Title

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Histone deacetylase-6 modulates the effects of 4°C platelets on vascular endothelial permeability

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Key Points

- 4°C storage of PLTs results in a diminished protective effect of the PLTs on vascular permeability.
- HDAC-6 inhibition attenuates the loss of function of 4°C-stored platelets in modulating vascular permeability.

Platelets (PLTs) stored at 4°C exhibit equivalent or superior hemostatic function compared with 22°C PLTs, but have shorter circulation times and a decreased ability to modulate vascular permeability. These differences may be due to morphological changes and storage-induced activation. Using a proteomics-based approach, we found that 4°C-stored PLTs express decreased α-tubulin, a key PLT structural protein. PLT activation is characterized by α-tubulin deacetylation, which is regulated by histone deacetylase-6 (HDAC-6). We hypothesized that inhibition of HDAC-6 in stored PLTs will improve their ability to regulate vascular permeability through reduced activation and α-tubulin deacetylation. In an in vivo model of vascular permeability, treatment of 4°C PLTs with the HDAC-6 inhibitor tubacin enhanced the vasculoprotective properties of untreated 4°C PLTs. 4°C PLT circulation, however, was unchanged by tubacin treatment, suggesting that circulation time may not be a critical factor in determining the vasculoprotective effects of PLTs. Assessing the factor content of stored PLTs revealed that angiopoietin-1 (Ang-1) increased in 4°C PLTs over time, which was further enhanced by tubacin treatment. In addition, angiopoietin-2, an inducer of vascular leak and antagonist of Ang-1, inhibited PLT barrier protection, suggesting involvement of the Tie-2 pathway. This study demonstrates that HDAC-6 inhibition with tubacin attenuates the diminished vasculo-protective properties of 4°C PLTs, and these properties may be independent of PLT circulation time.

Introduction

Platelets (PLTs) are transfused to attenuate bleeding and also serve as a prophylactic agent preventing vascular barrier compromise, vascular fragility, and hemorrhage in patients with thrombocytopenia.[1] In addition to the ability of PLTs to form clots and provide hemostasis, an important function of PLTs is their ability to regulate systemic vascular integrity and preserve organ perfusion and function.[2] In current blood banking practice, apheresis PLTs are stored at 22°C for up to 5 to 7 days with gentle agitation.[3] The short 5- to 7-day storage time and need for agitation create logistical issues in PLT availability particularly in rural or austere locations.[4,5] Another logistical issue with standard 22°C PLTs is the possibility of bacterial contamination.[6]

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Data are available on request from the corresponding author, Shibani Pati (shibani.pati@ucsf.edu).

The full-text version of this article contains a data supplement.

PLTs change as they are stored in bags before transfusion, resulting in a PLT storage lesion (PSL).[7,8] PSL at 22°C has been studied and is highlighted by structural and functional changes, including disk-to-sphere shape changes, breakdown to fragmented forms, spontaneous storage-induced PLT activation, degranulation and release of growth factors and cytokines, decreased pH, and increased aggregation.[9,10] Most importantly, PSL is associated with decreased efficacy in animal models and PLT function in vitro.[11]

Cold storage (4°C) of PLTs provides an attractive alternative to 22°C storage with increased storage time, reduced risk of bacterial contamination and reduced PSL.[3,12,13] This increased storage time may help reduce waste and increase the availability of PLT units. We and others have shown that 4°C PLTs preserve viability, hemostatic function, and possess a reduced risk of bacterial growth.[11,14-19] Furthermore, cold storage also preserves mitochondrial function, in vitro aggregation response, and clot formation.[20,21] However, in our previous studies, 4°C PLTs did not circulate for long or demonstrate equivalent vasculoprotective effects in comparison to 22°C PLTs in a mouse model of vascular permeability, hence suggesting that 4°C PLTs may not be an optimal PLT product.[11]

Optimizing PLT storage conditions for both 4 and 22°C PLTs can have broad-ranging clinical implications. One area of interest is how 4°C storage-induced changes in the PLT cytoskeleton relate to PLT function. There have been studies investigating the effects of cold exposure or chilling of PLTs on the cytoskeleton, which did not affect their hemostatic function. However, it is important to distinguish between a chilled PLT, which is exposed to the cold for a brief period and is not used clinically vs a PLT that has been stored in the cold for days.[22,23]

Protein acetylation and deacetylation mediate structural changes in the PLT marginal band, which is composed primarily of acetylated tubulin in nonactivated PLTs.[24] Upon activation, PLTs deacetylate proteins such as tubulins, which play a role in the regulation of PLT cytoskeletal changes that modulate PLT aggregation, clot formation, and clot retraction.[25] PLT histone deacetylase-6 (HDAC-6) is found in PLTs and is responsible for the reversible deacetylation of PLT tubulins.[24]

In our current studies, we sought to determine what changes occur in 4°C PLTs at the molecular level that result in their diminished capacity to attenuate vascular permeability. Using a proteomics-based approach, we found that 4°C PLTs contain decreased amounts of α-tubulin. Here, we study the effects of cold storage on PLT function, with a focus on changes in PLT tubulin acetylation and the ability of 4°C PLTs to regulate vascular endothelial integrity. We hypothesized that 4°C PLTs will display alterations in the posttranslational modification of cytoskeletal α-tubulin, contributing to their accelerated clearance from circulation and their diminished protective effects on the vascular barrier.[26] We further hypothesized that the modulation of cytoskeletal changes in 4°C PLTs could mitigate the PSL. Our study demonstrates that some of the diminished vasculoprotective changes in 4°C PLTs can be attenuated by acutely preserving α-tubulin acetylation through HDAC-6 inhibition. To our knowledge, this is the first study to examine cold storage and the effects of modulating PLT activation and vascular stability through HDAC-6 modulation.

Materials and methods

Preparation of human PLTs

Leukoreduced apheresis PLTs stored in plasma were obtained from Vitalant (Denver, CO). Each unit (n = 6) was split into 4 FDA-approved 25 mL PLT storage bags on day 1. Donor-to-donor variability was observed in endothelial barrier protection (2 unique donors tested). Hence, for the rest of the experiments, pooled PLTs derived from 3 unique donors, collected on the same day, which we called Pooled Donor 3, were used. This pooling method reduces donor-to-donor variability. Individual donor

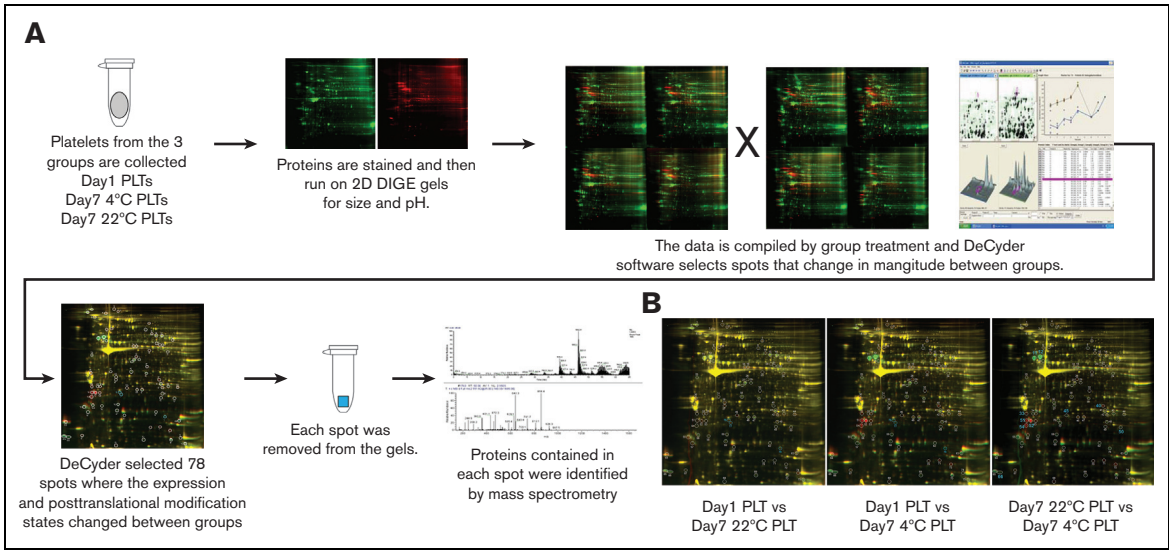

Figure 1. Significant storage-induced changes in PLT posttranslational protein modification. (A) 2D analytical gels were loaded with lysates from Day 1, Day 7 22°C, and Day 7 4°C PLTs. Protein targets that resulted in strong change in posttranslational modification were selected and identified by mass spectrometry. Green represents 1 storage group, red represents the other. An equal change in protein results in an overlap, creating a yellow color. Proteins that remained green or red indicate significant change as compared with the other group. (B) 2D analytical gels of each comparison combination.

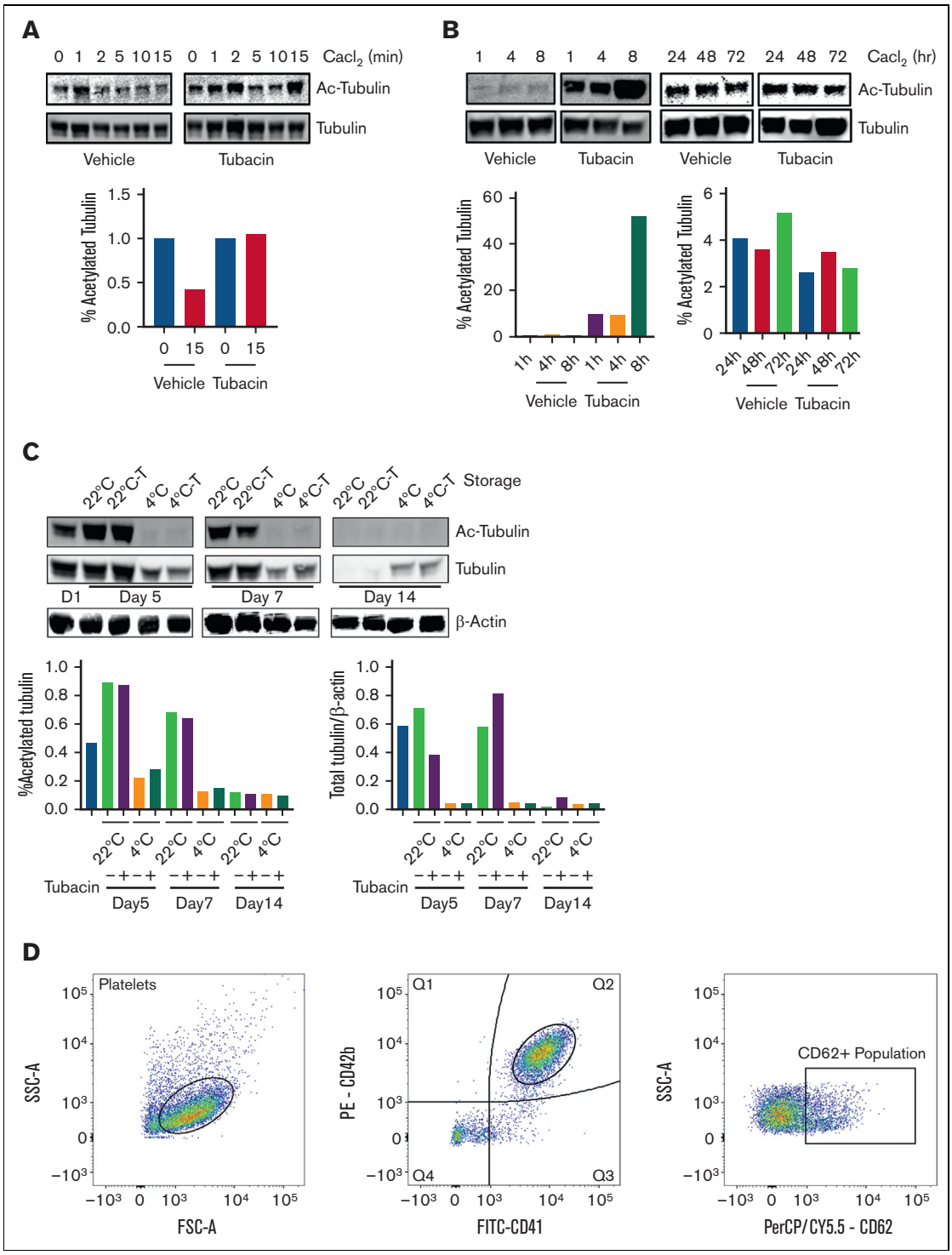

Figure 2. Storage-induced expression of PLT activation markers. (A-B) Western blot analysis of PLTs to assay tubulin acetylation. PLTs were pretreated with tubacin (10 μM) for 15 minutes before activation by $CaCl_2$ (1 mM) (A) Time course (0-15 minutes) of PLT activation in the presence of tubacin or vehicle (dimethyl sulfoxide). Bar graphs indicate densitometry ratios of acetylated tubulin/total tubulin. (B) Time course (1-8 hours, 24-72 hours) of PLT activation in the presence of tubacin or vehicle. Bar graphs indicate densitometry ratios of acetylated tubulin/total tubulin. (C) Time course (1-14 days) of PLTs stored at 22 or 4°C in the presence of tubacin or vehicle. Bar graphs indicate densitometry ratios of acetylated tubulin/total tubulin or total tubulin/β-actin. (D) Representative gating strategy of PLTs assayed by flow cytometry. All PLTs were first gated by forward and side scatter. From that population, cells expressing as double positive for CD41 and CD42b were selected. From that population, CD62P expression was determined. (E) Histograms of CD62P positive events for PLTs stored for 1, 7, and 14 days, at either 22 or 4°C, with or without tubacin treatment (10μM). Percent of CD62P-positive events indicate proportion of events collected from a population double positive for CD41 and CD42b. Three separate donor PLTs were tested, and a representative donor is shown.

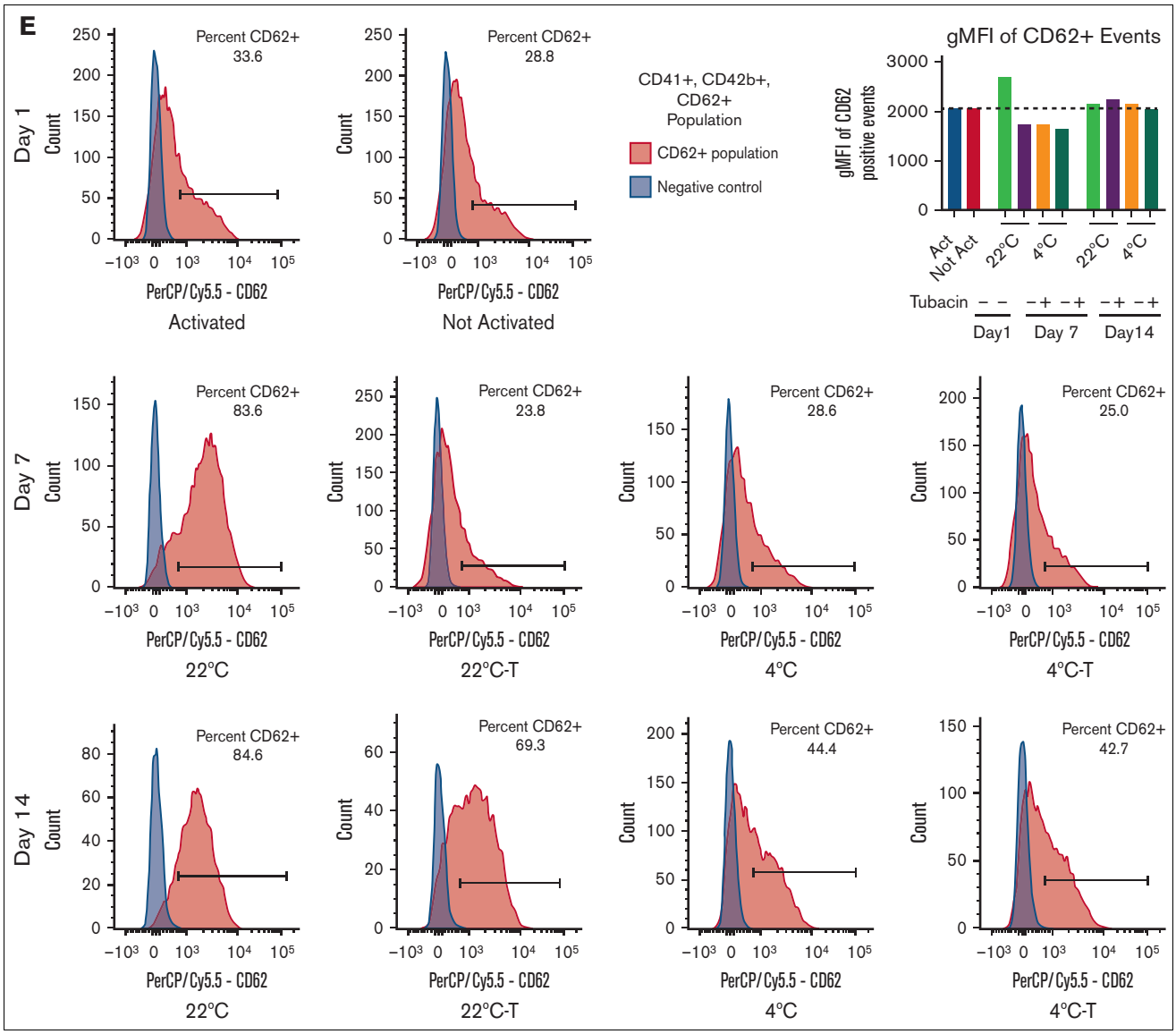

Figure 2 (continued)

replicates are shown in supplemental Figures. Two bags were stored at room temperature (22°C) and the other 2 at 4°C. All bags were gently agitated throughout storage. One of the 22°C bags and 1 of the 4°C bags were treated with 10 μM tubacin (Enzo Life Sciences, NC), creating 4 storage groups: 22°C, 22°C-T, 4°C, 4°C-T (supplemental Figure 1). PLTs were sampled, centrifuged at 800*g*, and then washed in phosphate-buffered saline to remove residual plasma before testing on days 1, 5, 7, and 14. This preparation was performed before administration in all assays. PLT pellets from each group were also stored at −80°C for Luminex analysis. All PLTs were tested for bacterial infection by Vitalant (Denver, CO) before shipping and found to be negative.

Two-dimensional difference in gel electrophoresis (2D-DIGE) and mass spectrometry

PLTs from 3 groups, Day 1, Day 7 22°C, and Day 7 4°C, were collected and sent to Applied Biomics (Hayward, CA) for 2D-DIGE and mass spectrometry analysis, as previously described, with details provided in supplemental Methods.[27]

Western blot analysis of PLTs

Western blot experiments were carried out as previously described, with details provided in supplemental Methods.[28] Pooled Donor 3 is represented in the main text (see Figure 2). A unique individual donor replicate is represented in supplemental Figure 3.

Flow cytometry of PLTs

PLTs stored at room temperature or 4°C with and without tubacin were collected at the specified time points and characterized by size and expression of human integrin-a2b (CD41) (BioLegend, San Diego, CA) and glycoprotein1b (CD42b) (BioLegend, San Diego, CA). PLT surface levels of P-selectin (CD62P) (BioLegend, San Diego, CA) were compared using the geometric mean

fluorescence intensity (gMFI) of expression. All events were analyzed using computer software (FlowJo, Version 9.7, Tree Star, Inc, Ashland, OR). Pooled Donor 3 is represented in the main text (see Figure 2). A unique individual donor replicate is represented in supplemental Figure 3.

Miles assay

The animal studies were performed with the approval of the Institutional Animal Care and Use Committee at UCSF. The experiments were conducted in compliance with the National Institutes of Health guidelines on the use of laboratory animals and the Department of Defense Animal Care and Use Review Office. The modified Miles assay (Miles AA, 1952) was performed in 8- to 10-week-old NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (Jackson Laboratories, Sacramento, CA) as described in supplemental Methods. To decrease donor variability, 3 donor PLTs were pooled before administration in this model.

Quantitation of endothelial barrier permeability

Human umbilical vein endothelial cells (HUVECs) were obtained from Promocell (Germany) and maintained using Growth Medium MVS (Promocell, Germany). The integrity of HUVEC monolayers was measured using an Electric Cell-substrate Impedance sensing System (ECIS) (ECIS 1600, Applied BioPhysics, Troy, NY), as previously described in supplemental Methods.[29] Please refer to the Preparation of Human PLTs section for the number of donors represented and tested. Because of the longitudinal aspect of this assay and donor PLTs received over a span of years, the biological data samples cannot be combined into a single figure for statistical analysis. Unique individual donor figures can be found in the supplemental Section.

Light transmission aggregometry

PLTs stored at 22 or 4°C were collected at the specified time points and analyzed on a Model 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-log, Havertown, PA). Briefly, 2 × 10^8 PLTs were primed with $CaCl_2$ (1 mM) for 1 minute before stimulation with the agonists collagen (1.0 μg/mL) or thrombin (0.1 U/mL). Any resulting aggregation was measured for 6 minutes, and the maximum aggregation was recorded as a percentage of the vehicle control (phosphate-buffered saline). Pooled Donor 3 is represented in the main text (see Figure 4D-E). A unique individual donor replicate is represented in supplemental Figure 4.

Immunostaining of endothelial tight and adherens junction protein expression

HUVECs were grown to confluence on collagen-coated coverslips before treatment. PLTs (50 × 10^6/mL) stored at 22°C, 22°C-T, 4°C, and 4°C-T were added to the HUVEC monolayer for 30 minutes, followed by a 0.2 U/mL thrombin challenge for 5 minutes at 37°C, and analyzed as previously described, with details provided in supplemental Methods.[30]

Scanning electron microscopy

PLTs from all 4 storage groups (22°C, 22°-T, 4°C, and 4°C-T) were washed and fixed in 4% paraformaldehyde, followed by serial dehydration in 25%, 50%, 75%, and 100% ethanol, and mounted onto ITO CTD coverslips (SPI, West Chester, PA), then coated with gold-palladium (3 nm). The PLTs were examined and imaged using an SEM (S4300, Hitachi, Tokyo, Japan) with an electron energy of 1.0 to 1.5 keV at 1000× and 10 000× magnification.

Luminex protein analysis

PLTs from all 4 storage groups (22°C, 22°C-T, 4°C, and 4°C-T) were washed and lysed with radio-immunoprecipitation assay buffer and diluted using diluents supplied by the manufacturer. A Luminex panel targeting the proteins angiogenin, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), brain derived neurotrophic factor (BDNF), CD40 ligand, endothelial growth factor, fibroblast growth factor, interferon-gamma, interleukin-1β (IL-1β), IL-10, IL-6, IL-8/CXCL8, platelet-derived growth factor-AA, platelet-derived growth factor-BB, thrombospondin-2, tumor necrosis factor-α, vascular endothelial growth factor-A, vWF-A2, thrombomodulin, PF4/CXCL4, and Tie-2 was created (R&D Systems, Minneapolis, MN). This multiple analyte assay was performed using a MAGPIX instrument, and the resulting data were analyzed using xPONENT software. Pooled Donor 3 is represented in the main text (see Figure 6A). A unique individual donor replicate is represented in supplemental Table 1.

Statistical analyses

Measures of transendothelial electrical resistance and gap junction measurements were analyzed by one-way analysis of variance (ANOVA), followed by Tukey multiple comparison tests. Evans Blue leakage quantification was analyzed by unpaired 1-tailed *t* test with Welch correction. Counts of CD41 to 42b double-positive events and gMFI of CD62P-positive events at both time points for circulation time of PLTs in the Miles assay were each analyzed by RM two-way ANOVA. Because we observed significant interaction between time and treatment, we performed individual one-way ANOVA to determine the effect of treatment at each of the time points. Counts of CD41-42b double-positive events at the 5 and 60 minute time points independently and gMFI of CD62P positive events at the 5 and 60 minute time points independently were each analyzed by one-way ANOVA followed by Tukey posthoc test. Differences between groups were considered significant with $P \leq .05$.

Results

2D-DIGE analysis reveals significant changes in post-translational modification in PLTs after storage

To elucidate the proteomic changes in 4 vs 22°C PLTs, we used a proteomic-based approach of 2D-DIGE followed by mass spectroscopy of differing proteins. In proteomic studies comparing 22 to 4°C PLTs at Day 7 of storage (Figure 1), 10 proteins demonstrated significant changes in expression between 22 and 4°C storage groups by 2D-DIGE analysis. The identity of these 10 proteins was revealed through mass spectrometry. The data were analyzed and expressed as fold change in protein modification between the following treatment groups: (1) Day 1 PLTs vs Day 7 22°C PLTs, (2) Day 1 PLTs vs Day 7 4°C PLTs, and (3) Day 7 22°C PLTs vs Day 7 4°C PLTs. Of the 10 proteins selected, 3 were forms of the cytoskeletal protein tubulin. Two of them, the tubulin beta chain and the tubulin alpha-1 chain, were dramatically diminished in posttranslational modifications in the 4°C PLT group as compared with Day 1 or 22°C Day 7 PLTs (Table 1). These studies prompted us to more closely investigate the role of α-tubulin deacetylation in the 4°C PSL.

Table 1. Mass spectrometry identified PLT proteins that expressed strong posttranslational modification after storage

Position	Protein	D7 22°C PLT/D1 PLT	D7 4°C PLT/D1 PLT	D7 4°C/D7 22°C
11	Tubulin β chain	−1.32	−16.94	−12.86
12	Tubulin α 1-β chain	−1.4	−21.95	−15.14
33	Calcineurin-β	1.05	4.14	3.94
40	Tubulin β1 chain	−1.11	6.3	6.98
45	Thioredoxin-dependent peroxide reductase	3.71	1.73	−2.15
50	Phosphatidylethanolamine-binding protein	1.08	−3.41	−3.69
51	Integrin α-IIβ	1.54	9.66	6.26
52	Lactoylglutathione	2.44	20.98	8.42
54	Proteasome subunit-β	−1.38	6.38	8.77
66	Profilin-1	−1.81	−3.57	−1.98

Protein targets selected by 2D gel analysis. Fold change in protein expression for each storage group comparison.

PLT α-tubulin acetylation is enhanced acutely by HDAC-6 inhibition

To study and compare protein acetylation in 4 and 22°C PLTs over time, we used a specific inhibitor to HDAC-6 (tubacin).[24,31] A collection of 3 unique donor PLTs were combined and used as a pooled sample. Upon activation with $CaCl_2$, PLTs exhibited a rapid deacetylation of α-tubulin. However, pretreatment with tubacin preserved the acetylated form of α-tubulin in the first 15 minutes (Figure 2A). Surprisingly, between 1 to 8 hours after activation, tubacin dramatically increased the level of acetylated α-tubulin above baseline, whereas the vehicle-treated PLTs continued to show deacetylated α-tubulin. At 24 to 72 hours after activation, after single tubacin treatment, levels of acetylated α-tubulin were similar to the vehicle-treated group (Figure 2B).

Long-term effects of single tubacin treatment were analyzed in these 4 PLT groups: (1) 22°C, (2) 22°C with tubacin (22°C-T), (3) 4°C, and (4) 4°C with tubacin (4°C-T). PLT counts in the storage bag decreased over time, where only 60% of the PLTs were retrievable after 14 days of storage, irrespective of storage conditions (supplemental Figure 2). A sample of PLTs was analyzed for α-tubulin acetylation at 5, 7, and 14 days of storage, and compared with Day 1 PLTs. At Days 5 and 7, 4°C PLTs demonstrated a faster deacetylation of α-tubulin and loss of total α-tubulin compared with 22°C and 22°C-T PLTs, suggesting they are more activated. Treatment with tubacin did not affect this loss of acetylation at the later time points of Days 5, 7 and 14, suggesting an acute effect only on α-tubulin deacetylation with single tubacin treatment. At 14 days of storage, acetylated α-tubulin expression is diminished similarly in both 22 and 4°C storage groups (Figure 2C). A separate, unique donor of PLTs was also analyzed and exhibited similar results (supplemental Figure 3).

Long-term storage of PLTs increases CD62P expression

The expression of CD62P was used as a marker of PLT activation and examined in stored PLTs by flow cytometry (Figure 2D). $CaCl_2$ treatment of Day 1 PLTs induced a slight increase in CD62P expression. However, Day 7 PLTs at 22°C showed increased expression of CD62P, whereas those in 4°C and tubacin-treated groups remained at levels similar to Day 1 (83.6%-28.8%), although their acetylation status indicated that they were more activated. Day 14 PLTs exhibited increased expression of CD62P, with both the 22°C and 22°C-T groups showing more CD62P than the 4°C and 4°C-T groups (Figure 2E).

HDAC-6 inhibition of PLTs stored at 4°C enables vasculoprotective ability in vivo

To assess the capacity of HDAC-6 inhibition to alter the vasculoprotective effects of 4°C PLTs, we used an established Miles model of vascular leak induced by vascular endothelial growth factor-A in immunocompromised NSG mice. Day 7 pooled PLTs were assessed in the model (Figure 3A). Day 1 PLTs significantly reduced permeability in vivo compared with those vehicle-treated (Figures 3B-C). Day 7 PLTs stored at 22°C significantly reduced permeability when compared with those saline-treated, but PLTs stored at 4°C did not, confirming our previous studies.[11] However, interestingly, 4°C-T PLT treatment resulted in a reduction in permeability as compared with saline treatment (Figures 3D-E).

Five minutes after injection, the number of circulating human PLTs stored at 22°C, 22°C-T, and 4°C were similar to those in Day 1 PLTs. In contrast, the 4°C-T PLTs decreased in circulation as compared with Day 1 PLTs. At 60 minutes, both 4°C and 4°C-T PLTs exhibited a significant reduction in numbers in circulation as compared with Day 1 PLTs (Figures 3F-H). Furthermore, the 4°C PLTs had decreased circulating numbers as compared with 22°C PLTs. At 5 minutes, Day 7 22°C and 22°C-T PLTs demonstrated higher expression of CD62P than Day 1 PLTs. Furthermore, 22°C-T PLTs had reduced CD62P expression as compared with 22°C PLTs. CD62P expression in 4°C and 4°C-T PLTs was similar to Day 1 PLTs but was reduced when compared with 22°C and 22°C-T (Figure 3J). At 60 minutes, both 4°C PLT groups showed lower expression of CD62P than Day 1 PLTs, with the 4°C-T group having reduced expression as compared with 4°C PLTs (Figures 3I-K). These data indicate that circulation times and CD62P expression is not related to the enhanced ability of the 4°C-T PLTs to attenuate vascular leak.

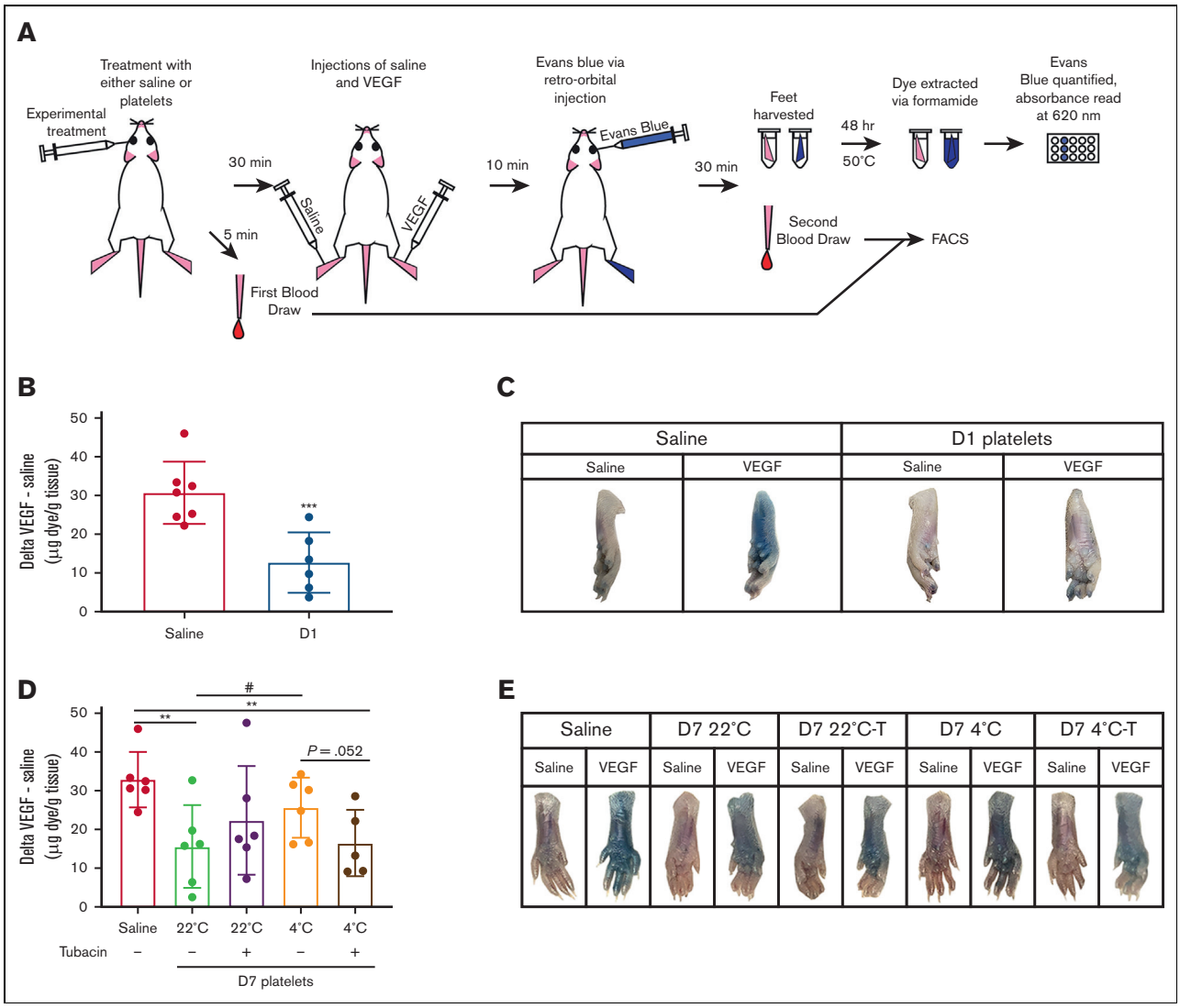

Figure 3. HDAC-6 inhibition restores vascular protective ability of 4°C PLTs in vivo. (A) Schematic showing Miles assay procedure. (B) Quantification of Evans Blue extravasation from mice treated with saline (n = 12) or Day 1 PLTs (n = 6). (C) Representative images of vascular leak of Evan's Blue dye in mouse paws treated with saline or Day 1 PLTs. (D) Quantification of Evans Blue extravasation from mice treated with saline (n = 10) or PLTs stored for 7 days at 22 or 4°C with or without tubacin (10 µM) treatment; 22°C (n = 6), 22°C-T (n = 6), 4°C (n = 6), 4°C-T (n = 6). *Indicates significant difference from saline group, as determined by Welch *t* test ($P < .01$). #Indicates significant difference between day 7 PLT groups, as determined by Welch *t* test ($P < .05$). (E) Representative images of vascular leak of Evans Blue dye in mouse paws treated with saline or day 7 PLTs in different storage conditions. (F) Flow cytometry analysis of human CD41/CD42b double-positive PLTs from each storage group, comparing 5 to 60 minutes of circulation after injection. Gating strategy from Figure 2D was used. D1 (n = 6), 22°C (n = 6), 22°C-T (n = 6), 4°C (n = 6), 4°C-T (n = 6); ($P = .05$ by RM-Two-way ANOVA). (G) Comparison of CD41/CD42b double-positive PLTs by storage group after 5 minutes of circulation (one-way ANOVA; $P = .0260$). (H) Comparison of CD41/CD42b double-positive PLTs by storage group after 60 minutes of circulation (one-way ANOVA; $P = .0048$). (I) gMFI of CD62P-positive PLTs from each storage group, comparing 5 to 60 minutes of circulation after injection. D1 (n = 6), 22°C (n = 6), 22°C -T (n = 6), 4°C (n = 6), 4°C-T (n = 6); (by RM-two-way ANOVA, significant interaction, $P < .0001$). (J) gMFI of CD62P-positive PLTs by storage group after 5 minutes of circulation (one-way ANOVA; $P < .0001$). (K) gMFI of CD62P-positive PLTs by storage group after 60 minutes of circulation (one-way ANOVA; $P < .0001$). *Indicates significant difference from Day 1 PLTs, whereas #indicates significant difference within Day 7 storage groups, as determined by one-way ANOVA (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$). Three separate donor PLTs were tested, and a representative donor is shown.

Effect of HDAC-6 inhibition on PLT aggregation and vascular protection in vitro

Consistent with our previous studies, Day 7 22°C PLTs showed a donor-dependent increase in barrier stability and attenuation of the effects of thrombin.[32] In vitro, in contrast to our in vivo findings, 4°C PLTs provided increased barrier protection (Figure 4A). When examining a pool of donors, Day 1 PLTs increased barrier resistance before and after challenge with thrombin (Figure 4B). In the unchallenged paradigm, Day 7 PLTs demonstrated an increased resistance in all groups, with the 4°C group providing significantly higher protection than its 22°C or 4°C-T counterparts (Figure 4C). All groups of Day 7 PLTs demonstrated protection against the thrombin challenge, with no significant differences between the

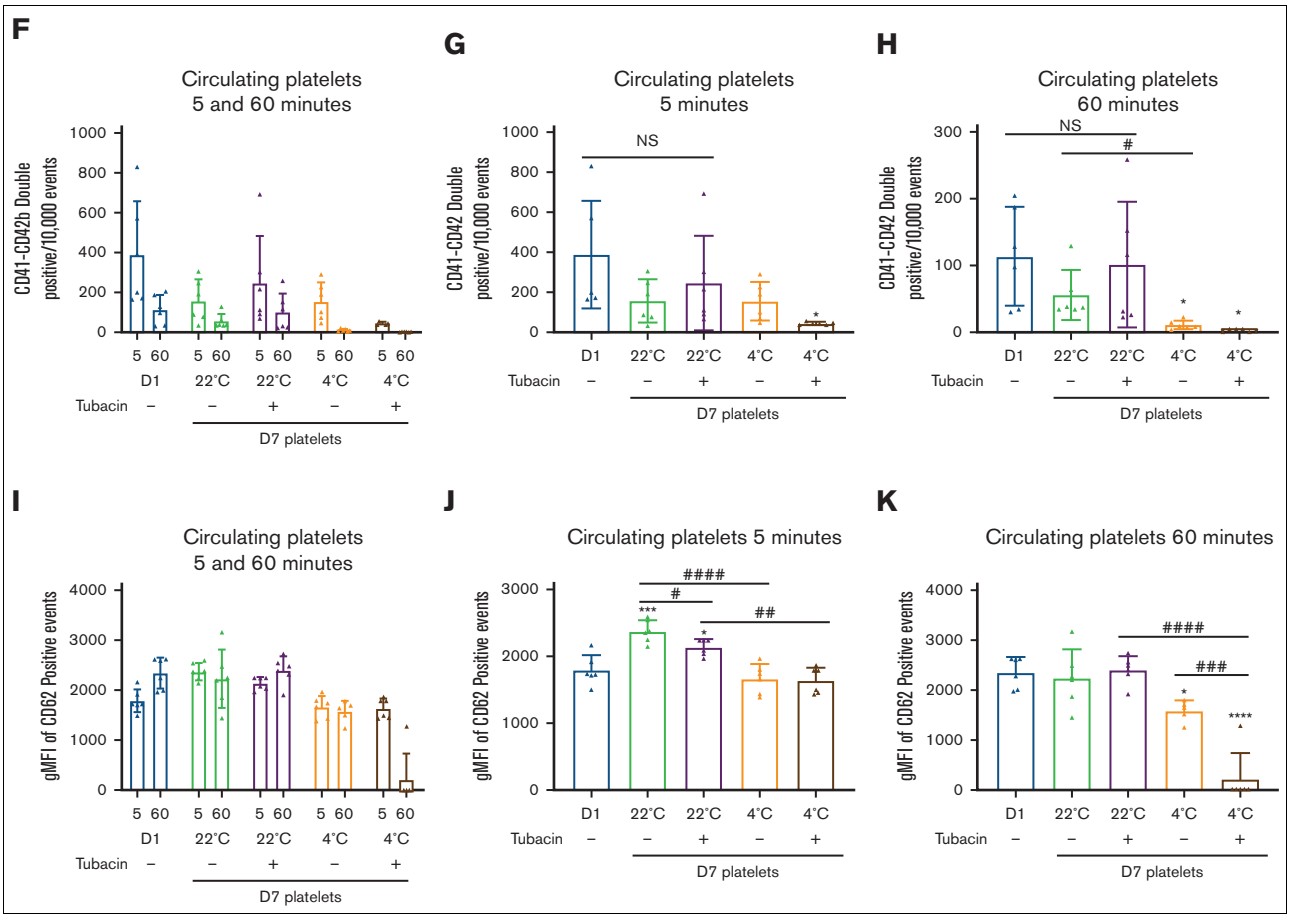

Figure 3 (continued)

groups (Figure 4C). At days 5 and 14 of storage, all groups of PLTs maintained the ability to attenuate the thrombin challenge (supplemental Figure 4A). In 2 other unique donors of PLTs, tubacin increased the protective ability of 22°C PLTs at days 5 and 7, but not at Day 14 (supplemental Figures 4B and 5). These data indicate differences in the in vitro and in vivo effects of 4°C and 22°C PLTs on vascular barrier protection. Using light transmission aggregometry, stimulation with collagen and thrombin induced an aggregation response in Day 7 PLTs similar to that seen in Day 1 PLTs in all storage groups with no differences noted (Figure 4E).

Maintenance of endothelial adherens and tight junctions is independent of storage conditions or HDAC-6 inhibition

To determine if HDAC-6 inhibition altered EC tight junction stability and adherens junctions, cocultures of PLTs and ECs were studied. Day 7 PLT treatment of the EC monolayer preserved the expression of VE-cadherin and ZO-1 and attenuated the level of F-actin mobilization (Figures 5A,C). Quantification of the gap distance created by the loss of VE-cadherin showed that PLTs from all storage groups were protective (Figure 5B). Similar results were seen with PLTs stored for 5 and 14 days (supplemental Figures 6 and 7), suggesting that enhanced EC junction protein expression was not the reason for the effects of tubacin on 4°C PLTs in vivo.

Storage of PLTs induces gross morphological change that is not altered by HDAC-6 inhibition

Using scanning electron microscopy, we imaged Day 1 and Day 7 PLTs stored at either 22 or 4°C. Stimulation of Day 1 PLTs with $CaCl_2$ induced a change in PLTs from a resting discoid to an active spheroid, exhibiting filopodia projections (arrows). This morphological change was accompanied by some PLT aggregation (arrows). After 7 days of storage, all PLT groups showed similar morphological alteration to an activated state (supplemental Figure 8).

Temperature and HDAC-6 inhibition affects protein factor expression of stored PLTs

To examine change in protein expression in stored PLTs, a Luminex panel was used to examine the levels of 19 growth factors, cytokines, and chemokines in a pooled PLT sample of 3 unique donors (Table 2). A separate unique donor PLT was also examined (supplemental Table 1). Throughout all treatment groups, the PLT protein content appeared to increase over time, including but not limited to angiogenin, endothelial growth factor, fibroblast growth factor, and Ang-1 (Figure 6A). This increase in Ang-1 appeared to be limited to PLTs stored at 4°C. Examination of the Ang-1/Ang-2 ratio also showed an increase in 4°C-T PLTs as compared with 4°C PLTs (Figure 6B). To determine the importance of the

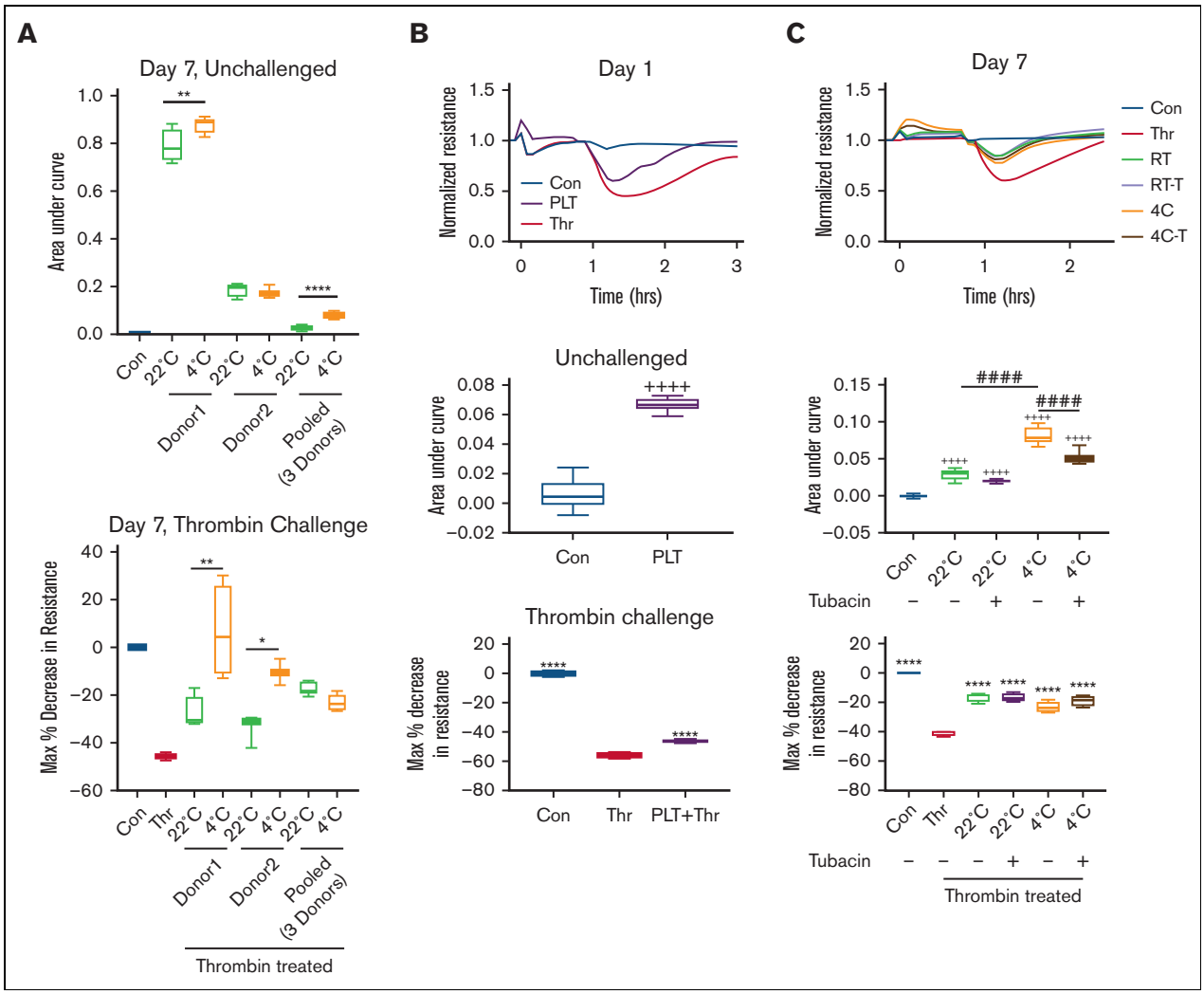

Figure 4. The effect of HDAC-6 inhibition on PLT endothelial barrier protection and PLT aggregometry. (A) Transendothelial Electrical Resistance (TEER) of HUVEC monolayers treated with PLTs isolated from 3 different donors, stored for 7 days at 22 or 4°C. Third donor group is a pooled sample of 3 donors. (B) ECIS traces and area under curve quantification show the mean treatment effects of day 1 PLTs (50 10^6 cells per mL) on the TEER of EC monolayers. Thrombin challenge (0.2 U/mL) was added 30 minutes after PLT treatment. (C) ECIS traces and area under curve quantification show the mean treatment effects of PLTs (50 × 10^6 cells per mL) stored for 7 days at 22 or 4°C, with or without tubacin (10 μM) treatment, on the TEER of EC monolayers. Thrombin challenge (0.2 U/mL) was added 30 minutes after PLT treatment. $^+$Indicates significant difference from control, *indicates significant difference from thrombin, whereas #indicates a significant difference between groups, as determined by one-way ANOVA ($P < .05$); n = 4 wells/treatment. (D) Representative traces of collagen (1.0 μg/mL)–induced PLT aggregometry, and quantification of the maximum amount of aggregation. (E) Representative traces of thrombin (0.1 U/mL)–induced PLT aggregation, and quantification of the maximum amount of aggregation. Three separate donor PLTs were tested, and a representative donor is shown. TEER, transendothelial electrical resistance.

angiopoietin-Tie2 pathway in the regulation of vascular permeability by PLTs, we investigated whether Ang-2, a competitive antagonist of the Tie-2 receptor, could block PLT regulation of endothelial monolayer permeability. Individually, Day 1 PLTs and Ang-1, but not Ang-2, a blocker of Tie-2, increased endothelial barrier resistance (Figure 6C). In thrombin-challenged cells, Day 1 PLTs and Ang-1 treatment independently attenuated EC barrier permeability, but as expected, treatment with Ang-2 was not protective against the thrombin challenge. The combination of Ang-1 and PLT treatment had no additive effect on barrier permeability, however, Ang-2 and PLTs in combination inhibited in part the PLT-mediated protection of the EC barrier. These data suggest that the Angiopoeitin-1/Tie-2 signaling pathway is an important pathway in the regulation of EC permeability by PLTs, and increased Ang-1 in 4°C-T PLTs may be 1 possible reason for the increased ability of 4°C-T PLTs to inhibit vascular permeability in vivo. A separate, unique donor PLT was also analyzed, which exhibited similar results (supplemental Figure 9).

Discussion

Here, we sought to understand how modulation of PLT microtubule dynamics may affect the PSL of 4°C PLTs and alter their ability to regulate vascular integrity. Using a proteomics-based approach, we identified time-dependent differences in the acetylation of α-tubulin between 4 and 22°C PLTs. Deacetylated α-tubulin is involved in

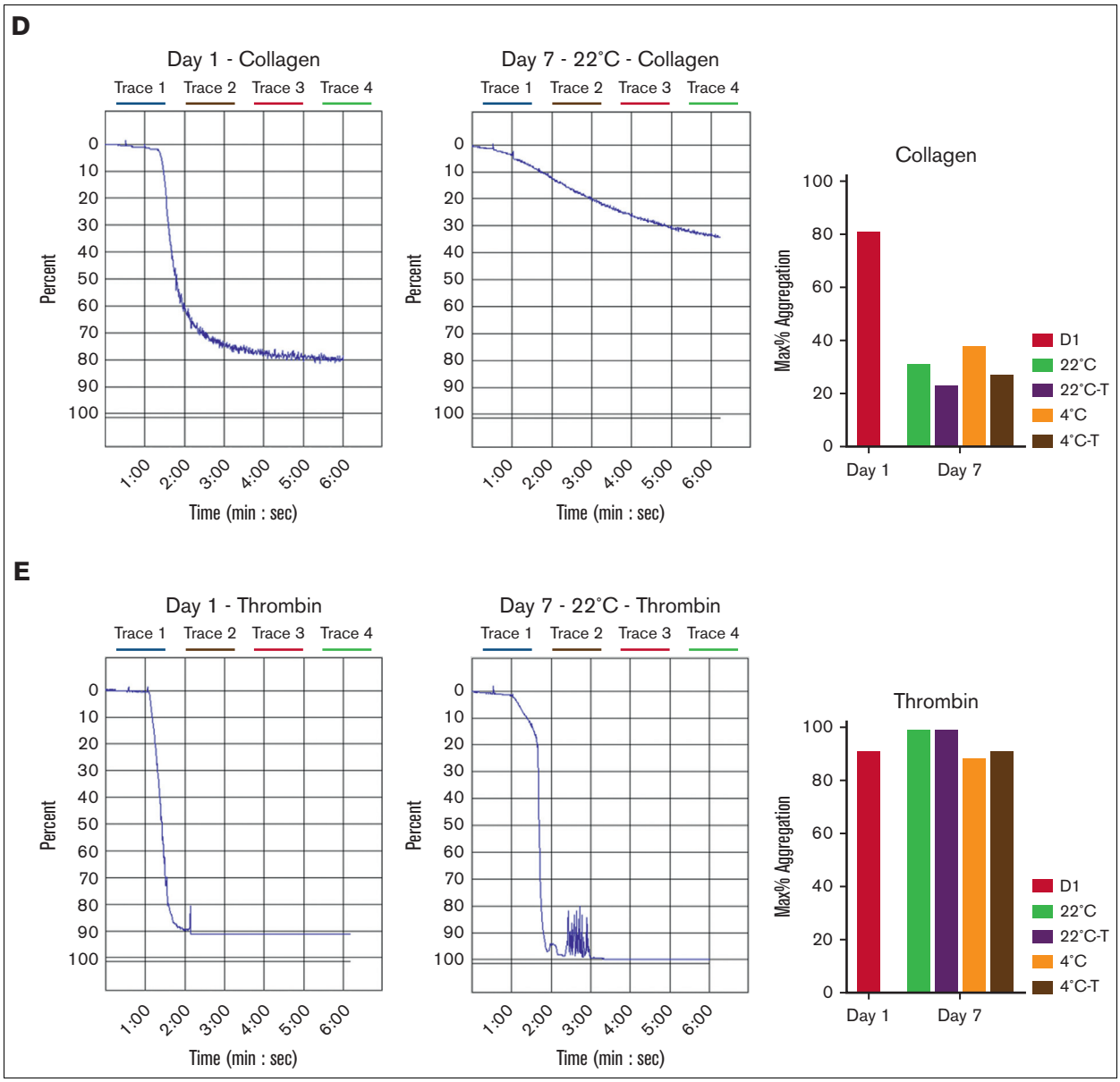

Figure 4 (continued)

PLT activation and serves as a marker for activated PLTs.[24] Consistent with our previous studies,[11] we found that 4°C PLTs demonstrated a diminished capacity to promote vascular stability compared with 22°C PLTs in vivo in a murine Miles assay of vascular leak, which could be attenuated by treatment with the HDAC-6 inhibitor tubacin (Figure 3). The mechanism underlying the differences in the vasculoprotective effects of tubacin on 4°C PLTS in vivo, after a single treatment of the PLT unit, do not appear to be directly related to extended prevention of tubulin deacetylation because the effect of tubacin on tubulin acetylation was observed to last for only the first 8 hours after treatment (Figure 2) of the PLT units and did not seem to affect total tubulin deacetylation at Days 5, 7, and 14. These data suggest that early changes (within 8 hours of storage) in the α-tubulin acetylation status of the PLTs can affect long-term functionality. Our attempts to better understand the mechanism behind the difference in functionality with tubacin revealed that there were no differences in PLT in vivo circulation times, PLT morphology, PLT aggregation ability, PLT surface marker expression, and preservation of endothelial adherens junctions in tubacin-treated PLTs (Figures 4 and 5). However, 4°C-tubacin–treated PLTs contained increased amounts of Ang-1, a highly potent vasculoprotective factor that binds to the endothelial Tie-2 receptor (Figure 6) and decreases vascular permeability. An increase in the Ang-1/Ang-2 ratio in 4°C-tubacin–treated PLTs may have induced the increased protection noted in vivo by tubacin treatment of 4°C PLTs on vascular permeability (Figure 3). Taken together, our data suggests that long-term storage of PLTs at 4°C is a viable option, and

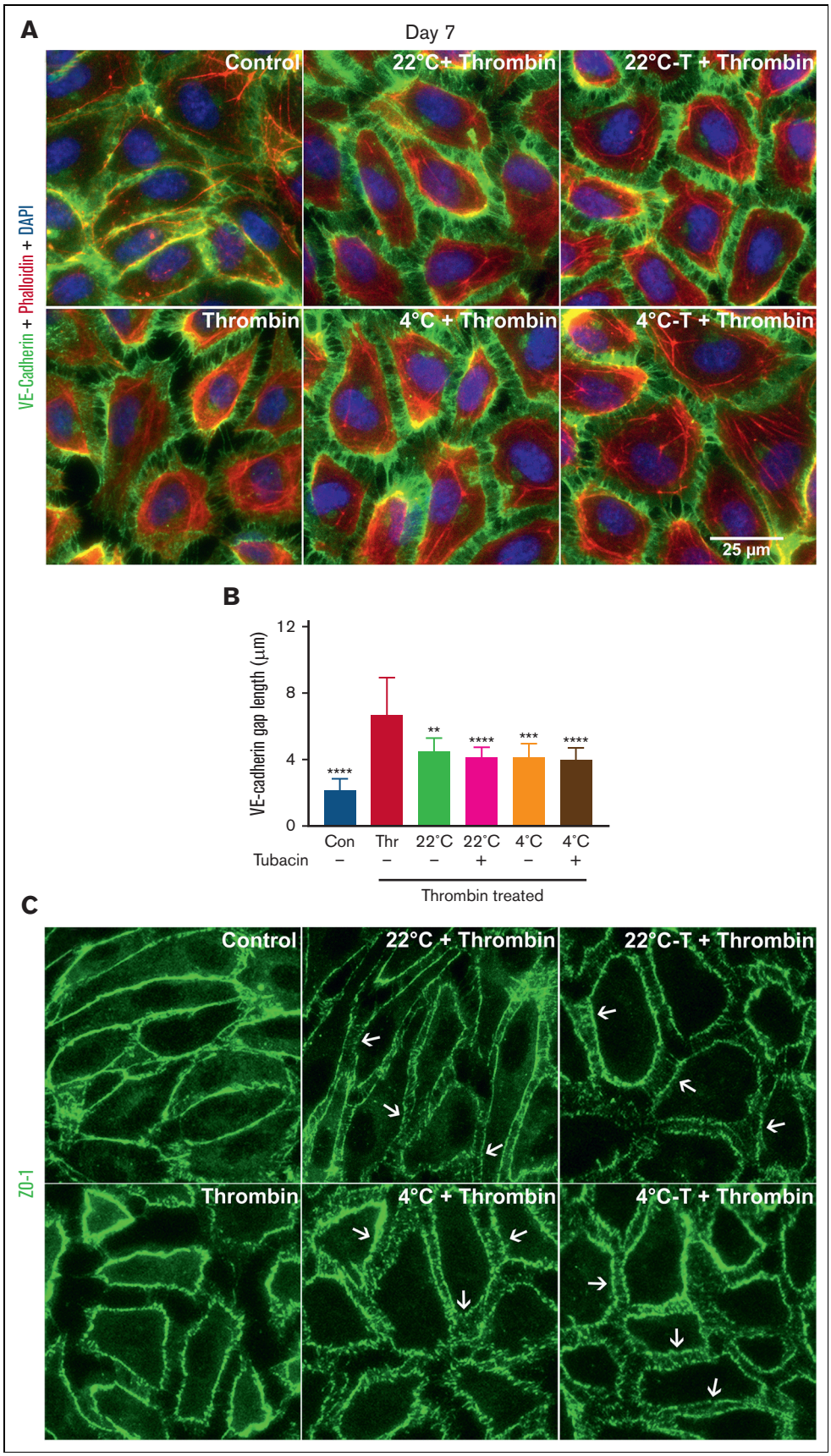
A
Day 7
Control
22°C+ Thrombin
22°C-T + Thrombin
Thrombin
4°C + Thrombin
4°C-T + Thrombin
25 µm
VE-Cadherin + Phalloidin + DAPI
B
VE-cadherin gap length (µm)
12
8
4
0
****
**
****
***
****
Con
Thr
22°C
22°C
4°C
4°C
Tubacin
–
–
–
+
–
+
Thrombin treated
C
Control
22°C + Thrombin
22°C-T + Thrombin
Thrombin
4°C + Thrombin
4°C-T + Thrombin
ZO-1

Figure 5.

single treatment of storage bags with tubacin can help attenuate diminished vascular protection in vivo of 4°C PLTs without affecting their clot-forming potential (Figure 4). Vascular protection is a critical function of PLTs in patients with thrombocytopenia, who are bleeding, that is often overlooked in the assessment of new PLT products and their suitability for transfusion.

We have previously shown that PLTs and PLT-derived extracellular vesicles restore EC junctions in vitro and decrease blood loss in a murine tail-snip model of hemorrhage.[29,32] We and others have reported that the vasculoprotective effect of 22°C PLTs in in vitro assays reduces with longer storage times.[33] Confirming our previously reported findings,[11] we observed that 22°C-stored PLTs performed better in attenuating vascular leak than 4°C-stored PLTs in vivo (Figure 3). It is hypothesized that 4°C PLTs perform poorly in vivo on vascular protection possibly because of their rapid clearance from circulation;[22] however, this does not seem to be the case in light of our findings with HDAC-6 inhibition in 4°C PLTs. Even though 4°C-tubacin–treated PLTs attenuated vascular leak in the mouse model, they displayed diminished circulation times and CD62P expression as compared with 22°C PLTs (Figure 3). Deficiencies in circulation time were the main reason for cold-stored PLTs being abandoned by blood banks during the 1970s, because circulation was thought to be critical to support vascular fragility and bleeding in patients with thrombocytopenia.[34] Our data suggest that the protective role of PLTs on vascular integrity may be independent of circulation time and activation state.

In our studies, there is an interesting disparity between our in vitro findings and in vivo findings relating to differences in function of 4 vs 22°C PLTs on endothelial barrier protection. In Figure 4, we find that 4°C-stored PLTs demonstrate an enhanced ability to increase EC monolayer impedance and decrease permeability in vitro, but this is not the case in vivo, where the 4°C PLTs were inferior in their barrier protective effects (Figure 3). In addition, treatment of stored PLTs with tubacin enhanced the vasculoprotective effect of the 4°C PLTs in vivo but not in vitro (Figures 3 and 4). In vitro, both 22°C PLTs and 4°C PLTs showed protection of the endothelial barrier with mixed results on the effects of tubacin (supplemental Figure 3). We found donor-dependent effects in the response, confirming our previous findings on the donor-dependent variability in PLT effects on vascular permeability.[11] We hypothesize that in vitro assessment of PLT function cannot accurately recapitulate the environment (ie, flow and shear stress, leukocytes, RBCs, plasma, and blood vessel walls) and milieu of cell-cell interactions present in vivo that may influence the function of the PLTs; hence, many established in vitro assays of PLT function may not accurately represent the differences and function of PLT products in patients who have undergone transfusion. At the same time, we recognize that a limitation of this study is that we are aiming to investigate human PLTs in animal models to remain translationally relevant, which can create a disparity because of cross-species interactions. In addition, the endogenous circulating mouse PLTs likely inhibit vascular leak, creating a baseline of protection in the mouse model; nonetheless, transfusion of human PLTs enhances this protection above baseline.

Although we observed an effect of PLT storage and tubacin treatment on endothelial permeability in vivo, no effect of tubacin was observed in assays of PLT aggregometry, an in vitro indication of the clot-forming ability of the PLTs (Figure 4). Aggregation in response to collagen was diminished in all groups at Day 7, independent of tubacin treatment. This contrasts with a recent study.[35] The difference in results between this study and ours could be due to the agonist used. Again, these data call into question the clinical significance of in vitro testing of PLT function with assays such as aggregometry.

By Luminex assay (Figure 6), we observed a drop in most of the PLT alpha granule proteins over time, but interestingly, proteins associated with vascular function and permeability demonstrated an increase over time in 4°C PLTs. It is possible that this increase is from altered degranulation, uptake from the plasma storage solution, or endogenous translation within 4°C PLTs. Ang-1 is particularly interesting because only the 4°C-tubacin–treated PLT groups demonstrated an increase in Ang-1 throughout storage. This protein is widely known to protect the endothelium by binding to the Tie-2 receptor on endothelial cells, which results in downstream signaling that regulates the cytoskeleton.[36] Ang-2 opposes Ang-1 activity by competing with Ang-1 for the Tie2-R, resulting in vascular permeability.[36] Hence, the ratio of the 2 proteins has been used in the field to gauge whether their combination is protective or stimulates vascular leak. A higher Ang1/Ang2 ratio is indicative of increased protection or inhibition of endothelial permeability. A number of papers on trauma and other disease conditions have found the ratio to be indicative of outcomes.[37-40] We find that the Ang1/Ang2 ratio (Figure 6B) is increased in 4°C-tubacin–treated PLTs, suggesting that the balance is tipped in favor of barrier protection. It is important to note that human Ang1 protein is protective against barrier leak in mouse endothelium, reinforcing the validity of human PLT treatment in mouse models.[41] In addition, a recent study has shown that Ang-1 also increases tubulin acetylation in endothelial cells.[42] As seen by the in vivo and in vitro assays, tubacin treatment did not completely rescue the diminished vasculoprotective effects, suggesting that other pathways may also play a role in PLT functionality on vascular protection.

Because of the renewed interest in 4°C storage of PLTs, clinical trials involving 4°C-stored PLTs have been performed and are being planned.[12] However, there is no clinical end point assessing the effects of 4°C PLTs on vascular integrity, a critical component of PLT function. Early HDAC-6 inhibition in 4°C PLTs mitigates the diminished effects of stored 4°C PLTs on vascular protection in vivo, suggesting that it may be a viable option for mitigation of the 4°C PSL. In future studies, we will aim to understand the mechanistic relationship between tubacin and Ang1 in relevant models of hemorrhage and injury and elucidate the role of increased Ang1 in the tubacin-treated human PLTs.

Figure 5. Stored PLTs maintain adherens and tight junctions independent of HDAC-6 inhibition. Representative images of HUVEC monolayers treated with PLTs (50 × 10^6/mL) stored for 7 days at 22 or 4°C, with or without tubacin (10 μM) treatment and subsequently challenged with thrombin (0.2 U/mL). HUVEC monolayers were stained for VE-cadherin and F-actin (A), or ZO-1 (C). Length of gaps between cells quantified from VE-cadherin–stained images (B). *Indicates significant difference from thrombin group, as determined by one-way ANOVA ($P < .05$). Three separate donor PLTs were tested, and a representative donor is shown.

Table 2. Factor concentration levels in PLTs stored over time and temperature

		Day5 (pg/ml)				Day7 (pg/ml)				Day14 (pg/ml)			
Protein	**Day 1 (pg/mL)**	**22°C**	**22°C-T**	**4°C**	**4°C-T**	**22°C**	**22°C-T**	**4°C**	**4°C-T**	**22°C**	**22°C-T**	**4°C**	**4°C-T**
Ang1	4 749.9	5 850.5	5 939.7	6 011.2	8 110.1	827.6	1 287.7	1 419.8	2 768.2	771.6	763.1	771.6	740.7
Ang2	399.9	800.2	901.7	1 067.7	726.1	839.2	716.4	835.3	628.6	657.9	450.9	593.	577.9
BDNF	3 354.1	3 816.9	3 739.4	3 947.0	5 406.8	465.2	1 212.4	774.3	2 148.1	408.3	665.2	444.1	640.7
vWF-A2	424.1	527.1	601.6	629.8	624.7	611.8	778.6	737.6	776.1	581.0	624.7	634.9	609.3
Thrombo moduli–	345.6	472.1	455.6	466.6	538.1	207.3	226.8	185.0	257.3	134.3	185.0	159.7	151.2
Thrombo spondin–	517.2	517.2	525.8	576.9	448.7	405.7	379.8	362.4	405.7	379.8	388.4	397.0	379.8
CD40	15 733.6	14 708.7	18 608.4	22 577.1	19 305.4	17 956.7	19 245.4	19 499.0	19 961.5	15 733.6	14 859.7	14 664.7	14 501.6
PDGF-AA	353.0	483.6	514.5	1 528.3	640.3	272.6	147.9	368.5	207.5	130.5	229.9	131.6	133.3
PDGF-BB	1 233.0	1 884.1	1 843.2	1 896.1	2 743.9	216.6	461.9	346.8	762.7	168.5	321.8	271.3	263.1
Vascular endothelial growth factor	393.9	216.3	266.7	332.2	252.6	323.4	342.8	357.0	329.0	242.8	257.3	249.5	245.1
Endothelial growth factor	40.7	164.9	123.4	102.0	87.2	96.8	108.7	88.2	101.8	132.3	149.9	140.9	133.7
Fibroblast growth factor	223.7	158.6	177.3	233.0	190.9	212.9	225.1	279.0	271.8	183.8	154.3	169.4	185.1
Tumor necrosis factor α	23.0	31.5	35.7	46.5	39.8	45.2	46.5	74.0	58.4	44.5	45.2	53.2	40.4
IFN-γ	59.1	92.9	112.9	134.4	122.8	116.2	168.8	139.3	201.3	149.2	178.6	185.1	149.2
IL-1β	59.1	20.9	29.1	28.2	28.2	32.7	34.5	36.3	47.5	30.9	42.4	32.7	29.1
IL-6	4.4	10.9	10.9	11.3	11.8	15.7	14.0	14.2	16.5	16.7	20.8	14.7	14.0
IL-10	3.5	9.8	14.2	16.5	14.2	32.6	38.6	28.4	32.1	31.8	29.5	30.3	27.2
CXCL8	80.7	55.5	70.6	77.3	69.6	99.3	103.0	107.8	93.7	94.6	93.4	85.6	72.1
CXCL12	649.1	556.6	558.7	721.3	645.8	679.2	677.3	683.0	657.5	615.6	645.8	624.9	584.8

PLTs stored for 1 to 14 days at 22 or 4°C, with or without tubacin (10 μM) treatment were assessed on a multiplex Luminex panel for the target proteins indicated.
IFN-γ, interferon-gamma; PDGF, platelet-derived growth factor.

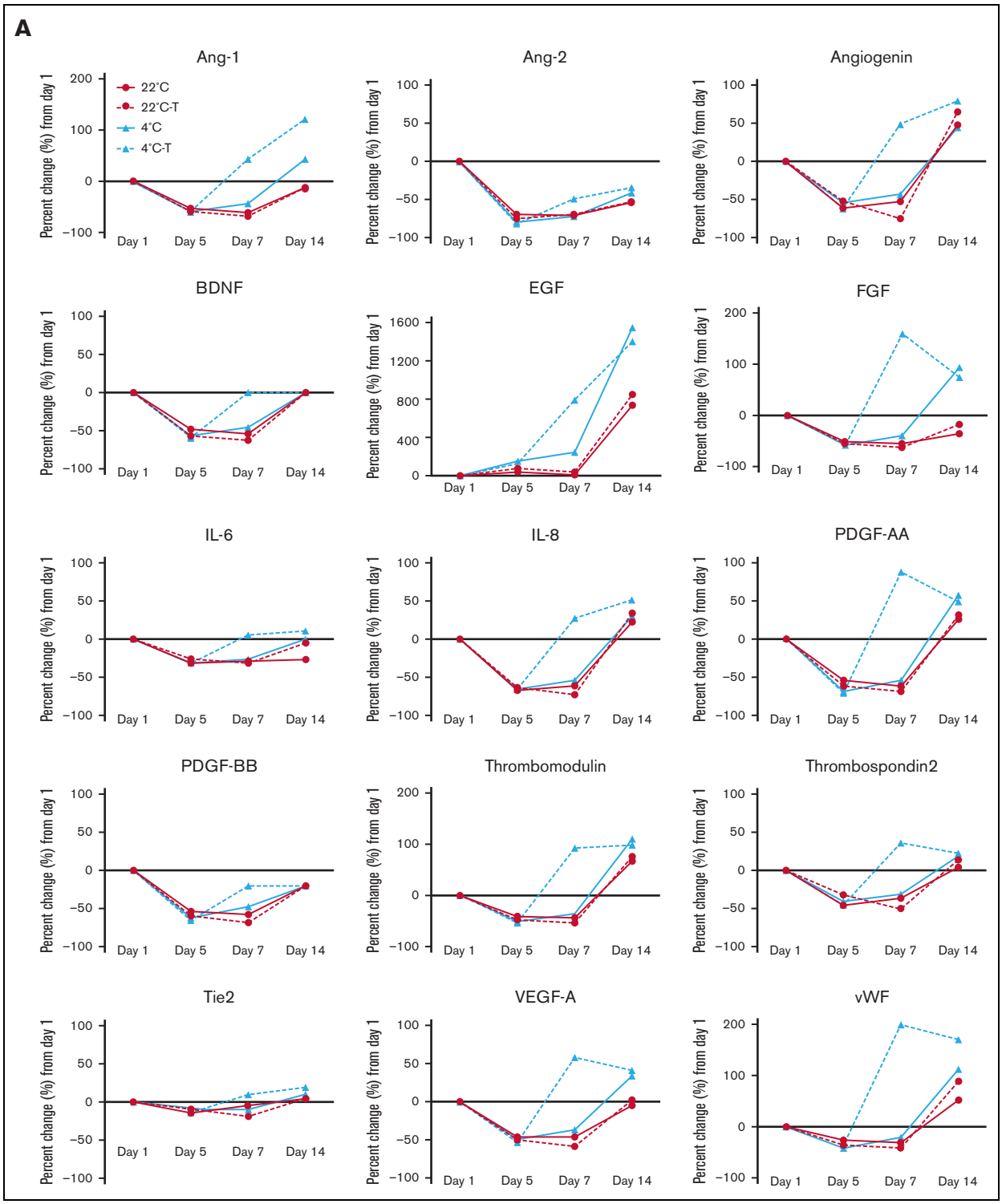

Figure 6. Storage of PLTs over time and temperature affects changes in PLT growth factor composition. (A) PLTs stored at 22 and 4°C, with or without tubacin (10 μM) treatment, were collected at Day 5, Day 7, and Day 14, and assessed on a multiplex Luminex panel for the target proteins indicated. (B) Comparison of Ang-1/Ang-2 ratio between 22°C and 22°C-T PLTs between 4°C and 4°C-T PLTs. (C) TEER quantification of Day 1 PLT treatment of HUVEC monolayers. PLTs (50×10^6/mL) were incubated with Ang-1 (1.0 μg/mL), or Ang-2 (1.0 μg/mL), before treatment of the HUVEC monolayer. HUVEC monolayers were challenged with thrombin (0.2 U/mL) 30 minutes after pretreatment. $^+$Indicates significant difference from control, *indicates significant difference from thrombin, *a* indicates significant difference from PLT, whereas #indicates significant difference between groups as determined by one-way ANOVA ($P < .05$); n = 4 wells per treatment. Three separate donor PLTs were tested, and a representative donor is shown. TEER, transendothelial electrical resistance.

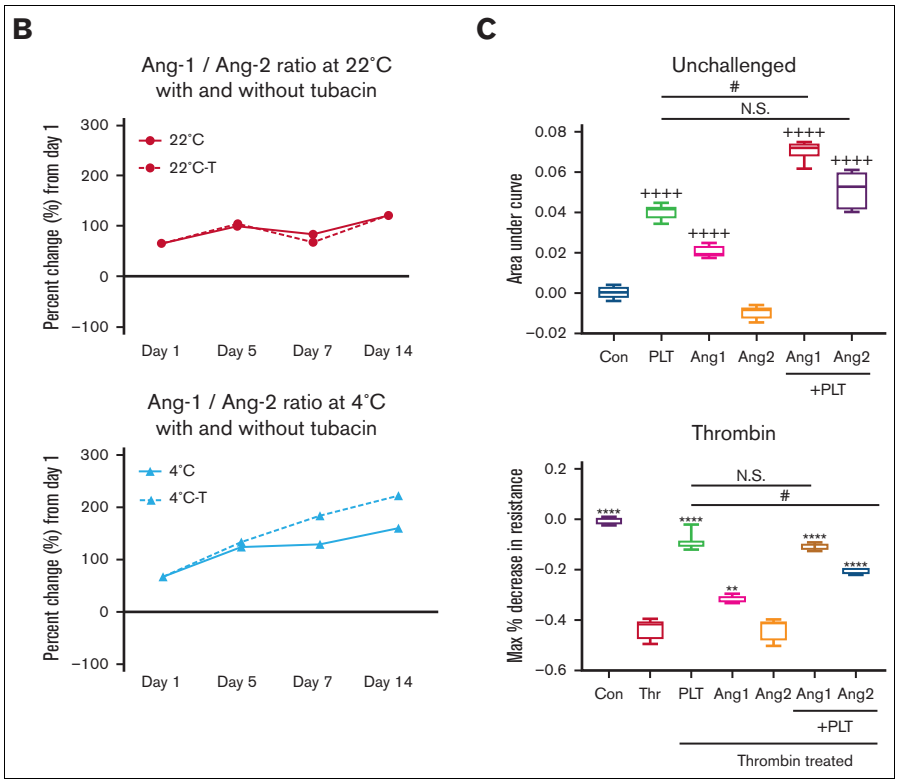

Figure 6 (continued)

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Authorship

Contribution: B.M. executed all in vitro assays, writing, and editing of the manuscript; A.T. performed analysis of all in vitro and in vivo experiments, writing, and editing of the manuscript; L.V. read and edited the manuscript, made figures, and performed staining for cell analysis and flow analysis; M.L. read and edited the manuscript and performed Miles assay; D.P. generated figures for in vivo model development and read the manuscript; A.N. graphed the protein granular content figure, read, and edited the manuscript; M.B. read and edited the manuscript for clinical relevance; A.P.C. performed analysis of data, critical reading, and editing of the manuscript; S.P. performed experiment conceptualization, supervision, writing and editing of the manuscript, and funding acquisition.

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