the random C-EL2 (C-EL2r) or keyhole limpet hemocyanin (KLH), inhibits α granule secretion. Platelets from vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were washed, before stimulation with 1 µmol/L U46619 (resting vs KLH, ***$P<0.0001$; KLH vs C-EL2, **$P<0.001$; C-EL2 vs C-EL2r, **$P<0.001$; KLH vs C-EL2r, P=not significant [NS]; resting vs C-EL2, P=NS; t test; A), 5 µmol/L ADP (resting vs KLH, **$P<0.001$; KLH vs C-EL2, P=NS; KLH vs C-EL2r, P=NS; C-EL2 vs C-EL2r, P=NS; t test; B), or 80 µmol/L thrombin receptor-activating peptide 4 (resting vs KLH, ***$P<0.0001$; KLH vs C-EL2, P=NS; KLH vs C-EL2r, P=NS; C-EL2 vs C-EL2r, P=NS; t test; C) for 3 minutes. Platelets were incubated with fluorescein isothiocyanate–conjugated CD62P antibody (for α granules), and the fluorescent intensities were measured by flow cytometry. The error bars in this figure represent SEM. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group.
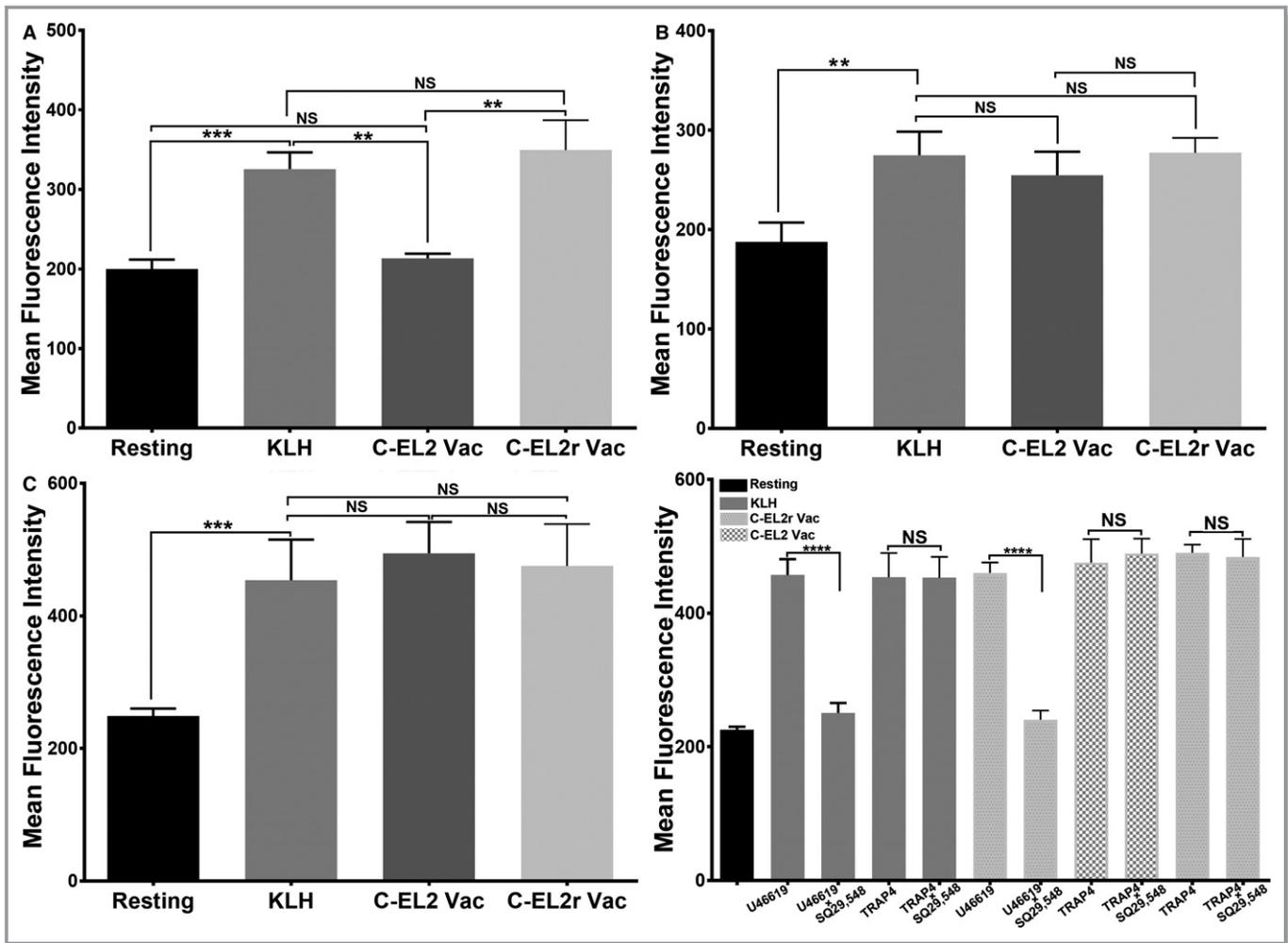
Figure 5. The C–terminus of the second extracellular loop vaccine (C-EL2 Vac) vaccine, but not the random C-EL2 (C-EL2r) or keyhole limpet hemocyanin (KLH), inhibits integrin activation. Platelets from vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were washed, before stimulation with 1 µmol/L U46619 (resting vs KLH, ***$P<0.0001$; KLH vs C-EL2, **$P<0.001$; C-EL2 vs C-EL2r, **$P<0.001$; KLH vs C-EL2r, P=not significant [NS]; resting vs C-EL2, P=NS; t test; A), 5 µmol/L ADP (resting vs KLH, **$P<0.001$; KLH vs C-EL2, P=NS; KLH vs C-EL2r, P=NS; C-EL2 vs C-EL2r, P=NS; t test; B), 80 µmol/L thrombin receptor-activating peptide 4 (TRAP4; resting vs KLH, ***$P<0.0001$; KLH vs C-EL2, P=NS; KLH vs C-EL2r, P=NS; C-EL2 vs C-EL2r, P=NS; t test; C), and 1 µmol/L U46619 or 80 µmol/L TRAP4 in the presence or absence of the thromboxane receptor antagonist SQ29,548 (KLH/U46619 vs KLH/U46619+SQ29,548, ***$P<0.0001$; KLH/TRAP4 vs KLH/TRAP4+SQ29,548, P=NS; C-EL2r Vac/U46619 vs C-EL2r Vac/U46619+SQ29,548, ***$P<0.0001$; C-EL2r Vac/TRAP4 vs C-EL2r Vac/TRAP4+SQ29,548, P=NS; C-EL2 Vac/TRAP4 vs C-EL2 Vac/TRAP4+SQ29,548, P=NS; t test; D), for 3 minutes. Platelets were incubated with fluorescein isothiocyanate–conjugated JON/A antibody, and the fluorescent intensities were measured by flow cytometry. The error bars in this figure represent SEM. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group.

hampered TPR-focused drug discovery. Furthermore, we believe that resolution of these issues is crucial for the development of anti–thromboxane-based therapeutic strategies. To this end, we have previously shown that the TPR ligand-binding domain resides in the C-terminal segment of the second extracellular loop (C-EL2) of the receptor protein,[11,41] and that an antibody targeted against this domain inhibits platelet function in vitro[47] and protects against thrombogenesis in vivo.[19] We also showed that this antibody does not interfere with physiological hemostasis.[19] Aspirin remains the only clinically approved agent for therapeutic interventions that target this pathway, but its

use is associated with many limitations, including severe adverse effects (eg, bleeding) and resistance, among others.[25,26,28,29,58] Based on these considerations, there remains interest in developing more selective and new/novel means for targeting the TXA$_2$/TPR pathway (namely, agents with TPR antagonistic activity, because they would be expected to exhibit a better/safer pharmacological profile and perhaps be more effective in managing thrombosis-based diseases). More important, evidence obtained using mouse models suggests that, in certain disease states, therapeutic intervention using a TPR antagonist, with or without aspirin, is superior to aspirin therapy alone.[59]
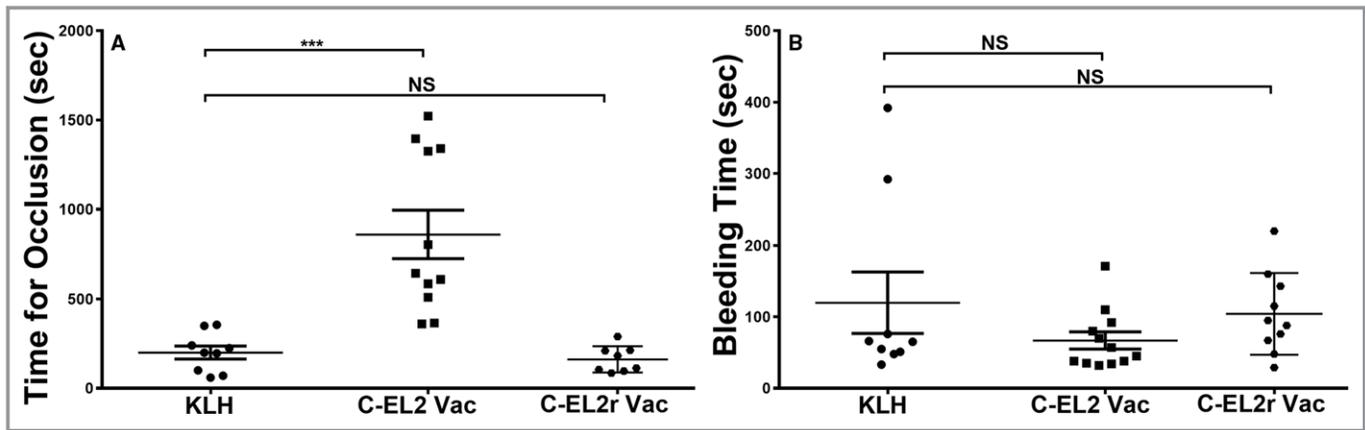
Figure 6. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac) prolongs the time for occlusion, but not the tail bleeding time, whereas the random C-EL2 (C-EL2r) or keyhole limpet hemocyanin (KLH) has no effect. A, Vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were subjected to the ferric chloride–induced thrombosis, and the time for occlusion was measured (C-EL2, ***$P<0.001$, Mann-Whitney test; or C-EL2r, P=not significant [NS], Mann-Whitney test). Each point represents the occlusion time of a single animal (C-EL2, n=11; C-EL2r, n=9; and KLH, n=8). B, Vaccinated mice (ie, C-EL2, C-EL2r, or KLH) were subjected to the tail bleeding time assay, as described in the Methods section. Each point represents the bleeding time of a single animal (C-EL2, P=NS, Mann-Whitney test, n=11; C-EL2r, P=NS, Mann-Whitney test, n=9; and KLH, n=8).

In the present article, we sought to examine the antiplatelet activity of a TPR-based vaccine in which the cognate peptide of the aforementioned antibody (ie, C-EL2) serves as an antigen/immunogen, in mice. Notably, none of the antagonists that were developed for TPR has received approval by the Food and Drug Administration; and they face important shortcomings and limitations because of frequent administration, high doses, and high costs.[18,37,38,55,56,60] In contrast, much like our passive immunization studies,[19,47] we believe that active vaccination is likely to offer a promising approach for the treatment of thrombosis. Therefore, we initially examined the ability of this vaccine to exert antiplatelet activity by using aggregometry experiments. Our results indicated that C-EL2 vaccine did exert inhibitory effects on TPR-mediated platelet aggregation, and that it did so in a selective manner (no effects on other activation pathways). On the other hand, platelets vaccinated with the random C-EL2 (C-EL2r) or the KLH control did not exhibit any inhibitory effects on the aggregation response, regardless of the agonist used. Furthermore, the C-EL2 vaccine, unlike C-EL2r and KLH, was found to block glycoprotein IIb-IIIa activation, which is consistent with the attenuated aggregation response. As for the platelet secretion response, we observed inhibition of the dense and α granule release, in the C-EL2–vaccinated platelets. We finally sought to investigate whether the inhibitory effects we observed under in vitro experimental conditions would translate into an antithrombotic activity in vivo. Indeed, our data revealed that our immunized mice had prolonged time for thrombus occlusion, thereby supporting its utility as antithrombotic agent. To confirm that these inhibitory effects derive from the production of a C-EL2 antibody (ie, "success" of the immunization process), in these mice, ELISA experiments were performed. Our results showed that vaccination with the C-EL2 peptide did result in generation of a TPR antibody (C-EL2Ab) and collectively support the notion that this antibody interacts with the C-EL2 domain, and specifically blocks TPR-mediated platelet activation. In support of the specific nature of our approach, the C-EL2 vaccine was found not to affect the expression levels of major integrin receptors (namely, glycoproteins IIb-IIIa, Ib, and VI). The TPR antagonist SQ29,548 did not exert any inhibitory effects on TRAP4-induced glycoprotein IIb-IIIa activation in any of the immunizations, whereas it inhibited the response mediated by TPRs in the KLH and C-EL2r vaccinations.

The formation of platelet-leukocyte aggregates has been previously shown to play a significant role in thrombosis.[46,53] Moreover, TPR activation is known to enhance platelet-leukocyte complex formation,[54] suggesting that this event is a contributor to TPR-dependent thrombogenesis. On this basis, we examined whether immunization with the C-EL2 peptide would exert any inhibitory effects on platelet-leukocyte aggregate formation. Indeed, it was found that TPR-induced platelet-leukocyte aggregate formation is inhibited in blood from the C-EL2–vaccinated mice.

We believe that the proposed C-EL2 TPR vaccine therapeutic approach, as supported by data from our previously characterized C-EL2Ab,[19,47] would be expected to be superior to a classic antagonist because its effects would, most likely, be predominantly limited to platelet TPRs. This is because the distribution of antibodies to compartments other than the vascular is, in general, restricted because of poor penetration
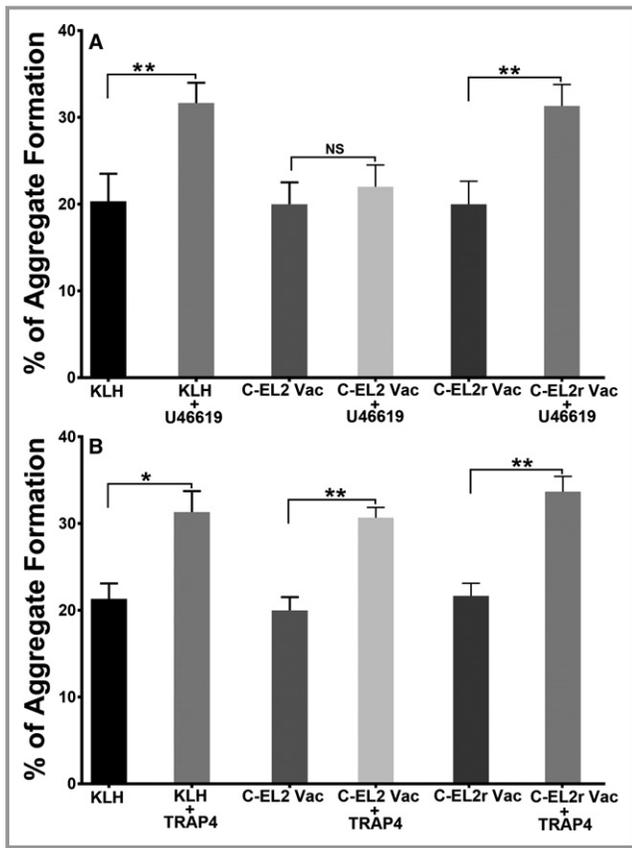
Figure 7. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac) inhibits thromboxane A$_2$ receptor–induced, but not thrombin receptor-activating peptide 4 (TRAP4)–induced, platelet-leukocyte aggregate formation. A, Blood from vaccinated mice (ie, C-EL2, random C-EL2 [C-EL2r], or keyhole limpet hemocyanin [KLH]) was incubated with anti–P-selectin (platelet marker) and anti-CD11b (leukocyte marker) antibodies, before incubation with or without U46619 (1 μmol/L). Events double positive for CD11b and P-selectin identified platelet-leukocyte aggregates and were recorded as a percentage of a total of 10 000 gated leukocytes (KLH vs KLH+U46619, **P<0.001; C-EL2 Vac vs C-EL2 Vac+U46619, P=not significant [NS]; C-EL2r Vac vs C-EL2r Vac+U46619, **P<0.0015). B, Blood from vaccinated mice (ie, C-EL2, C-EL2r, or KLH) was incubated with anti–P-selectin (platelet marker) and anti-CD11b (leukocyte marker) antibodies, before incubation with or without TRAP4 (80 μmol/L). Events double positive for CD11b and P-selectin identified platelet-leukocyte aggregates and were recorded as a percentage of a total of 10 000 gated leukocytes (KLH vs KLH+TRAP4, *P<0.05; C-EL2 Vac vs C-EL2 Vac+TRAP4, **P<0.001; C-EL2r Vac vs C-EL2r Vac+TRAP4, **P<0.001). The error bars in this figure represent SEM. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 8 mice per group.

of the endothelial cell layer.[61–63] Thus, these approaches may allow us to address bleeding time and thrombosis under conditions of selective modulation of the platelet TPRs, but not those TPRs of the smooth muscle[64–66] (which are known to affect bleeding time). Strikingly perhaps, but as predicted,

the C-EL2–immunized mice possessed a tail bleeding time that was no different from the controls (C-EL2r and KLH), indicating that it does not interfere with hemostasis. In other words, much like in the case of KLH and C-EL2r vaccine, the C-EL2 vaccine (ie, the generated antibody) does not block TPRs of the smooth muscle, thereby allowing them to mediate vasoconstriction in response to injury, which limits blood loss and allows the bleeding to stop "normally." Thus, the development of a C-EL2–derived vaccine that actively immunizes patients against TPR and effectively and safely lowers platelet reactivity would be expected to have clinical applications and be highly desirable as a means for the prevention and/or treatment of thrombotic events. It also should offer an advantage in patients' compliance, particularly in individuals with high risk of thrombosis, and serve as a cost-effective alternative and/or supplement. This approach, unlike aspirin, would not be expected to be associated with gastric ulcers or promote the production of isoprostanes (eg, 8-iso Prostaglandin F$_{2\alpha}$).[67,68] The latter were found to be potential markers of oxidative stress and mediators of platelet activation.[30,69] In terms of other measures of safety, there was no apparent toxicity in the mice vaccinated with the C-EL2 peptide (and controls). The vaccinated mice appeared normal; there was no change in their hematological profile, weight, breathing pattern, or level of activity; and there were no obvious inflammation lesions/signs at the vaccination sites. Nevertheless, the potential toxicity of C-EL2 vaccine needs to be investigated further.

Although vaccine development is a complex and challenging process, peptide-based vaccines (eg, C-EL2) provide several advantages in comparison to conventional vaccines.[12,59] Peptide vaccines are safer and more economic,[12,59] when compared with traditional vaccines that are made of dead or attenuated pathogens,[3] inactivated toxins, and recombinant subunits. Peptide vaccine production is also relatively inexpensive because of the ease of production and simplistic composition.[12,59] In addition, peptide vaccines avoid the inclusion of unnecessary components possessing high reactogenicity to the host, such as lipopolysaccharides, lipids, or toxins.[12,59] Vaccination-based approaches for targeting G-protein–coupled receptors have been gaining much attention recently,[70] and recent clinical trials supported the effectiveness of a cocaine dependence vaccine[71] and an angiotensin II–based vaccine (in hypertensive patients),[72] as well as their safety for humans.

In summary, these studies constitute the first investigation of a vaccine-based antithrombotic agent and of immunization-based inhibition of TPR function in vivo. Moreover, they also provide novel information about a potential target site (ie, C-EL2) for therapeutic intervention. Given the biological activity of our C-EL2 immunization (eg, protection against thrombosis), an anti-TPR/antithrombosis vaccine should be developed.
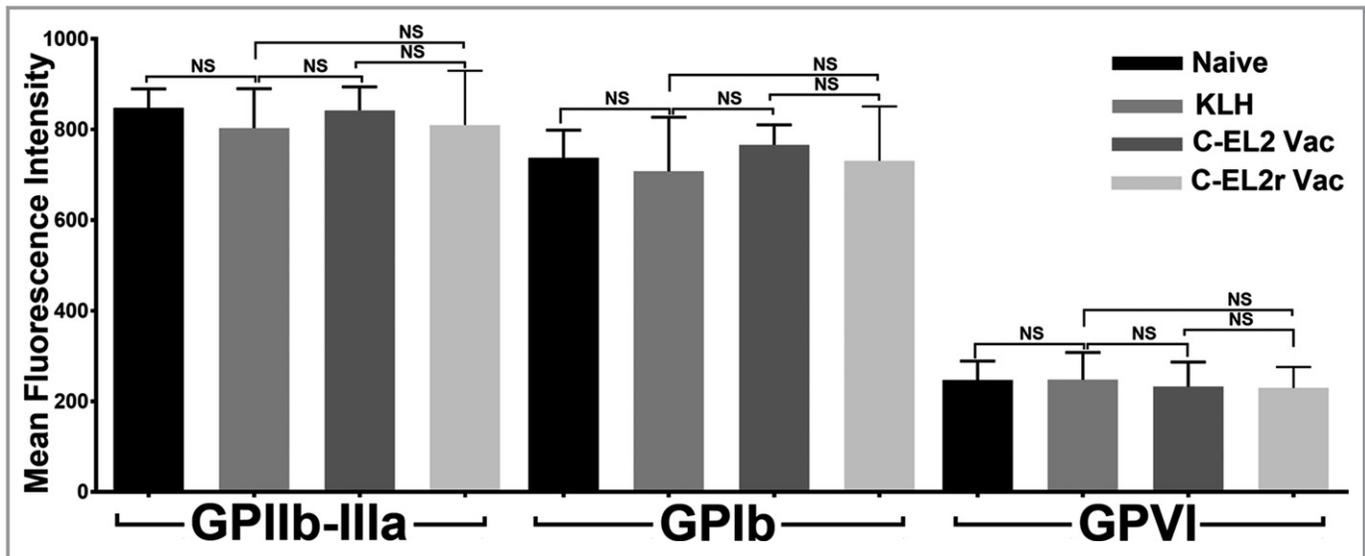
Figure 8. The C-terminus of the second extracellular loop vaccine (C-EL2 Vac) does not affect the expression levels of major platelet integrins. Platelets from nonvaccinated/naïve and from vaccinated mice (ie, C-EL2, random C-EL2 [C-EL2r], or keyhole limpet hemocyanin [KLH]) were washed and incubated with antibodies against glycoprotein IIb-IIIa (GPIIb-IIIa), GPIb, and GPVI (*P*=not significant [NS]), and the fluorescent intensities were measured by flow cytometry. The error bars in this figure represent SEM. Each experiment was repeated 3 times using 3 separate groups, with blood pooled from 6 mice per group.

Finally, the identification of a functionally active TPR sequence will significantly aid molecular modeling study predictions for organic derivatives that possess in vivo activity. This is an important consideration because, despite the clear involvement of TPR signaling in occlusive vascular disease, aspirin is still the only clinically effective drug for the prevention of TPR-mediated platelet activation. Thus, the availability of a pharmacologically effective non-aspirin derivative or C-EL2–derived vaccine with anti-TPR activity could have widespread therapeutic applications, especially given the limitations of current thromboembolic therapy (eg, resistance and bleeding associated with clopidogrel [Plavix] and/or aspirin).

Collectively, our findings make significant contributions to our understanding of the role of C-EL2 in TPR-dependent platelet activation and may lay down the foundation for the clinical application of the C-EL2 peptide as a novel/new pharmacological agent/vaccine for the treatment and prevention of multiple thrombotic disease states.

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Disclosures

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