

Inhibition of GPIb- α -mediated apoptosis signaling enables cold storage of platelets

Irene Marini,^{1,2} Lisann Pelzl,^{1,2} Yoko Tamamushi,¹ Chiara-Tanita Maettler,¹ Andreas Witzemann,¹ Karina Althaus,^{1,2} Stefanie Nowak-Harnau,² Erhard Seifried³ and Tamam Bakchoul^{1,2}

¹Institute for Clinical and Experimental Transfusion Medicine, Medical Faculty of Tübingen, Tübingen; ²Center for Clinical Transfusion Medicine Tübingen and ³Institute of Transfusion Medicine and Immunohematology, German Red Cross Blood Transfusion Service Baden-Württemberg-Hessen, Frankfurt, Germany

Correspondence: T. Bakchoul
tamam.bakchoul@med.uni-tuebingen.de

Received: December 14, 2022.

Accepted: June 15, 2023.

Early view: June 22, 2023.

<https://doi.org/10.3324/haematol.2022.282572>

©2023 Ferrata Storti Foundation

Published under a CC BY-NC license



Abstract

Cold storage of platelets has been suggested as an alternative approach to reduce the risk of bacterial contamination and to improve the cell quality as well as functionality compared to room temperature storage. However, cold-stored platelets (CSP) are rapidly cleared from the circulation. Among several possible mechanisms, apoptosis has been recently proposed to be responsible for the short half-life of refrigerated platelets. In the present study, we investigated the impact of apoptosis inhibition on the hemostatic functions and survival of CSP. We found that blocking the transduction of the apoptotic signal induced by glycoprotein Ib (GPIb)- α clustering or the activation of caspase 9 does not impair CSP functionality. In fact, the inhibition of GPIb- α clustering mediated-apoptotic signal by a RhoA inhibitor better conserved δ granule release, platelet aggregation, adhesion and the ability to form stable clots, compared to untreated CSP. In contrast, upregulation of the protein kinase A caused a drastic impairment of platelet functions and whole blood clot stability. More importantly, we observed a significant improvement of the half-life of CSP upon inhibition of the intracellular signal induced by GPIb- α clustering. In conclusion, our study provides novel insights on the *in vitro* hemostatic functions and half-life of CSP upon inhibition of the intracellular cold-induced apoptotic pathway. Our data suggest that the combination of cold storage and apoptosis inhibition might be a promising strategy to prolong the storage time without impairing hemostatic functions or survival of refrigerated platelets.

Introduction

Transfusion of platelet concentrates (PC) is an essential medical approach to treat bleeding in thrombocytopenic patients.¹ Currently, PC are stored at room temperature (RT, 22-24°C). At this storage condition PC might be associated with increased risk of bacterial contamination,² post-transfusion septic reaction^{3,4} and platelet storage lesions (PSL).⁵ Therefore, the storage time of PC is restricted to 4-7 days, depending on the national guidelines.¹ Nevertheless, the risk of transmission of bacterial infection still remains high.^{2,6} Moreover, recent clinical studies reported on detrimental effects in patients who received RT-stored PC.^{7,8} In this context, alternative storage conditions like cold storage have been considered. In fact, cold storage might reduce the risk of bacterial contaminations, protect cells from storage lesions and extend products' shelf-life.^{9,10} However, clinical appli-

cations of cold-stored platelets (CSP), even if characterized by better functionality,⁹⁻¹⁴ have been abandoned due to concerns regarding poor recovery and survival compared to RT.¹⁵ In fact, as reported in a recent radiolabeling study, the survival of autologous CSP upon 5 days of storage was significantly lower (around 20%) compared to platelets stored for 7 days at RT (around 50%).¹⁶ However, this drawback of CSP might be compensated by better functionality.

Several groups have investigated potential mechanisms underlying the observed short half-life of CSP. One of the first proposed mechanisms was the desialylation of glycoproteins (GP).¹⁷ However, a clinical study showed that reconstitution of sialylation does not prevent the accelerated clearance of CSP.¹⁸ This indicates that alternative mechanisms, which do not require desialylation, trigger the fast elimination of refrigerated platelets. Interestingly, we and others detected increased apoptosis levels in pla-

telets upon cold storage compared to RT suggesting that the apoptotic pathway might contribute to the reduced half-life of CSP.^{9,16,19} In fact, cold storage was shown to trigger clustering of GPIb- α ²⁰ on the platelet surface leading to apoptosis.^{19,21} Interestingly, it has been reported that the presence of an apoptosis inhibitor (p38 inhibitor) reduces the expression of apoptotic markers, like Bax and Bak, in pathogen-inactivated PC stored at RT but it did not improve platelet survival *in vivo*.²²

In the present work, we aimed to verify the efficacy of three compounds that have been shown to inhibit the apoptotic intracellular signal at different levels by targeting three key regulatory proteins (Figure 1; *Online Supplementary Appendix*). Our hypothesis was that all compounds could reduce cold-induced apoptosis. Nevertheless, since the proteins targeted by the inhibitors are involved in essential mechanisms of platelet functionality we thought to investigate potential desired/undesired effects of each inhibitor on different cell functions in order

to screen them and select the most promising one to perform *in vivo* studies.

Methods

Preparation of platelet concentrates

PC were collected using the apheresis device TRIMAAccel 7.0 (TERUMO BCT, Munich, Germany). Briefly, 166 mL platelet-rich plasma (PRP) were collected and resuspended in 271 mL additive solution (PASIII, Machropharm, Germany). Next, three inhibitors were added to inhibit the apoptotic signals. The first compound (G04) targets the RhoA GTPase, which has been shown to play an essential role in the transduction of the outside/inside signal downstream of GPIb- α .²³ Furthermore, we tested a compound (forskolin), which upregulates the protein kinase A (PKA), via adenylyl cyclase (AC), that is known to control the apoptotic pathway by inhibiting the pro-apoptotic protein

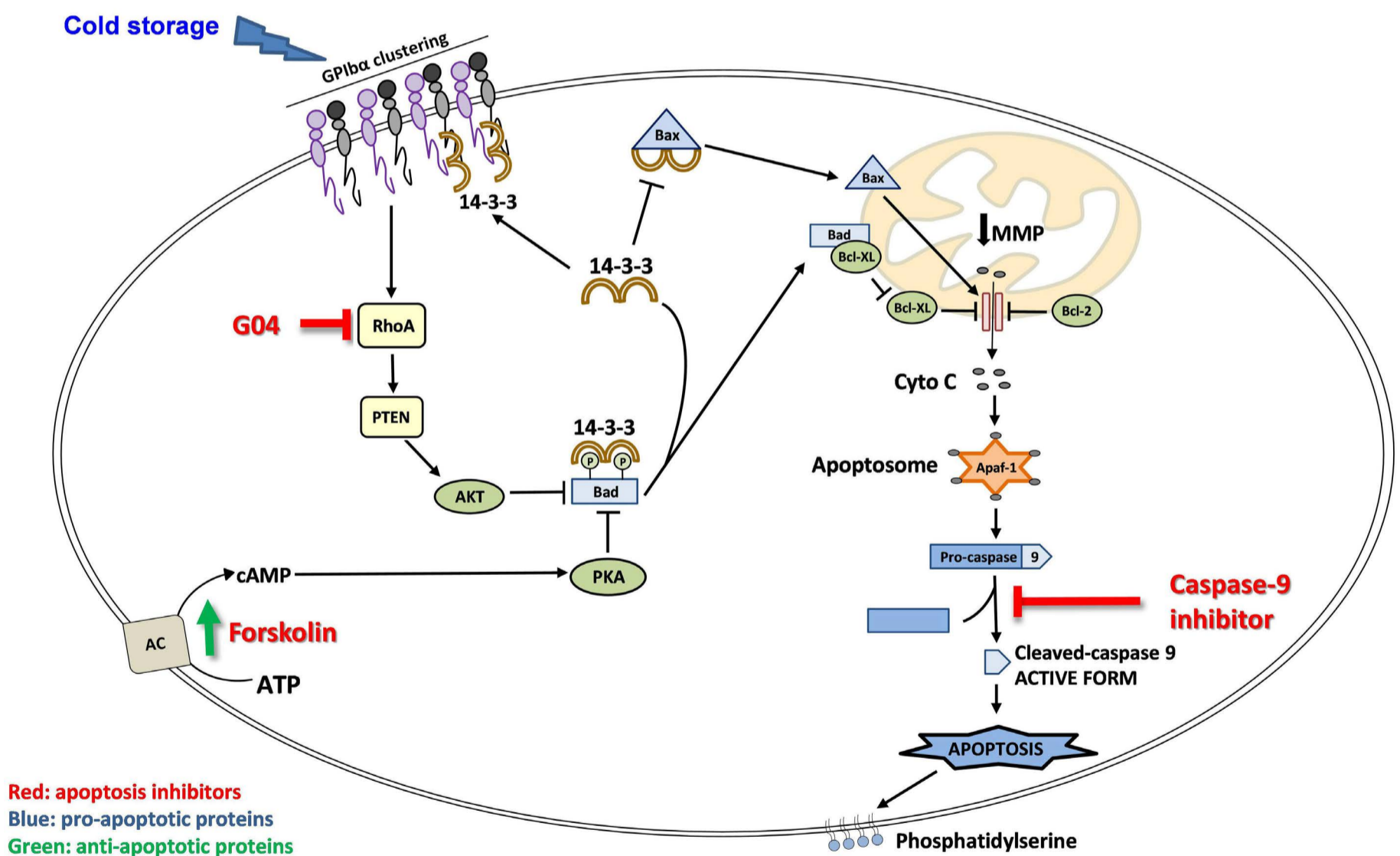


Figure 1. Schematic illustration of the intrinsic apoptotic pathway and the corresponding targets of the apoptosis inhibitors used in the present study. It is presumed that cold storage of platelets induces clustering of the glycoprotein Ib- α (GPIb- α) leading to apoptosis which in turn triggers the reduction of the mitochondrial membrane potential (MMP) and phosphatidylserine externalization. In the present study we used the following apoptosis inhibitors: G04 which inhibits RhoA binding its guanine exchange factor domain. Forskolin that enhances the functionality of adenylyl cyclase (AC) inducing conformational change upon its binding and increasing the production of cyclic adenosine monophosphate (cAMP). The latter, triggers the activation of protein kinase A (PKA) which in turn inhibits the pro-apoptotic protein Bad by phosphorylation. Caspase-9 activation is blocked by a covalent irreversible binding with the Caspase-9 inhibitor which prevents the autocatalytic cleavage of the pro-caspase-9. Cyto C: cytochrome C.

Bad upon phosphorylation (Figure 1).²⁴ The last tested compound (Z-LEHD-fmk) prevents the autocatalytic cleavage of caspase-9 that is necessary to begin the caspase cascade (Figure 1).²⁵ The following final concentrations were used: 150 μM G04 (RhoA Inhibitor, Millipore Corp., Darmstadt, Germany), 0.75 μM forskolin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 40 μM caspase-9 inhibitor²⁶ (Z-LEHD-fmk, BD Biosciences, San Jose, USA). Apoptosis inhibitors were added to different apheresis bags and stored at 4°C under agitation. See the *Online Supplementary Appendix* for further details.

Detection of platelet apoptosis

In order to detect changes in the mitochondrial membrane potential (MMP), the tetramethylrhodamine ethyl ester (TMRE) assay kit (Abcam, Cambridge, UK) was used and the externalization of phosphatidylserine (PS) was determined using Annexin-V (Immunotools, Friesoyhte, Germany) staining. See the *Online Supplementary Appendix* for further information.

Assessment of platelet function

In order to analyze platelet activation, CSP were stimulated with thrombin receptor-activating peptide-6 (TRAP-6, 10 μM , HART Biologicals, Hartlepool, UK) and incubated with CD62-P (CLB-Thromb/6, Beckman Coulter, Krefeld, Germany) or CD63 (CLB-Gran/12, Beckman Coulter) and measured by flow cytometry (FC) (Navios, Beckman-Coulter, Krefeld, Germany). Light transmission platelet aggregation assay was performed using a four-channel aggregometer (LABiTec, Labor BioMedical Technologies, Ahrensburg, Germany) to investigate the aggregation ability in response to 20 μM TRAP-6, 1.0 mg/mL ristocetin (HART Biologicals, Hartlepool, UK) or NaCl (Braun, Melsungen, Germany). Next, the adhesion ability of CSP was assessed as previously described using coverslips (Corning, New York, USA) coated with 100 mg/mL fibrinogen (Sigma Aldrich, Munich, Germany) or 5% human serum albumin (Grifols, Berlin, Germany).⁹ CSP were allowed to seed on coverslips in the presence of 10 μM TRAP-6. Platelet clot retraction was assessed upon incubation with 7.4 μM CaCl_2 (Sigma Aldrich, Darmstadt, Germany) and thrombin 10 U/mL (Roche, Mannheim, Germany). Thromboelastography was performed using the ClotPro analyzer (Haemonetics, Munich, Germany) according to the manufacturer's instructions. See the *Online Supplementary Appendix* for further information concerning each assay.

In vivo studies

In order to determine the survival and recovery of human CSP, we used an NSG (NOD [non-obese diabetic] Scid Gamma) mouse model. The experiment procedure was performed as previously described with minor modifications.⁹ See the *Online Supplementary Appendix* and *Online*

Supplementary Figures S5 and S6 for additional information.

Ethics and statistical analysis

All studies involving human subjects were approved by the Ethics Committees of the University Hospital of Tübingen. Animal studies were approved by the State Animal Ethics Committees of Baden-Württemberg. Statistical analyses were performed using GraphPad Prism 9.4.1 (GraphPad Software, La Jolla, USA). See the *Online Supplementary Appendix* for additional details.

Results

Inhibition of cold-induced platelet apoptosis

In order to verify the efficacy of the apoptosis inhibitors during cold storage, two of the key steps of the apoptotic pathway were analyzed, the reduction of MMP and the PS externalization. We found that CSP started showing apoptotic phenotype from storage day 4, as suggested by the significant decrease of MMP compared to day 1 (MMP mean fluorescence intensity [MFI] mean \pm standard error of the mean [SEM]: buffer day 1 vs. day 4, 30.00 \pm 0.61 vs. 24.33 \pm 1.26, $P=0.0076$; Figure 2A-C, respectively). This effect was time-dependent (MMP MFI mean \pm SEM: buffer day 4 vs. day 7, 24.33 \pm 1.26 vs. 9.74 \pm 1.05, $P=0.0001$; buffer day 7 vs. day 10, 9.74 \pm 1.05 vs. 3.76 \pm 0.29, $P=0.0081$; Figure 2A-C, respectively). The inhibition of RhoA (G04, Figure 2A, D), activation of PKA (forskolin, Figure 2B, E) and down-regulation of the autocatalytic cleavage of caspase-9 (Figure 2C, F) induced a significant reduction of the apoptotic signal. The mitochondrial integrity was better maintained on day 7 and 10, as indicated by the significant higher levels of MMP, in comparison to untreated cells (MMP MFI mean \pm SEM: G04 vs. buffer day 7, 17.65 \pm 1.64 vs. 9.74 \pm 1.05, $P=0.0091$; day 10, 6.81 \pm 0.53 vs. 3.76 \pm 0.29, $P=0.0080$; forskolin vs. buffer day 7, 12.92 \pm 1.23 vs. 9.73 \pm 1.05, $P=0.0434$; day 10, 4.81 \pm 0.37 vs. 3.76 \pm 0.29, $P=0.0404$; caspase-9 inhibitor vs. buffer day 7, 12.45 \pm 0.83 vs. 9.74 \pm 1.05, $P=0.0384$; day 10, 5.08 \pm 0.61 vs. 3.76 \pm 0.29, $P=0.0477$; Figure 2A-C, respectively).

Similarly, treatment of CSP with apoptosis inhibitors revealed a significant reduction of the percentage of cells exposing PS starting from storage day 7 compared to cells stored in buffer (% apoptotic cells, mean \pm SEM: G04 vs. buffer day 7, 9 \pm 3% vs. 26 \pm 6%, $P=0.0218$; day 10, 39 \pm 5% vs. 67 \pm 6%, $P=0.0023$; forskolin vs. buffer day 7, 7 \pm 1% vs. 26 \pm 6%, $P=0.0084$; day 10, 48 \pm 4% vs. 67 \pm 6%, $P=0.0319$; caspase-9 inhibitor vs. buffer day 7, 10 \pm 3% vs. 26 \pm 6%, $P=0.0105$; day 10, 48 \pm 5% vs. 67 \pm 6%, $P=0.0371$; Figure 2D-F, respectively). Of note, after 10 days of storage all treated CSP showed increased PS externalization, compared to storage day 7. Nevertheless, the percentage of cells ex-

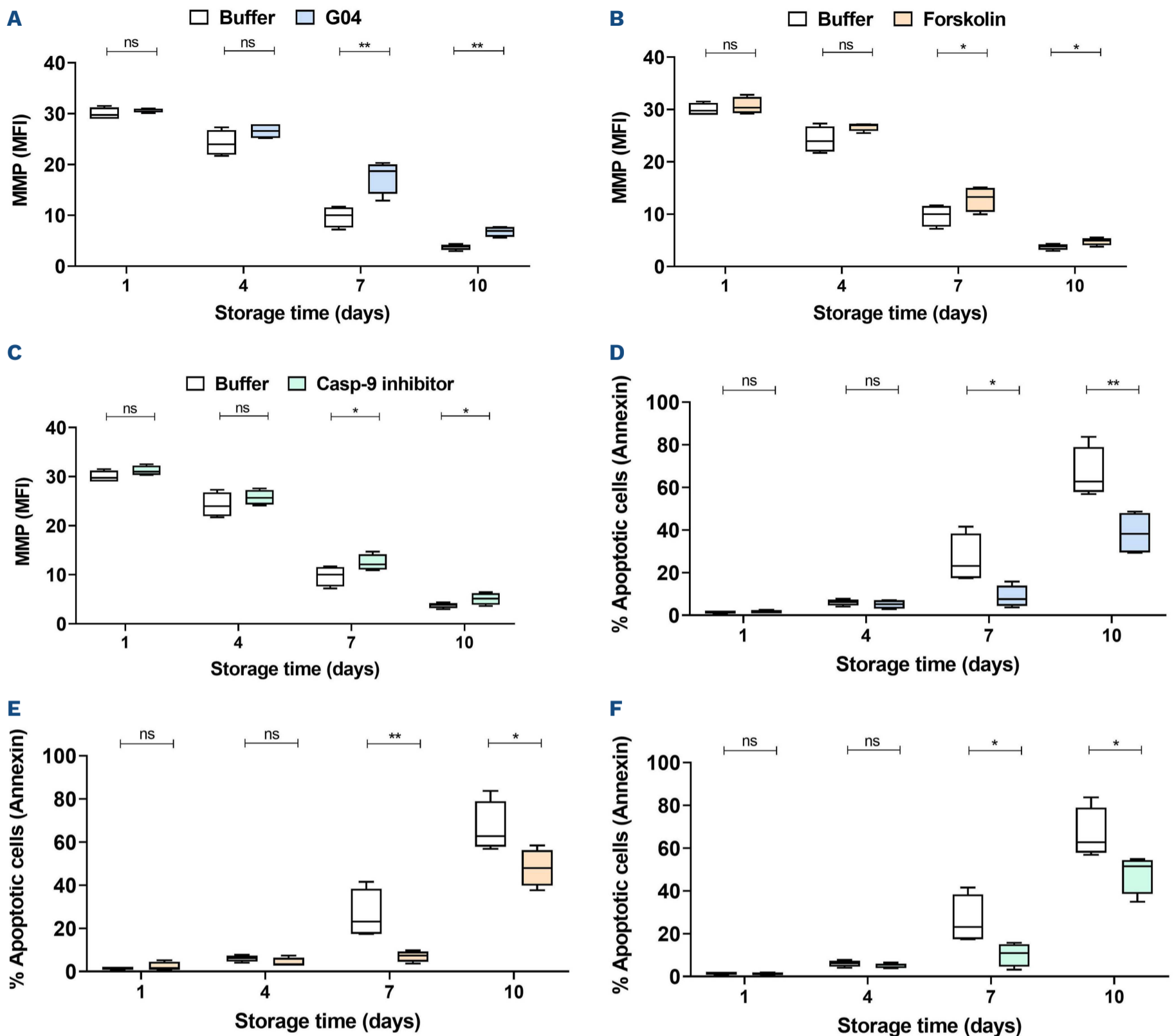


Figure 2. Apoptosis inhibition during cold storage of platelet concentrates. The mitochondrial membrane potential (MMP; A-C) and the percentage of apoptotic cells (% Annexin-positive cells; D-F) of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) were detected by flow cytometry after 1, 4, 7 and 10 days of cold storage, respectively. MFI: mean fluorescence intensity. Data are shown as box and whiskers \pm standard error of the mean. * $P < 0.05$; ** $P < 0.01$; ns: not significant, $N = 4$.

posing PS on day 10 was significantly lower in the presence of the inhibitors than cells stored in buffer (Figure 2D-F).

These results indicate that pretreatment of PC with apoptosis inhibitors can prevent the cold-induced apoptosis.

Inhibition of GPIIb- α clustering signal maintains β granule release better and preserves platelet aggregation and agglutination in cold-stored platelets

The impact of apoptosis inhibition on platelet activation and aggregation was analyzed using FC and aggregometry,

respectively. First, the release of α (CD62-P) and δ (CD63) granules content was determined upon TRAP-6 stimulation (Figure 3). The inhibition of either GPIIb- α clustering signal, induced by G04 incubation, or caspase-9 showed unchanged α granule secretion compared to untreated-CSP (Figure 3A, C). On the contrary, the incubation with the PKA agonist induced a significant reduction of the α granule secretion, already after 1 day of cold storage (fold increase [FI] CD62-P mean \pm SEM: forskolin vs. buffer day 1, 2.03 ± 0.24 vs. 4.18 ± 0.74 , $P = 0.0487$; day 4, 0.91 ± 0.16 vs. 2.25 ± 0.39 , $P = 0.0229$; Figure 3B).

Upon apoptosis inhibition with G04 a significant increase in the release of δ granule content was detected, in a time-dependent manner, compared to untreated-CSP (FI CD63 mean \pm SEM: G04 vs. buffer day 1, 4.54 ± 0.79 vs. 2.19 ± 0.22 , $P=0.0394$; day 7, 2.70 ± 0.46 vs. 1.53 ± 0.04 , $P=0.0466$; day 10, 1.90 ± 0.20 vs. 1.19 ± 0.17 , $P=0.0133$; Figure 3D). Upregulation of PKA as well as caspase-9 inhibition significantly impaired the TRAP-6-induced δ granule se-

cretion on storage day 4 and 7 (FI CD63 mean \pm SEM: forskolin vs. buffer day 4, 1.38 ± 0.20 vs. 2.04 ± 0.18 , $P=0.0333$; day 7, 1.04 ± 0.01 vs. 1.53 ± 0.04 , $P=0.0005$; caspase-9 inhibitor vs. buffer day 7, 1.14 ± 0.10 vs. 1.53 ± 0.04 , $P=0.0058$; Figure 3E and F, respectively).

Furthermore, we found that the inhibition of apoptosis induced by both G04 and caspase-9 show similar aggregation and agglutination ability upon stimulation with

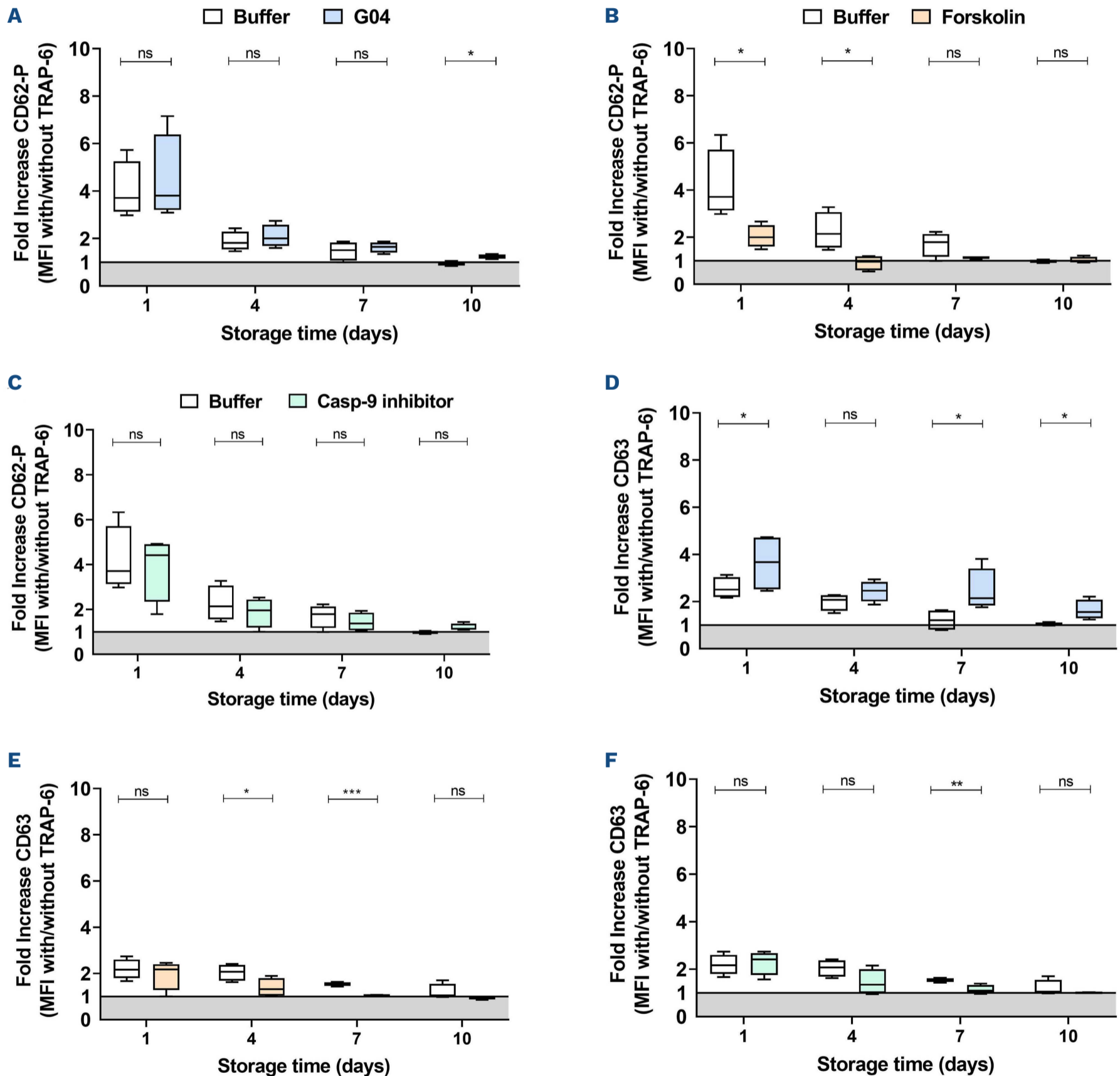


Figure 3. The impact of apoptosis inhibition on α (CD62-P) and δ (CD63) granules release from cold-stored platelets. The expression of CD62-P (A-C) and CD63 (D-F) on platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) was analyzed after 1, 4, 7 and 10 days of cold storage, respectively. The expression of both markers was detected by flow cytometry after stimulation with TRAP-6 (10 μ M). Data are shown as box and whiskers \pm standard error of the mean. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; ns: not significant, N=4. MFI: mean fluorescence intensity.

TRAP-6 and ristocetin compared to untreated cells (Figure 4A, D and 4C, F, respectively). Of note, enhanced aggregation and agglutination were detected after 10 days of cold storage only in the presence of the inhibitor G04 compared to cells stored in buffer (% maximal aggregation mean \pm SEM: G04 vs. buffer day 10, after TRAP-6, 53 \pm 9% vs. 19 \pm 9%, $P=0.0192$; after ristocetin, 83 \pm 4% vs. 63 \pm 7%, $P=0.0108$; Figure 4A, D, respectively). Similarly to the activation results (Figure 3B, E), upregulation of PKA caused a drastic reduction of the aggregation ability in response to TRAP-6 in comparison to untreated-CSP, already after 1 day of storage, with a time-dependent trend (% maximal

aggregation mean \pm SEM: forskolin vs. buffer, day 1, 90 \pm 3% vs. 98 \pm 2%, $P=0.0311$; day 4, 15 \pm 5% vs. 91 \pm 8%, $P<0.0001$; day 7, 13 \pm 1% vs. 54 \pm 13%, $P=0.0275$; Figure 4B, respectively). The same undesired effect was detected upon ristocetin stimulation but only on storage day 7 (% maximal agglutination mean \pm SEM: forskolin vs. buffer, 58 \pm 6% vs. 86 \pm 5%, $P=0.0045$, Figure 4E).

Taking together, these data indicate that inhibition of GPIb- α clustering-mediated signal, induced by G04 incubation, better maintains δ granule release of CSP without affecting the α granule secretion as well as the aggregation and agglutination abilities.

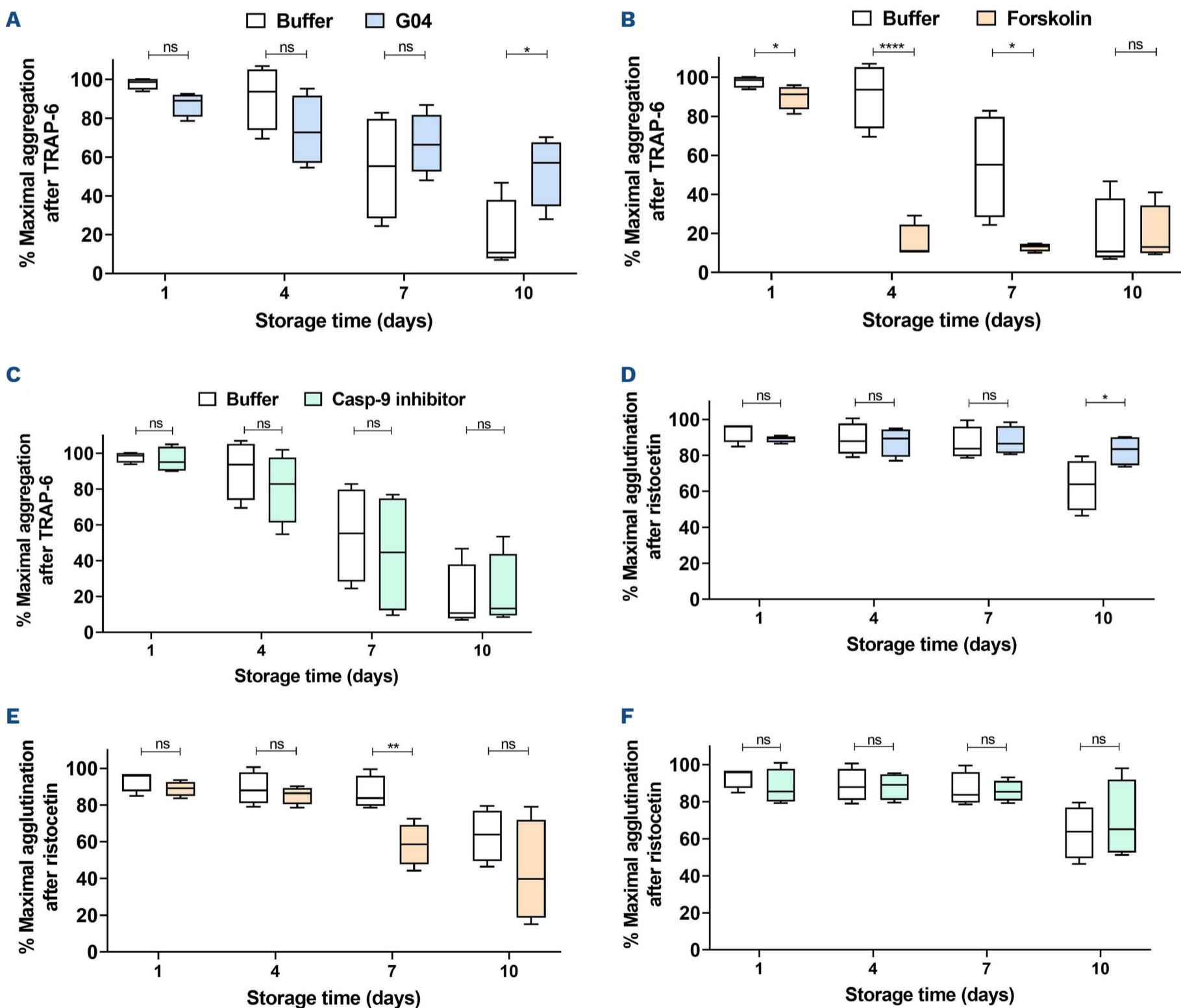


Figure 4. Aggregation and agglutination of cold-stored platelets upon apoptosis inhibition. The maximal aggregation (A-C) and agglutination (D-F) abilities of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) were measured after stimulation with the inductors TRAP (20 μ M) and ristocetin (1 mg/mL), respectively. Data are shown as box and whiskers \pm standard error of the mean. * $P<0.05$; ** $P<0.01$; **** $P<0.0001$; ns: not significant, $N=4$.

The impact of apoptosis inhibition on cold-stored platelet adhesion

Analyzing the adhesion ability of CSP to fibrinogen we found that the total number of adherent cells detected upon inhibition of RhoA was significantly higher in comparison to untreated-CSP on storage day 4 (number of adherent cells/field mean \pm SEM: G04 vs. buffer, day 4, 93 ± 17 vs. 49 ± 13 , $P=0.0493$; Figure 5A). Similar adhesion ability was observed upon upregulation of PKA and inhibition of caspase-9 compared to cells stored in buffer, after 4 and 7 days of cold storage (Figure 5A, B). Platelet adhesion is a multistep process, which leads to

a complex reorganization of the cytoskeleton resulting in the formation of filopodia/lamellipodia ending with complete spreading of the cells. Therefore, we analyzed the morphology of CSP by quantifying the percentage of the different spreading patterns (type 1: resting cells; type 2: cells with filopodia; type 3: cells with lamellipodia and type 4: fully spread platelets; Figure 5C, D). Surprisingly, the higher number of total adherent cells observed on storage day 4 after RhoA inhibition (Figure 5A) was not correlated with a higher percentage of activated cells (type 2, 3 and 4) compared to cells stored in buffer (Figure 5C); whereas, the upregulation of PKA

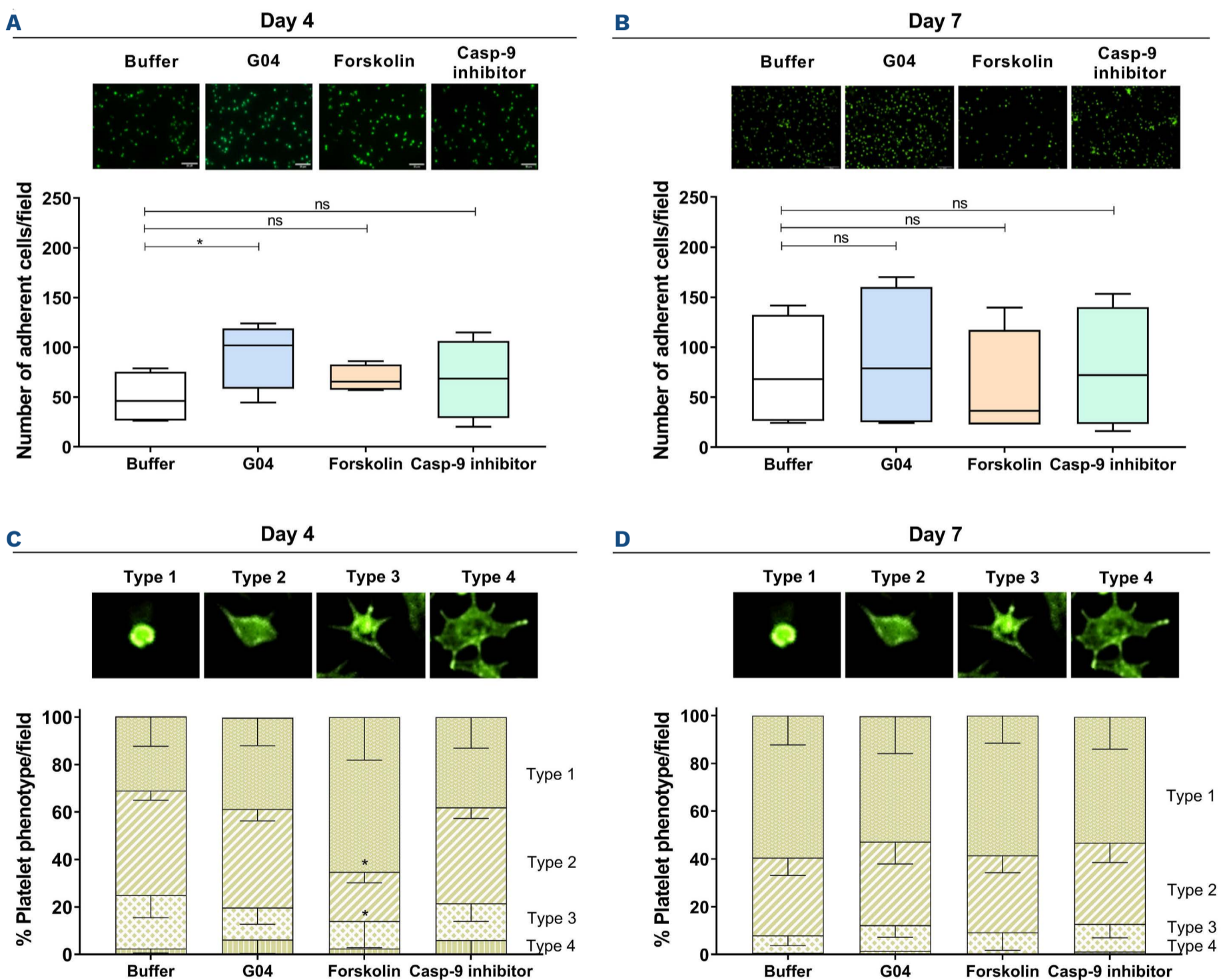


Figure 5. Cold-stored platelet adhesion to fibrinogen upon apoptosis inhibition. The adhesion ability of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 (Casp-9) inhibitor (green symbols) was measured after TRAP-6 stimulation on storage day 4 (A, C) and 7 (B, D), respectively. The number of adherent cells (A, C) and the percentage of the different platelet phenotypes (type 1: resting cells; type 2: cells with filopodia; type 3: cells with lamellipodia and type 4: fully spread cells; C, D) were quantified from 6 different microscopic fields per coverslips, respectively. Representative immunofluorescence images of adherent cells (A and B; scale bar: 20 μ m) and of the platelet phenotypes (C and D). Green signal, glycoprotein IIb/IIIa (A-D). Data are shown as box and whiskers \pm standard error of the mean. * $P<0.05$; ns: not significant. C and D, if not indicated the data were not significant. N=4.

induced a significant shift of cell morphology from type 2 to type 1 (resting platelets), in comparison to untreated-CSP on day 4 (% platelet phenotype/field mean \pm SEM: forskolin vs. buffer type 1, $65\pm 18\%$ vs. $31\pm 13\%$, $P=0.0433$ and type 2, $21\pm 5\%$ vs. $44\pm 4\%$, $P=0.0176$; Figure 5C, respectively). Of note, the inhibition of caspase-9 showed similar spreading phenotypes compared to untreated-CSP on storage day 4 (Figure 5C). Finally, comparable total number of adherent cells as well as spreading patterns were observed in all treated-CSP in comparison to cells stored in buffer on storage day 7 (Figure 5B, D).

These data suggest that inhibition of cold-induced GPIIb- α clustering signaling better conserved the adhesion ability of CSP, upon 4 days of storage.

Effect of apoptosis inhibition on the kinetic of clot formation

In order to deeper investigate the hemostatic functions of treated-CSP, we analyzed the platelet clot formation performing a clot retraction assay. We found that inhibition of either GPIIb- α clustering or caspase-9 did not impair clot retraction in comparison to cells stored in buffer (Figure 6A, B). In contrast, the extent of platelet clot retraction was almost completely abolished in the presence of the PKA agonist (% clot retraction mean \pm SEM: forskolin vs. buffer day 4, $1\pm 0\%$ vs. $88\pm 1\%$, $P<0.0001$

and day 7, $1\pm 0\%$ vs. $91\pm 1\%$, $P<0.0001$; Figure 6A, B, respectively).

Since clot retraction is a crucial mechanism to stabilize thrombi, we investigated the kinetics of whole blood clot formation performing an thromboelastography assay. We designed an experimental setting that mimics platelet transfusion in thrombocytopenic patients. Briefly, we produced platelet-depleted full blood samples from healthy donors and spiked these samples with CSP. We found that CSP treated with RhoA or caspase-9 inhibitor maintain similar ability to form clots, in terms of maximum clot firmness (MCF) and maximum lysis (ML) compared to cells stored in buffer at 4°C (Figure 7A, D and 7C, F, respectively). In contrast, a significant decrease of the MCF on day 4 (MCF mean \pm SEM: forskolin vs. buffer day 4, 45.66 ± 2.93 vs. 58.02 ± 1.12 , $P=0.0173$; Figure 7B) and increase of ML were detected after incubation with the PKA activator indicating reduced stability of the clots (% ML mean \pm SEM: forskolin vs. buffer day 1, $4\pm 0.3\%$ vs. $23\pm 2\%$, $P=0.0006$; day 4, $2\pm 1\%$ vs. $24\pm 4\%$, $P=0.0076$; day 7, $2\pm 0.6\%$ vs. $14\pm 4\%$, $P=0.0206$; day 10, $2\pm 0.6\%$ vs. $19\pm 4\%$, $P=0.0169$; Figure 7E, respectively).

Taken together our data indicate that the inhibition of apoptosis by blocking cold-induced GPIIb- α - or caspase-9-mediated signals does not affect the contribution of CSP to clot formation or stability.

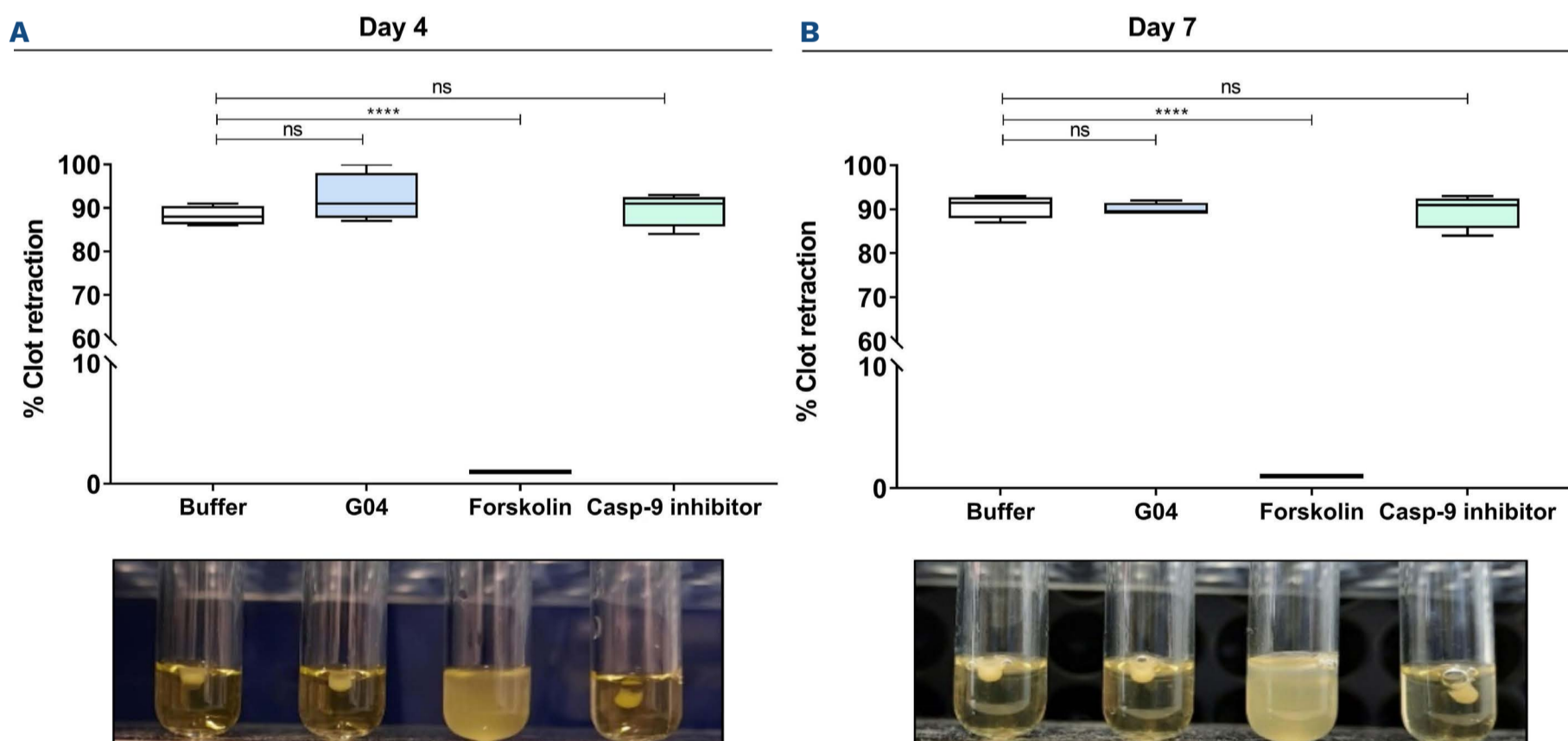


Figure 6. Clot retraction ability of platelet concentrates after cold-induced apoptosis inhibition. The percentage of clot retraction of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 (Casp-9) inhibitor (green symbols), was analyzed after TRAP-6 stimulation on storage day 4 (A) and 7 (B), respectively. The clot surfaces were calculated as percentage of retraction area compared to the total area. For forskolin a virtual value of 1% was reported in the graphics. Lower panel: representative pictures taken after 1 hour. Data are shown as box and whiskers \pm standard error of the mean. **** $P<0.0001$; ns: not significant, $N=4$.

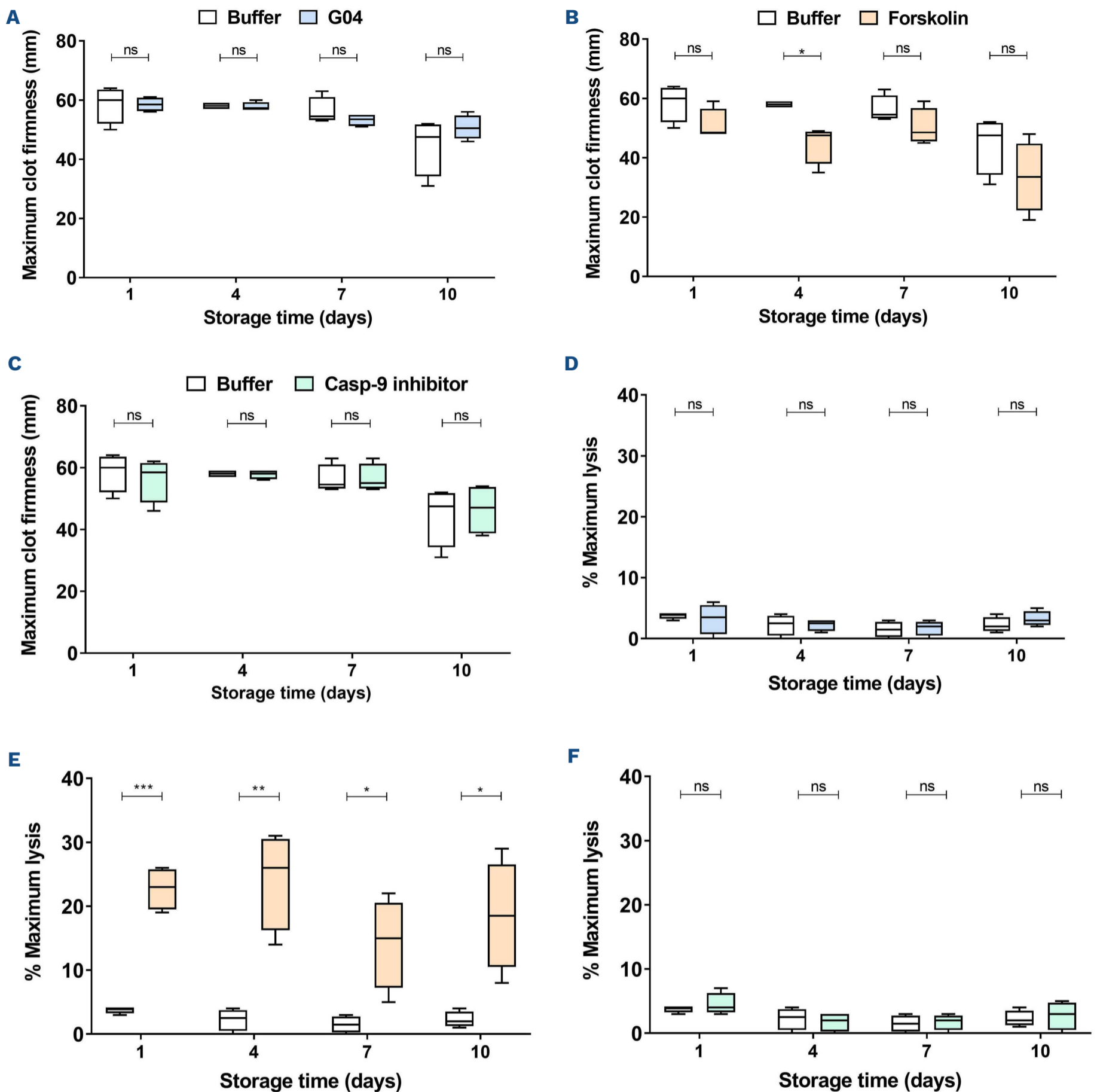


Figure 7. The impact of cold-induced apoptosis inhibition on the kinetic of clot formation. The maximum clot firmness (A-C) and the percentage of maximum lysis (D-F) of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 (Casp-9) inhibitor (green symbols) were measured performing thromboelastography assay (extrinsic test), respectively. Data are shown as box and whiskers \pm standard error of the mean. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns: not significant, $N = 4$.

Preventing the transduction of the cold-induced GPIIb- α clustering signal reduces the fast clearance of cold-stored platelets *in vivo*

Based on the results of our *in vitro* analyses, which suggest the RhoA inhibitor G04 as the most promising compound, and in accordance with the principles of the 3R

(replacement, reduction and refinement) for animal research, we investigated whether the survival of CSP could be improved by preventing cold-induced GPIIb- α clustering signal transduction. After 7 days of storage, CSP were administrated to the mice and the survival was analyzed 1, 2, 5 and 24 hours (h) post injection. As shown in Figure

8 significantly higher percentage of treated-CSPs was detected in the mouse circulation 2 and 5 hours (h) post injection in comparison to cells stored in buffer (% survival of human platelets mean \pm SEM: G04 vs. buffer, 2 h, $47 \pm 10\%$ vs. $26 \pm 6\%$, $P=0.0387$; 5 h, $40 \pm 7\%$ vs. $18 \pm 3\%$, $P=0.0355$; Figure 8). Of note, comparable recovery after injection was observed between platelets stored in buffer and with the RhoA inhibitor (see the *Online Supplementary Figure S6*).

These data indicate that the inhibition of cold-induced apoptosis by blocking the GPIIb- α clustering signal improves the survival of CSP.

Discussion

In the present study, we investigated the impact of apoptosis inhibition on the hemostatic functions and life span of CSP. Giving the specific target of each inhibitor (Figure 1; *Online Supplementary Appendix*), we found that G04 might be the most promising one, as this agent provided the best compromise between apoptosis inhibition and side effects on platelet functions. We presume that this is due to the fact that G04 blocks the cold-induced apoptosis at an early stage (outside/inside signal transduction). Therefore, it might better maintain the platelet integrity and functionality during cold storage, compared to the other tested inhibitors that target downstream proteins (Figure 1). Interestingly, we found that the inhibition of apoptosis induced by G04, which prevents the transduction of the cold-induced GPIIb- α clustering signal, improves δ granule release and platelet response to agonists. Furthermore, the ability of platelets to adhere to fibrinogen and to form a stable clot upon exposure to thrombin was conserved. More importantly, we observed a significant improvement of the survival of CSP upon inhibition of the intracellular signal induced by GPIIb- α clus-

tering indicating promising potential for clinical use.

The apoptotic phenotype of CSP was detectable in our study already after 4 days of storage. The blockade of cold-induced apoptosis signaling pathway at three different stages (GPIIb- α clustering signal, PKA as well as caspase-9; Figure 1) showed a protective effect as determined by testing MMP and PS surface externalization. First, we inhibited the transduction of the outside/inside signal induced by GPIIb- α a clustering by blocking the RhoA guanine exchange factor binding domain, which maintains the protein in its inactive form (RhoA-bound GDP).²⁷ In the presence of the inhibitor, we detected better CSP activation, aggregation and adhesion as well as similar clot retraction and thrombus stability in comparison to untreated cells. Given the key role of RhoA in the cytoskeletal assembly events regulating platelet functionality,^{28,29} our findings might appear unexpected. This may be explained by the fact that other members of the Rho GTPase family, like Rac1, Cdc42, RhoB and RhoC, have redundant functions in the regulation of platelet functionality.^{27,30} Therefore, one or more of these proteins might compensate the inhibition of RhoA. This hypothesis is supported by a previous study, where the RhoA inhibitor does neither affect either Rac1 nor Cdc42 functions.²⁷ Another possible explanation might be a protective effect regarding the accumulation of PSL. The inhibition of GPIIb- α signaling at a very early stage might decrease PSL preserving cell integrity and reducing the formation of exhausted platelets.

Next, we used an agent (forskolin) which upregulates PKA via AC activation. One of the downstream targets of PKA is the proapoptotic protein Bad, which is sequestered upon phosphorylation into to the cytoplasm.³¹ By this mechanism the translocation of Bad to the mitochondrial membrane is prevented and the apoptosis signaling is inhibited. Despite the well-recognized role of PKA in the intrinsic apoptosis pathway, the impact of the modulation

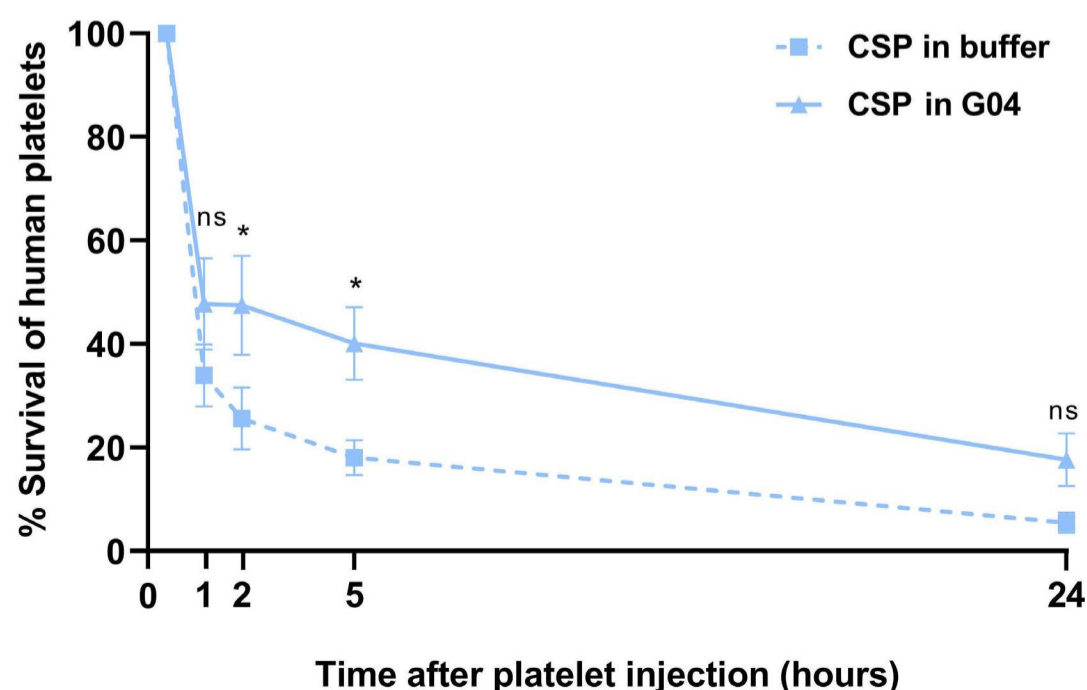


Figure 8. Survival of cold-stored platelets upon inhibition of cold-induced GPIIb- α clustering signal transduction. Cold-stored platelets (CSP) stored for 7 days in buffer (full squares and dashed blue line) or with G04 (RhoA inhibitor; full triangles and continues blue line) were added to the NSG mouse circulation via the lateral tail vein. Survival of human platelets in the mouse circulation was analyzed by flow cytometry by collecting murine blood 1, 2, 5 and 24 hours post injection. Data are shown as mean \pm standard error of the mean. * $P<0.05$; ns: not significant; N=4.

of its activity on platelet functions still remains controversial, likely due to the wide range of downstream targets regulated by PKA-phosphorylation. Several groups observed that enhanced PKA activity prevents apoptosis induction increasing platelet life span *in vivo*.^{24,32} One study showed, however, that PKA activation correlates with enhanced apoptosis and reduced thrombin-induced platelet activation.³³ In the present work, CSP treated with the PKA agonist showed sufficient prevention of apoptosis after cold storage but caused a drastic impairment of cell functions. This is likely due to a higher phosphorylation of one of the PKA downstream targets, vasodilator-stimulated phosphoprotein (VASP), which is known to negatively regulate platelet functionality.^{34,35} Another important finding of our study is that the clot stability (clot retraction and resistance to fibrinolysis) was impaired in PKA agonist-treated CSP. This finding might indicate disadvantages for clinical applications of this compound.

In the third part of our *in vitro* study, we blocked the cold-induced apoptotic signal at a late stage by inhibiting the autocatalytic cleavage of caspase-9 (Figure 1). We found comparable platelet responsiveness between caspase-9 inhibitor treated-platelets and cells stored in buffer at 4°C. These data are in line with a previous study that showed normal functionality of caspase-9-depleted murine platelets.³⁶

Taking together our *in vitro* data indicate that apoptosis and activation signaling pathways in CSP might be better dissected if the signal could be blocked at an early stage. The better responsiveness in the presence of RhoA inhibitor (*Online Supplementary Table S1*) suggests additional protective effect against apoptosis-independent PSL.

RhoA inhibitor has been recently tested in mice.³⁷ In this study, RhoA inhibitor was administered daily intraperitoneally at 40 mg/kg for 7 days. All animals survived and no adverse events were observed indicating the safety of this compound. Despite this observation and considering the expected dilution effect after PC transfusion, the removal of G04 from CSP to reduce potential systemic toxic effects still remain a relevant question; in particular in the absence of a clinical study on its safety.

Our *in vitro* results showed better platelet functions and cell integrity upon G04 incubation, compared to untreated CSP or those that have been treated with forskolin or caspase-9 inhibitor (*Online Supplementary Table S1*). Therefore, we thought to perform an *in vivo* study and we observed a significantly higher number of circulating CSP 2 and 5 h after injection, when cells were treated with RhoA inhibitor compared to buffer. This finding shows that the presence of the inhibitor effectively extends the half-life of CSP. Our *in vivo* results are in line with a recent study that reported improved survival of CSP after 14 days of storage upon inhibition of p38MAPK,³⁸ which is known to be involved in the apoptotic signal in PC.³⁹

Nevertheless, our study has some limitations. Although better functionality and survival of RhoA inhibitor-treated CSP were observed, we did not verify their *in vivo* functionality. Therefore, further investigations testing the platelet functions *in vivo* are needed to address this crucial question. Moreover, since the aim of the present study was to screen three apoptosis inhibitors for their efficacy and impact on platelet functions and half-life, we focused on the individual effect. Future studies should, however, investigate the effects of combination of inhibitors during cold storage. Keeping in mind that the final goal would be to add reagent/s to enhance CSP survival without affecting their functionality, the possibility to combine compounds might be a promising approach that should be addressed in future studies. Furthermore, we reported that the higher functionality of refrigerated platelets was even more pronounced upon inhibition of the signal transduction induced by GPIb- α clustering. It could be argued that increased risk of thrombosis might exist for patients receiving these products. Even if this consideration is correct and legitimate, it can be speculated that in some clinical cases like active bleeding upon injury in thrombocytopenic patients, transfusion of CSP with better functionality would be more efficient to treat bleeding compared to PC stored at RT. Although robust data from clinical trials with a significant number of patients are still missing, a recent small pilot study investigated the safety and feasibility of CSP in patients during cardiothoracic surgery. CSP have been shown to be still functional after 14 days of storage and no significant difference in clinical outcome was observed compared to standard products. These data indicate toward the feasibility of CSP application to treat perioperative bleeding.⁴⁰

In conclusion, our study provides novel insights on the *in vitro* hemostatic functions and half-life of CSP upon inhibition of cold-induced apoptotic signaling pathways. Our findings indicate that the combination of cold storage and apoptosis inhibition might provide a promising strategy to prolong the storage time without affecting cell functionality or reducing platelet survival. Nevertheless, further analysis and clinical studies are still needed to evaluate whether the use of these products might also give better patient outcome.

Disclosures

TB has received research funding from CoaChrom Diagnostica GmbH, DFG, Robert Bosch GmbH, Stiftung Transfusionsmedizin und Immunhämatologie e.V.: Ergomed, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden-Württemberg, has received lecture honoraria from Aspen Germany GmbH, Bayer Vital GmbH, Bristol-Myers Squibb GmbH & Co., Doctrina Med AG, Meet The Experts Academy UG, Schoechl medical edu-

ation GmbH, Mattsee, Stago GmbH, Mitsubishi Tanabe Pharma GmbH, Novo Nordisk Pharma GmbH, has provided consulting services to Terumo, has provided expert witness testimony relating to heparin induced thrombocytopenia (HIT) and non-HIT thrombocytopenic and coagulopathic disorders. All of these are outside the current work. TB and ES together with DRK Blutspendedienst Baden-Württemberg-Hessen have a pending patent application on the use of apoptosis inhibitors for cold storage of blood platelets. Other authors declare no competing financial interests.

Contributions

IM, KA, SN-H, ES and TB designed the study. IM, LP, YT, C-TM and AW performed the experiments. IM, LP and TB analyzed the data and interpreted the results. IM and TB wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

The authors would like to thank Karoline Weich, Julian Duerr and the team of the blood donation center of Tübingen for their excellent technical support.

Funding

This study was supported by a grant from the German Red Cross, Blutspendedienst Baden-Württemberg-Hessen, to I.M. (Forschungs- und Entwicklungsprojekt, Projekt Nr. F+E_2018_016) and a grant for young scientist from the University Hospital of Tübingen to IM (Junior Antrag, Fortuene-Antrag Nr. 2706-0-0).

Data-sharing statement

Data generated from this study are available from the corresponding author upon reasonable request.

References

- Schiffer CA, Bohlke K, Delaney M, et al. Platelet transfusion for patients with cancer: American Society of Clinical Oncology Clinical Practice Guideline Update. *J Clin Oncol*. 2018;36(3):283-299.
- Ketter PM, Kamucheka R, Arulanandam B, Akers K, Cap AP. Platelet enhancement of bacterial growth during room temperature storage: mitigation through refrigeration. *Transfusion*. 2019;59(S2):1479-1489.
- Li JW, Brecher ME, Jacobson JL, et al. Addressing the risk of bacterial contamination in platelets: a hospital economic perspective. *Transfusion*. 2017;57(10):2321-2328.
- Center for Biologics Evaluation and Research. U.S. Food and Drug Administration. Fatalities reported to FDA following blood collection and transfusion: annual summary for fiscal year 2017. www.fda.gov/media/124796. Accessed September 2017.
- Cho J, Kim H, Song J, et al. Platelet storage induces accelerated desialylation of platelets and increases hepatic thrombopoietin production. *J Transl Med*. 2018;16(1):199.
- Ramirez-Arcos S, DiFranco C, McIntyre T, Goldman M. Residual risk of bacterial contamination of platelets: six years of experience with sterility testing. *Transfusion*. 2017;57(9):2174-2181.
- Baharoglu MI, Cordonnier C, Al-Shahi Salman R, et al. Platelet transfusion versus standard care after acute stroke due to spontaneous cerebral haemorrhage associated with antiplatelet therapy (PATCH): a randomised, open-label, phase 3 trial. *Lancet*. 2016;387(10038):2605-2613.
- Curley A, Stanworth SJ, Willoughby K, et al. Randomized trial of platelet-transfusion thresholds in neonates. *N Engl J Med*. 2019;380(3):242-251.
- Marini I, Aurich K, Jouni R, et al. Cold storage of platelets in additive solution: the impact of residual plasma in apheresis platelet concentrates. *Haematologica*. 2019;104(1):207-214.
- Yang J, Yin W, Zhang Y, et al. Evaluation of the advantages of platelet concentrates stored at 4 degrees C versus 22 degrees C. *Transfusion*. 2018;58(3):736-747.
- Koessler J, Klingler P, Niklaus M, et al. The impact of cold storage on adenosine diphosphate-mediated platelet responsiveness. *TH Open*. 2020;4(3):e163-e172.
- Nair PM, Pandya SG, Dallo SF, et al. Platelets stored at 4 degrees C contribute to superior clot properties compared to current standard-of-care through fibrin-crosslinking. *Br J Haematol*. 2017;178(1):119-129.
- Johnson L, Vekariya S, Wood B, Tan S, Roan C, Marks DC. Refrigeration of apheresis platelets in platelet additive solution (PAS-E) supports in vitro platelet quality to maximize the shelf-life. *Transfusion*. 2021;61(Suppl 1):S58-S67.
- Miles J, Bailey SL, Obenaus AM, et al. Storage temperature determines platelet GPVI levels and function in mice and humans. *Blood Adv*. 2021;5(19):3839-3849.
- Murphy S, Gardner FH. Effect of storage temperature on maintenance of platelet viability - deleterious effect of refrigerated storage. *N Engl J Med*. 1969;280(20):1094-1098.
- Stolla M, Bailey SL, Fang L, et al. Effects of storage time prolongation on in vivo and in vitro characteristics of 4 degrees C-stored platelets. *Transfusion*. 2020;60(3):613-621.
- Jansen AJ, Josefsson EC, Rumjantseva V, et al. Desialylation accelerates platelet clearance after refrigeration and initiates GPIIb/IIIa metalloproteinase-mediated cleavage in mice. *Blood*. 2012;119(5):1263-1273.
- Wandall HH, Hoffmeister KM, Sorensen AL, et al. Galactosylation does not prevent the rapid clearance of long-term, 4 degrees C-stored platelets. *Blood*. 2008;111(6):3249-3256.
- van der Wal DE, Du VX, Lo KS, Rasmussen JT, Verhoef S, Akkerman JW. Platelet apoptosis by cold-induced glycoprotein Iba1 clustering. *J Thromb Haemost*. 2010;8(11):2554-2562.
- Chen W, Druzak SA, Wang Y, et al. Refrigeration-induced binding of von Willebrand factor facilitates fast clearance of refrigerated platelets. *Arterioscler Thromb Vasc Biol*. 2017;37(12):2271-2279.
- Gitz E, Koekman CA, van den Heuvel DJ, et al. Improved platelet survival after cold storage by prevention of glycoprotein Iba1 clustering in lipid rafts. *Haematologica*. 2012;97(12):1873-1881.
- Stivala S, Gobbato S, Infanti L, et al. Amotosalen/ultraviolet A pathogen inactivation technology reduces platelet activatability, induces apoptosis and accelerates clearance. *Haematologica*. 2017;102(10):1650-1660.
- Dutting S, Gaits-Iacovoni F, Stegner D, et al. A Cdc42/RhoA regulatory circuit downstream of glycoprotein Iba1 guides transendothelial platelet biogenesis. *Nat Commun*. 2017;8:15838.

24. Zhao L, Liu J, He C, et al. Protein kinase A determines platelet life span and survival by regulating apoptosis. *J Clin Invest.* 2017;127(12):4338-4351.
25. Lebois M, Josefsson EC. Regulation of platelet lifespan by apoptosis. *Platelets.* 2016;27(6):497-504.
26. Lopez JJ, Salido GM, Gomez-Arteta E, Rosado JA, Pariente JA. Thrombin induces apoptotic events through the generation of reactive oxygen species in human platelets. *J Thromb Haemost.* 2007;5(6):1283-1291.
27. Shang X, Marchioni F, Sipes N, et al. Rational design of small molecule inhibitors targeting RhoA subfamily Rho GTPases. *Chem Biol.* 2012;19(6):699-710.
28. Flevaris P, Stojanovic A, Gong H, Chishti A, Welch E, Du X. A molecular switch that controls cell spreading and retraction. *J Cell Biol.* 2007;179(3):553-565.
29. Pleines I, Hagedorn I, Gupta S, et al. Megakaryocyte-specific RhoA deficiency causes macrothrombocytopenia and defective platelet activation in hemostasis and thrombosis. *Blood.* 2012;119(4):1054-1063.
30. Rowley JW, Oler AJ, Tolley ND, et al. Genome-wide RNA-seq analysis of human and mouse platelet transcriptomes. *Blood.* 2011;118(14):e101-111.
31. Nagy Z, Smolenski A. Cyclic nucleotide-dependent inhibitory signaling interweaves with activating pathways to determine platelet responses. *Res Pract Thromb Haemost.* 2018;2(3):558-571.
32. Xiao W, Zhou K, Yang M, et al. Carbamazepine induces platelet apoptosis and thrombocytopenia through protein kinase A. *Front Pharmacol.* 2021;12:749930.
33. Rukoyatkina N, Butt E, Subramanian H, et al. Protein kinase A activation by the anti-cancer drugs ABT-737 and thymoquinone is caspase-3-dependent and correlates with platelet inhibition and apoptosis. *Cell Death Dis.* 2017;8(6):e2898.
34. Benz PM, Laban H, Zink J, et al. Vasodilator-stimulated phosphoprotein (VASP)-dependent and -independent pathways regulate thrombin-induced activation of Rap1b in platelets. *Cell Commun Signal.* 2016;14(1):21.
35. Massberg S, Gruner S, Konrad I, et al. Enhanced in vivo platelet adhesion in vasodilator-stimulated phosphoprotein (VASP)-deficient mice. *Blood.* 2004;103(1):136-142.
36. White MJ, Schoenwaelder SM, Josefsson EC, et al. Caspase-9 mediates the apoptotic death of megakaryocytes and platelets, but is dispensable for their generation and function. *Blood.* 2012;119(18):4283-4290.
37. Francis TC, Gaynor A, Chandra R, Fox ME, Lobo MK. The selective RhoA inhibitor Rhosin promotes stress resiliency through enhancing D1-medium spiny neuron plasticity and reducing hyperexcitability. *Biol Psychiatry.* 2019;85(12):1001-1010.
38. Skripchenko A, Gelderman MP, Vostal JG. P38 mitogen activated protein kinase inhibitor improves platelet in vitro parameters and in vivo survival in a SCID mouse model of transfusion for platelets stored at cold or temperature cycled conditions for 14 days. *PLoS One.* 2021;16(5):e0250120.
39. Chen Z, Schubert P, Culibrk B, Devine DV. p38MAPK is involved in apoptosis development in apheresis platelet concentrates after riboflavin and ultraviolet light treatment. *Transfusion.* 2015;55(4):848-857.
40. Strandenes G, Sivertsen J, Bjerkvig CK, et al. A pilot trial of platelets stored cold versus at room temperature for complex cardiothoracic surgery. *Anesthesiology.* 2020;133(6):1173-1183.