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# Amotosalen/ultraviolet A pathogen inactivation technology reduces platelet activatability, induces apoptosis and accelerates clearance

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## ABSTRACT

Amotosalen and ultraviolet A (UVA) photochemical-based pathogen reduction using the Intercept™ Blood System (IBS) is an effective and established technology for platelet and plasma components, which is adopted in more than 40 countries worldwide. Several reports point towards a reduced platelet function after Amotosalen/UVA exposure. The study herein was undertaken to identify the mechanisms responsible for the early impairment of platelet function by the IBS. Twenty-five platelet apheresis units were collected from healthy volunteers following standard procedures and split into 2 components, 1 untreated and the other treated with Amotosalen/UVA. Platelet impedance aggregation in response to collagen and thrombin was reduced by 80% and 60%, respectively, in IBS-treated units at day 1 of storage. Glycoprotein Ib (GpIb) levels were significantly lower in IBS samples and soluble glyocalicin correspondingly augmented; furthermore, GpIbα was significantly more desialylated as shown by *Erythrina Cristagalli Lectin* (ECL) binding. The pro-apoptotic Bak protein was significantly increased, as well as the MAPK p38 phosphorylation and caspase-3 cleavage. Stored IBS-treated platelets injected into immune-deficient nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice showed a faster clearance. We conclude that the IBS induces platelet p38 activation, GpIb shedding and platelet apoptosis through a caspase-dependent mechanism, thus reducing platelet function and survival. These mechanisms are of relevance in transfusion medicine, where the IBS increases patient safety at the expense of platelet function and survival.

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## Introduction

Platelet transfusion is a cornerstone in today's medicine in general, and more particularly in hemato-oncology, as illustrated by the 1.3 million platelet units transfused annually in the USA and more than 2.9 million in Europe.<sup>1-3</sup> One of the major challenges in transfusion medicine is the reduction of pathogen transmission by blood products, in particular for platelet components, since they need storage at room temperature.<sup>4</sup> To circumvent the problem of pathogen contamination of blood products, pathogen inactivation (PI) technologies have been developed and routinely implemented in blood transfusion centers worldwide, including the USA, France and Switzerland.<sup>5-7</sup>

One such technology, the IBS (Cerus Corporation, Concord, CA, USA), employs a synthetic psoralen (amotosalen, S-59) and UVA light to induce cross-linking of DNA and ribonucleic acid (RNA) molecules, thus blocking replication and pathogen

proliferation<sup>8</sup> and rendering  $\gamma$ -irradiation for graft-versus-host disease (GvHD) prophylaxis unnecessary. Several studies on the efficacy of non-pathogen-reduced *versus* IBS-treated platelets reported no cases of transfusion transmitted infections or transfusion associated GvHD, together with a reduction of other transfusion reactions. On the other hand, some reduction in platelet function, platelet count increments (CI) and corrected count increment (CCI) have been described.<sup>9,10</sup> Although 1 trial showed an increase in clinically irrelevant World Health Organization (WHO) grade 2 bleeding,<sup>10</sup> other studies did not find an increase in bleeding, thus confirming the safety of the IBS technology.<sup>9,11–15</sup> However, evaluating platelet function and survival *in vivo* is a challenging task due to the multiple and heterogeneous clinical and pharmacological factors affecting platelet function in patients.

Some reports suggest that all pathogen inactivation systems, including the IBS, aggravate the platelet storage lesion (PSL) and reduce the platelet function *in vitro*; the molecular mechanism behind these observations, however, is unclear.<sup>16–18</sup> Abonnenc *et al.* reported a reduced aggregation response to low-dose TRAP and collagen in IBS-treated platelets, a finding confirmed in the study by Picker *et al.*<sup>19,20</sup> The latter also described an increased glycolytic flux after pathogen reduction technology (PRT), with lactate accumulation and increased acidity. Schubert and Chen reported an increased phosphorylation of several intracellular kinases and higher caspase activity after riboflavin and ultraviolet B (UVB) treatment (Mirasol), which could be reverted by pre-treatment with specific p38 inhibitors.<sup>21,22</sup>

The study herein was undertaken in order to test the hypothesis that the IBS leads to reduced platelet activatability in response to certain agonists (i.e., collagen, thrombin and von Willebrand Factor [vWF]), increased platelet apoptosis and, consequently, enhanced clearance from the circulation. We therefore compared platelet function and parameters of apoptosis and clearance of untreated and IBS-treated human platelets in a large number of *in vitro* and *in vivo* tests in an immune-deficient mouse model (NOD/SCID). In addition, we analyzed the physiopathological pathway(s) involved.

## Methods

### Platelet collection and processing

Platelet apheresis units (AU) were collected from 25 volunteers at the Regional Blood Transfusion Service of the Swiss Red Cross of Basel, Switzerland. A table with the AU characteristics is provided in the *Online Supplementary Material*. The study was approved by the Institutional Review Board and each donor provided written informed consent. Of the 2 AU obtained from each donor, 1 was kept untreated (non-IBS) and the other treated with IBS on day 1 after collection according to the standard procedure (IBS).<sup>8</sup> In some cases (n=10), 3 bags were obtained by splitting the apheresis product from 1 donor: 1 kept untreated (non-IBS), and 2 IBS-treated, of which 1 was injected with a sterile solution of the p38 inhibitor SB203580 (Sigma-Aldrich; final concentration 20  $\mu$ M, n=5), or the sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA, Calbiochem, final concentration 150  $\mu$ M, n=5) and the other with an equal volume of vehicle (ethanol). Both were left to incubate overnight before undergoing the IBS procedure. All AU contained about 1/3 plasma and 2/3 the platelet additive solution InterSol (Fenwal, Lake Zurich, IL, USA)

and were stored at standard blood banking conditions (22  $\pm$  2°C under gentle agitation).

### Adhesion to collagen and vWF under flow

Adhesion in the microfluidic chamber (Fluxion Biosciences, San Francisco, CA, USA) was performed on citrate, calcein-stained platelets (4  $\mu$ M calcein AM, Enzo Life Sciences) at low and high shear rates (10 dyn/cm<sup>2</sup> and 100 dyn/cm<sup>2</sup>, respectively).<sup>23</sup> For detailed protocol see the *Online Supplementary Methods*.

### *In vivo* platelet survival in NOD/SCID mouse

Platelets from untreated and IBS-treated AU were incubated with calcein as described above, then pelleted (340 relative centrifugal force (RCF), 10 min) and resuspended in 0.9% sodium chloride (NaCl) at 4x10<sup>9</sup>/ml. Eight-week old NOD/SCID male mice (Charles River, France) were injected intravenously with 100  $\mu$ l of the platelet suspension.<sup>24</sup> Thirty minutes, 2 hours and 5 hours after injection, a 10  $\mu$ l blood sample was taken from the tail tip and mixed with Aster Jandl anticoagulant, centrifuged (125 RCF, 8 min), and 100  $\mu$ l of the supernatant was immediately analyzed on a Fortessa LSR II (BD Biosciences).<sup>25</sup> The 30 minutes sample was set as 100%, and the percentages of human platelets in circulation at 2 and 5 hours were calculated accordingly. Following the final blood sampling, the animals were euthanized and the spleens excised and frozen in optimal cutting temperature (O.C.T) medium (Tissue-Tek O.C.T, Sakura Finetek Europe, AJ Alphen aan den Rijn, The Netherlands). All animal experiments were approved by and in strict compliance with the local Veterinary Office (animal licenses 174/2011 and 035/15).

### Impedance aggregometry, flow cytometry, ELISA and Western blotting

Detailed protocol for additional methods (impedance platelet aggregometry, flow cytometry staining, Western blotting (WB), glycoalbumin enzyme-linked immunosorbent assay (ELISA), immunofluorescence staining) can be found in the *Online Supplementary Methods*.

### Statistical analyses

Results are mean  $\pm$  SEM. Data were analyzed by paired, two-tailed Student's *t*-test, one- or two-way analysis of variance (ANOVA), followed by Bonferroni *post hoc* test as appropriate. A *P*-value of less than 0.05 was considered significant. All calculations were performed with GraphPad Prism 5.04 (GraphPad Software Inc., San Diego, CA, USA).

## Results

### Amotosalen and UVA photochemical treatment reduces platelet aggregation

Untreated and IBS-treated samples were analyzed for aggregation in response to different doses of collagen and thrombin (Figure 1). At day (d)1 of storage, IBS-treated platelets showed a maximum collagen-induced aggregation of 20.5% (5  $\mu$ g/ml collagen) and 45.2% (10  $\mu$ g/ml collagen) compared to the non-IBS samples set as 100% (n=20, *P*<0.0001; Figure 1A,B). In response to thrombin, the IBS platelets showed 40.2% of aggregation compared to the non-IBS samples with a lower dose (0.25 U/ml, n=20, *P*<0.0001; Figure 1C), but did not show a significant difference with a high dose (0.5 U/ml; Figure 1D).

To further evaluate the effects on shear-induced aggregation, platelets were analyzed under low (10 dyn/cm<sup>2</sup>) and high (100 dyn/cm<sup>2</sup>) shear on collagen and human

vWF-coated channels, respectively. The platelet-covered area from IBS samples showed a 47% reduction in collagen compared to non-IBS (from 38'561  $\mu\text{m}^2$  to 20'718  $\mu\text{m}^2$ ,  $n=17$ ; Figure 1E), and a 65% reduction on vWF (from 2490  $\mu\text{m}^2$  to 866  $\mu\text{m}^2$ ,  $n=17$ ; Figure 1F), which reached a borderline significance for the area under the curve (AUC) for collagen ( $P=0.05$ ; Figure 1G), and was significant for vWF ( $P=0.01$ ; Figure 1H).

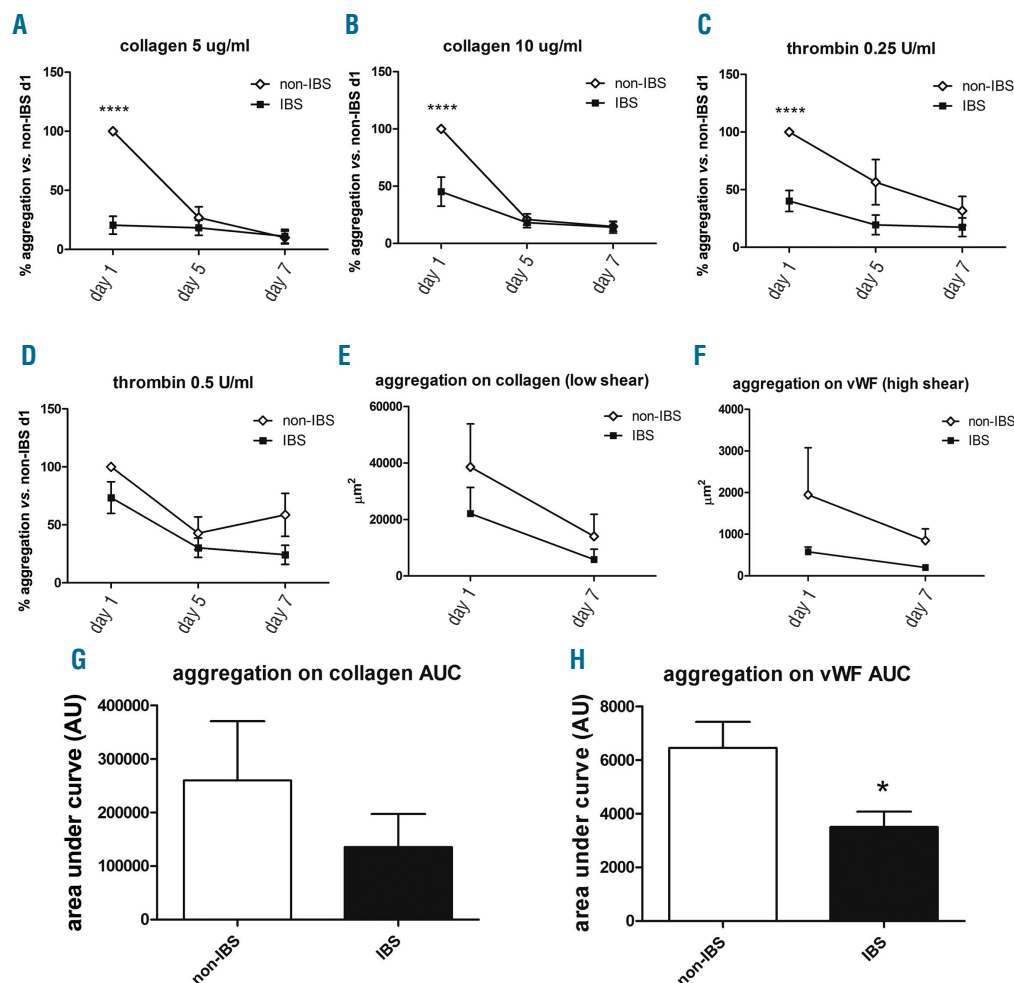
#### Amotosalen and UVA photochemical treatment induce desialylation and cleavage of GpIb $\alpha$ , the release of glyocalicin and p38 phosphorylation

While flow cytometric analyses for Annexin V and PAC-1 binding and P-selectin exposure showed no significant difference in IBS samples compared to the untreated controls (Online Supplementary Figure S1A-C), IBS platelets had a significantly lower expression of the vWF receptor GpIb $\alpha$  (mean fluorescence intensity (MFI) d1: 2258.9 non-IBS, 1937.4 IBS,  $n=20$ ,  $P=0.01$ ; Figure 2A), which could contribute to the reduced platelet aggregation on immobilized vWF observed under flow. Consistently, the amount

of the N-terminal fragment of GpIb $\alpha$  (glycocalicin) in the supernatant was significantly increased by 20% in IBS samples (Figure 2B). This result was confirmed upon adjustment of the platelet count per unit (Glycocalicin Index; Figure 2C). Since the MAPK p38 is directly involved in TNF- $\alpha$  converting enzyme (TACE) activation and GpIb shedding, we compared p38 phosphorylation in IBS and non-IBS platelet lysates and found it to be significantly increased in the IBS samples (Figure 2D).

#### Amotosalen and UV light increases Bak protein level and induces apoptosis

On account of the key role played by the proteins of the Bcl-2 family in determining platelet lifespan *in vivo*,<sup>27</sup> we analyzed the expression of Bak and Bcl-XL in non-IBS and IBS platelets. The level of the anti-apoptotic protein Bcl-XL was unchanged (*data not shown*) but that of the pro-apoptotic Bak was significantly increased in the IBS platelets (Figure 2E). In order to confirm that the increased level of Bak was inducing platelet apoptosis, we determined the amount of cleaved caspase-3 in platelet



**Figure 1. IBS treatment reduces platelet function.** Platelet aggregation in response to collagen (A and B) and thrombin (C and D) at days 1, 5 and 7 of storage from untreated (non-IBS,  $\diamond$ ) versus IBS-treated ( $\blacksquare$ ) AU. Lower panel: platelet aggregation under flow on collagen at 10 dyn/cm<sup>2</sup> (E) and vWF at 100 dyn/cm<sup>2</sup> (F) was also reduced in IBS samples compared to untreated platelets. Area under the curve (AUC) for the aggregation on collagen reached a borderline significance (G;  $P=0.05$ ), while it was statistically significant for vWF (H).  $n=20$ , \* $P<0.05$ , \*\*\*\* $P<0.0001$ . Graphs show mean $\pm$ SEM. vWF: von Willebrand Factor; IBS: Intercept Blood System; AU: apheresis unit.

lysates; as shown in the blot and the relative quantification, it was significantly increased in the samples treated with the IBS as compared to the non-IBS controls (Figure 2F). Immunofluorescence staining of fixed, permeabilized platelets confirmed an increased Bak intensity for the IBS platelets (Figure 2G).

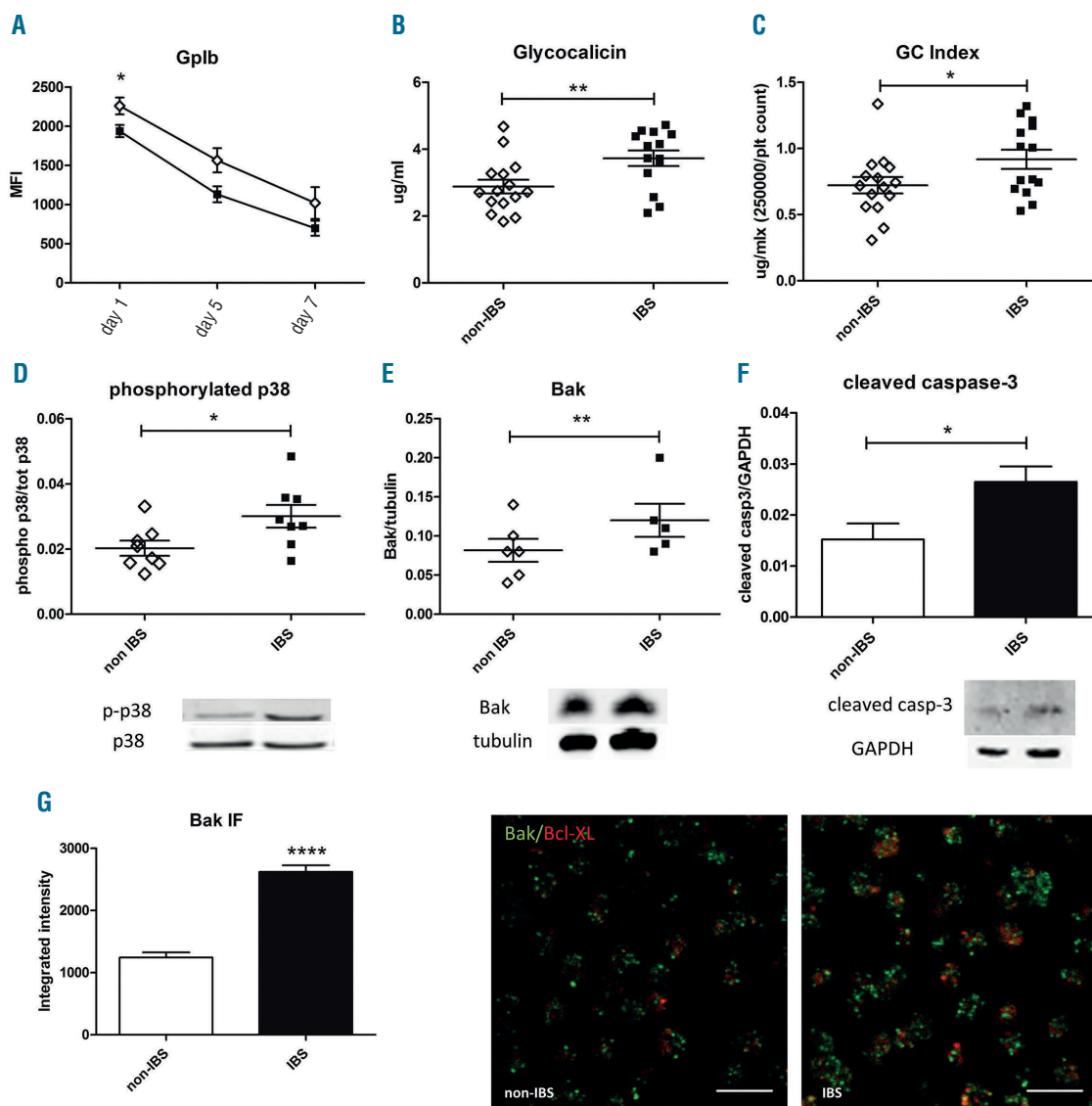
### IBS treatment reduces platelet survival *in vivo* in NOD/SCID mice

Next, we tested the physiological relevance of our findings *in vitro* on platelet survival *in vivo*. The AUC for platelet survival over 5 hours was significantly lower for the IBS platelets (Figure 3A,B), demonstrating reduced platelet half-life due to accelerated clearance *in vivo* upon transfusion. Taking the first time point (30 min post-injec-

tion) as 100%, 34.4% non-IBS platelets were still circulating compared to 28.2% IBS at 2 hours ( $n=15$ ,  $P>0.05$ ); at 5 hours, they were 26.1% *versus* 11.5%, respectively ( $P=0.05$ ; Figure 3A).

The area of fluorescent platelets in spleens from injected mice was significantly increased in mice receiving IBS platelets compared to those injected with untreated platelets (Figure 3C and corresponding micrographs in the bottom panel). Under these conditions, we could not detect platelet clearance in the liver of these mice (*data not shown*).

Since it has been reported that GpIb $\alpha$  levels correlate with platelet survival,<sup>28</sup> we analyzed its correlation with the *in vivo* survival of non-IBS and IBS platelets and found a significant positive correlation between GpIb levels and



**Figure 2. IBS induces GpIb shedding from platelets, p38 activation and apoptosis through Bak.** MFI of surface GpIb $\alpha$  measured by flow cytometry is significantly reduced in IBS platelets at day 1 of storage (A) and correspondingly increased in the supernatant when measured by ELISA (B) ( $n=15$ ,  $P<0.01$ ). (C) Glycocalicin (GC) concentration from AU supernatant, normalized to the platelet count to give the GC Index, was significantly increased in IBS-treated AU;  $n=15$ ,  $P=0.03$ . Western blot quantification of phosphorylated p38 (D), (E) Bak expression and (F) caspase-3 cleavage in platelet lysates from untreated or IBS-treated AUs. (G) Immunofluorescence staining of fixed/permeabilized platelets for Bak (green) and Bcl-XL (red) and relative quantification for Bak. Scale bar = 5  $\mu$ m. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\*\* $P<0.0001$ . IBS: Intercept Blood System.

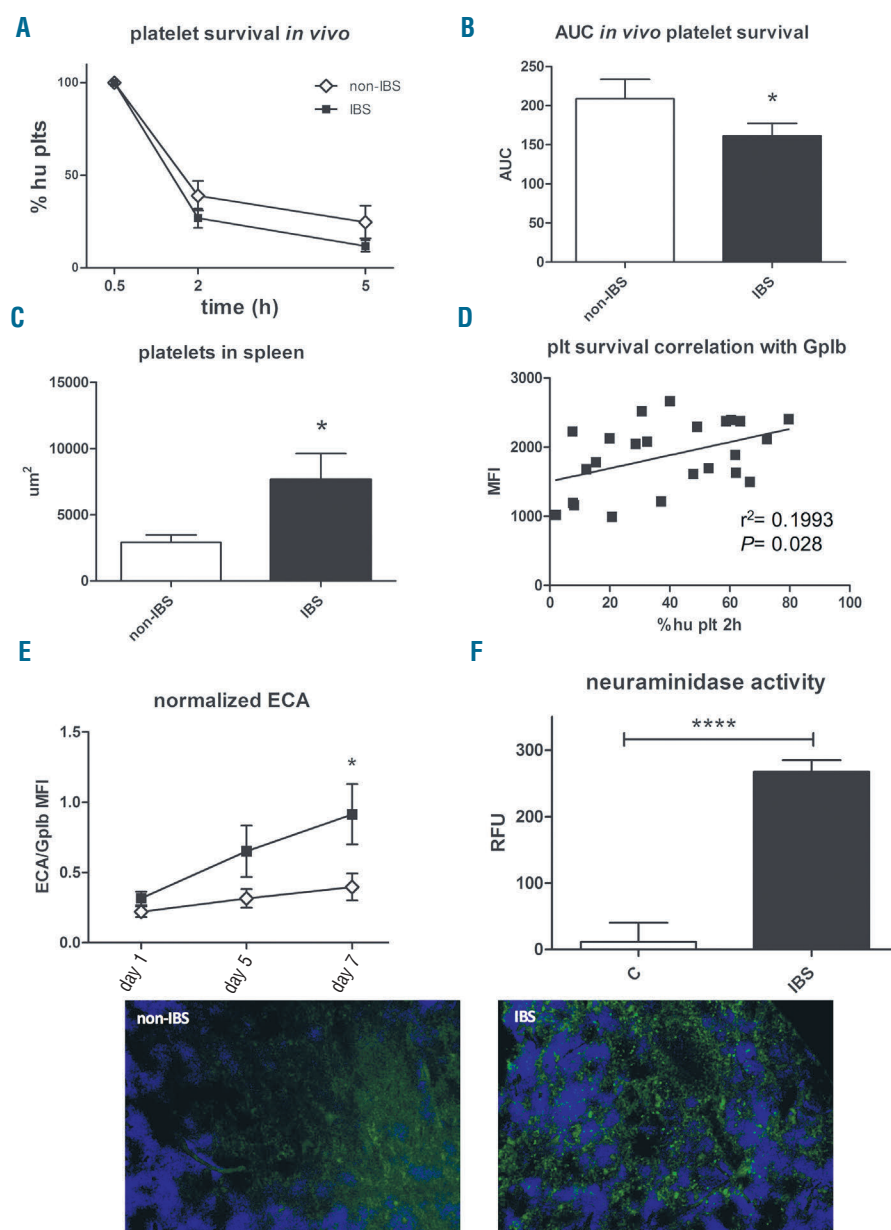


survival at 2 hours post-injection ( $r^2=0.1993$ ,  $P=0.028$ ; Figure 3D). It has been recognized that sialic acid on the heavily glycosylated GpIb $\alpha$  plays a relevant role in platelet clearance;<sup>26</sup> therefore, we analyzed our samples for desialylation of platelet surface proteins by the fluorescein isothiocyanate (FITC)-conjugated *Erythrina Cristagalli* Lectin (ECL) binding in flow cytometry experiments (Figure 3E). *Erythrina Cristagalli* Agglutinin Lectin (ECA) binds to unsialylated galactose ( $\beta$ 1-4) on N-acetyl-glucosamine (GlcNAc) and the ECA-binding level is inversely proportional to the level of sialylation. Concordant to our hypothesis, ECA binding was significantly higher in IBS-treated platelets compared to non-IBS samples, even after normalization to GpIb levels to account for the increased receptor shedding in IBS samples ( $n=6$ ,  $P=0.006$ ; Figure 3E). In order to confirm that GpIb $\alpha$  desialylation was due to an increased neuraminidase exposure/release following Amotosalen/UVA, a specific neuraminidase activity assay

was performed on the supernatants from untreated or IBS-treated samples. Cleavage of the specific neuraminidase substrate was significantly higher in supernatants from samples that underwent the Amotosalen/UVA procedure, demonstrating a release of neuraminidase from platelets following the IBS (Figure 3F;  $P<0.001$ ). Staining of fixed, non-permeabilized platelets for Neu1 revealed a higher fluorescence intensity for IBS-treated samples compared to control ones, while the fluorescence intensity was equivalent following platelet permeabilization to reveal total (surface and internal) Neu1 (Online Supplementary Figure S4).

### UV light without Amotosalen is sufficient to induce an increase in apoptotic Bak protein through mRNA translation

Platelets don't have a nucleus but they contain messenger (m)RNA and are capable of translation and protein



**Figure 3. Platelet clearance *in vivo* in NOD/SCID mice is increased in IBS samples.** (A) NOD/SCID mice were injected intravenously with fluorescently labeled platelets (untreated  $\diamond$ , or IBS-treated,  $\blacksquare$ ) and the % of circulating human platelets calculated after 2 and 5 hours;  $n=10$ . (B) Area under the curve (AUC) for the overall platelet survival of non-IBS versus IBS platelets;  $*P=0.02$ . (C) Spleens from mice injected with untreated or IBS-treated platelets were analyzed for the area of fluorescently-labeled platelets;  $n=10$ ,  $*P=0.018$ . (D) Correlation analysis of GpIb $\alpha$  levels and platelet survival 2 hours post-injection in mice. ( $r^2=0.1993$ ,  $P=0.028$ ). (E) Desialylation of platelets by FITC-conjugated ECA lectin staining and flow cytometry analysis, normalized to GpIb levels;  $n=6$ ,  $*P<0.05$ . (F) Neuraminidase activity was tested in supernatants from control and IBS samples and was found to be significantly increased after Amotosalen/UVA treatment;  $n=18$ ,  $****P<0.001$ . Bottom panel: representative microphotograph (4x magnification) of spleen from mice injected with fluorescent non-IBS or IBS platelets (platelet: green, nuclei: blue). Scale bar = 100  $\mu$ m. Plotted are mean  $\pm$ SEM. IBS: Intercept Blood System; plt: platelet; ECA: *Erythrina Cristagalli* Agglutinin; MFI: mean fluorescence intensity.

synthesis.<sup>29</sup> We hypothesized, therefore, that the increase in Bak protein after IBS was due to the translation of BAK specific mRNA. Immunoprecipitation of eukaryotic initiation factor 4E (eIF4E) followed by quantitative PCR showed that the relative expression of BAK was increased significantly 24 hours after irradiation, demonstrating an increased association of the specific BAK mRNA with eIF4E ( $n=4$ ,  $P=0.01$ ; Figure 4A). This result was confirmed at the protein level, because WB analysis of the platelet lysates showed an increased Bak level 24 hours after UV irradiation compared to non-UV platelets ( $n=9$ ,  $P=0.009$ ; Figure 4B). Blockade of mRNA translation with the protein synthesis inhibitor cycloheximide (10  $\mu\text{g/ml}$  final concentration) was able to block the increase in Bak protein after UV irradiation ( $n=3$ ,  $P=0.01$ ; Figure 4C).

#### Inhibition of p38 restores Gplb levels but does not rescue platelet survival

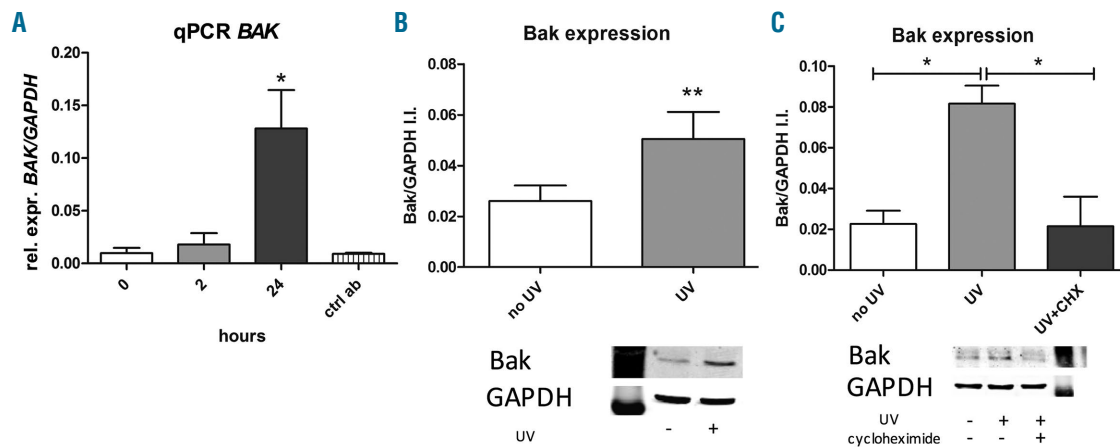
Due to the increased p38 phosphorylation observed in the IBS platelets, we reasoned that inhibition of p38 would block the adverse effects caused by the Amotosalen/UVA treatment. However, when injected into NOD/SCID mice, platelets pre-treated with the p38 inhibitor did not survive better as compared to untreated platelets, as shown in Figure 5A. At 2 hours post-injection, 28.5% of SB203580 platelets were circulating compared to 26.4% IBS vehicle-treated and 41.8% untreated platelets, respectively; at 5 hours, there were 5.08% of the SB203580 samples *versus* 5.7% of the IBS platelets and 32.2% for the untreated ones, respectively ( $n=5$ ,  $P>0.05$ ; Figure 5A). Analysis of Gplb $\alpha$  expression on untreated, IBS vehicle and IBS-SB203580 platelets revealed that the receptor cleavage was indeed blocked in the SB203580 samples, with receptor levels similar to the untreated samples (Figure 5B). Loss of sialic acid from platelet receptors was also prevented by the p38 inhibitor (Figure 5C). Aggregation to collagen and thrombin, however, was not different from the vehicle-treated IBS samples (*data not shown*).

Interestingly, we found that levels of the pro-apoptotic Bak were increased in the SB203580 samples, as shown by WB analysis of platelet lysate (*Online Supplementary Figure S2A*) and by immunofluorescence staining (*Online Supplementary Figure S2B* and *corresponding microphotograph*). Additionally, we found increased levels of cleaved caspase-3, suggesting that the increment in Bak leads to platelet apoptosis (Figure 5D).

#### Exploratory analysis of potential mechanisms with the neuraminidase inhibitor DANA

It has been proposed that desialylation of platelet receptors may in part regulate platelet survival, with the heavily glycosylated Gplb $\alpha$  being a major contributor.<sup>30,31</sup> Since we observed increased desialylation of platelets after IBS treatment (Figure 3E), we hypothesized that pre-treatment of platelets with the neuraminidase inhibitor DANA could block sialic acid loss and increase platelet survival. As shown in Figure 6A, 2 hours after injection 31.8% non-IBS platelets were circulating compared to 20.3% IBS-treated platelets. Pre-treatment with DANA was able to restore the circulating platelet value back to 31.4% during the first 2 hours ( $n=5$ ,  $P>0.05$ ; Figure 6A). However, this effect was partly lost at the later time point (5 hours), when circulating platelets from the DANA sample were 14.2% compared to 22.9% non-IBS and 10.4% IBS-treated, respectively (Figure 6A), possibly as a result of platelet washing causing removal of the inhibitor before the injection. Gplb $\alpha$  levels in the DANA samples were similar to those in IBS samples at all days of storage tested (Figure 6B). We also analyzed the level of desialylation and found that pre-incubation with DANA protected platelets from sialic acid loss, with levels of ECL binding similar to that of the untreated control at all days tested (Figure 6C).

Analysis of p38 phosphorylation, Bak expression and caspase-3 cleavage by WB did not show any significant difference between the samples (Figure 6D-F) in this series of experiments, and the limited number of donors (as per



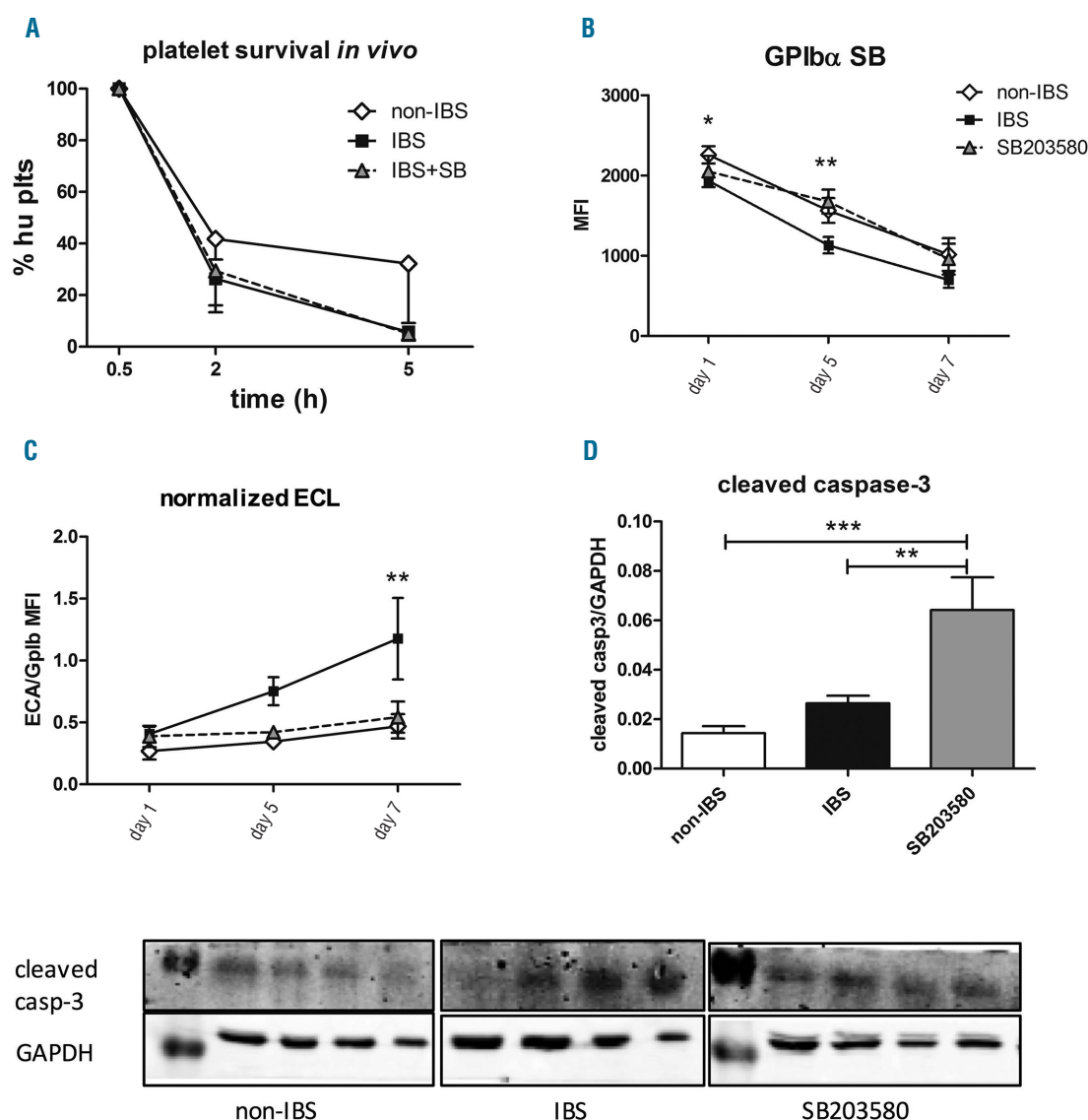
**Figure 4. UV irradiation of platelets induces Bak protein expression through mRNA translation.** Platelets were isolated and resuspended in 40% plasma/60% Intersol and kept either untreated (no UV) or UVA-irradiated (UV). (A) eIF4E was immunoprecipitated 2 or 24 hours after UV irradiation with a specific or a control antibody, and Bak quantitative PCR performed on the isolated RNA from the IP. 24 hours after UV irradiation, specific Bak RNA in complex with eIF4E is significantly increased ( $n=4$ ,  $*P=0.01$ ). (B) Bak protein levels normalized to GAPDH are increased 24 hours after UV irradiation, reflecting an increased protein synthesis ( $n=9$ ,  $**P=0.009$ ). (C) Platelets non irradiated or irradiated after pre-treatment with cycloheximide (10  $\mu\text{g/ml}$ ) were analyzed for Bak protein expression. Cycloheximide blocked Bak increase induced by UV ( $n=3$ ,  $*P=0.01$ ). qPCR: quantitative polymerase chain reaction.

ethical approval) did not allow us to increase the number of samples. In aggregation experiments, the response of DANA samples to collagen and thrombin was not different from that of the IBS samples (*data not shown*).

## Discussion

The study herein analyses in depth the structural and functional consequences induced by Amotosalen/UVA treatment using the IBS and the underlying mechanisms. We provide evidence of diminished platelet function, i.e., reduced aggregation and adhesion under flow, and reduced platelet survival *in vivo* by increased apoptosis

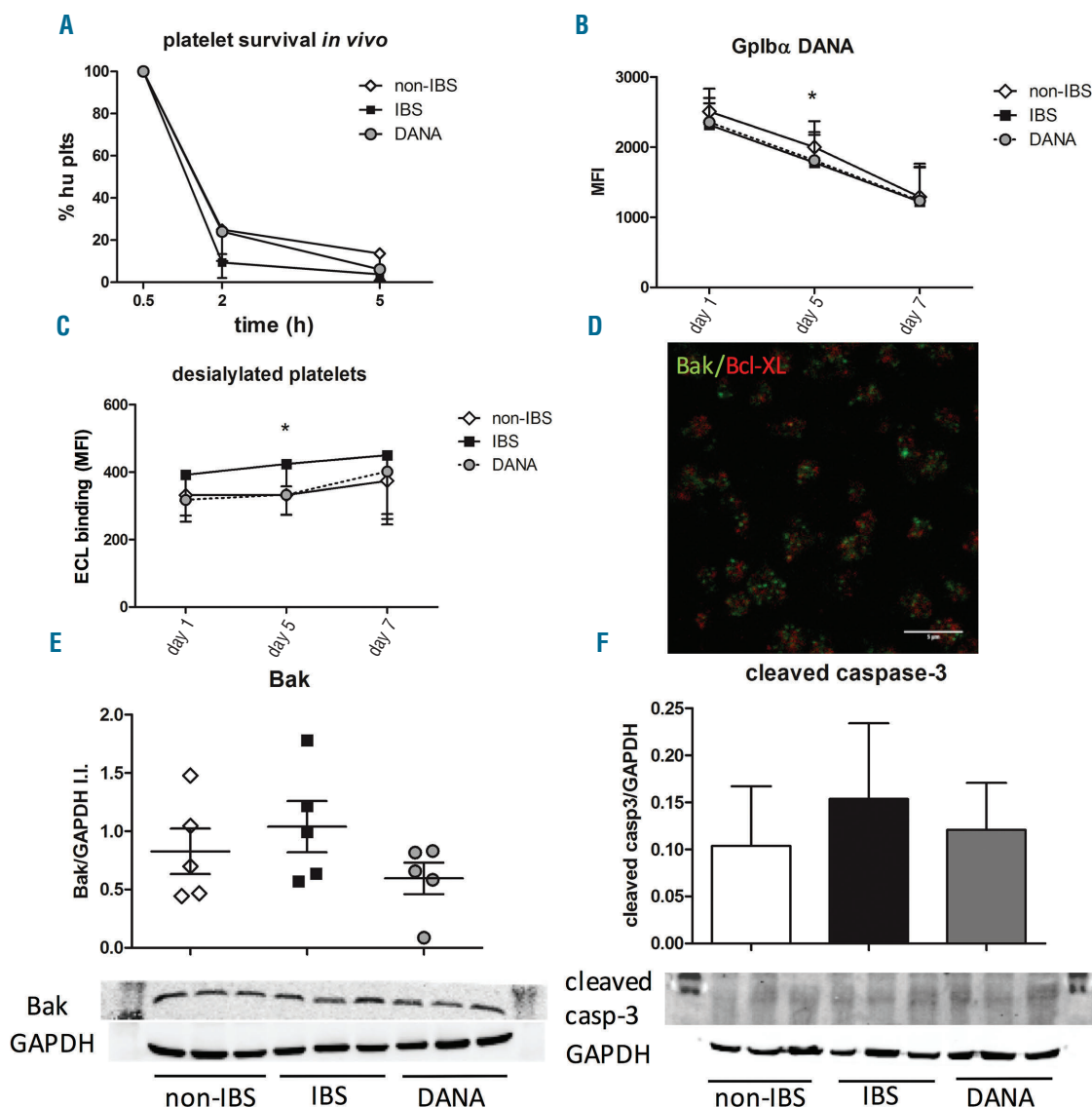
through Bak upregulation and a caspase-dependent pathway. We propose mechanisms based on our data (Figure 7) and potential interventions to reverse them. Besides the significantly reduced platelet response to physiological agonists in aggregometry, we found a reduced adhesion to vWF and collagen under flow after IBS treatment from the first day of storage (Figure 1), implicating a direct and rapid effect of the IBS on platelet function. This pattern is explained at the molecular level with a significant loss (about 20%) of surface GpIb $\alpha$  in IBS-treated platelets, and, accordingly, the corresponding accumulation of the cleaved glycoprotein in the supernatant plasma/Intersol (Figure 2A,B). The reduced aggregation over collagen could be explained by a reduced ability of platelets to



**Figure 5. Pre-incubation of platelets with SB203580 does not improve platelet survival and exacerbates apoptosis.** (A) Platelets from AU untreated, IBS-treated and pre-treated with the p38 inhibitor SB203580 (final concentration 20  $\mu$ M) were injected i.v. into NOD/SCID mice, and their survival in the circulation analyzed by flow-cytometry over 5 hours. The p38 inhibitor did not improve platelet survival compared to untreated platelets. (B) GPIb $\alpha$  expression levels in platelets pre-treated with SB203580 were comparable to untreated platelets at all days of storage; \* $P=0.04$  IBS vs. non-IBS; \*\* $P=0.01$  IBS vs. SB203580. (C) When levels of desialylation were measured by FITC-conjugated ECA lectin binding, SB203580 was able to inhibit desialylation compared to IBS platelets at all days of storage; \*\* $P<0.01$  non-IBS vs. IBS. (D) Pre-treatment of platelets with SB203580 induced caspase-3 cleavage;  $n=5$ , \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . IBS: Intercept Blood System; plt: platelet; hu: human; ECA: *Erythrina Cristagalli* Agglutinin; MFI: mean fluorescence intensity; ECL: *Erythrina Cristagalli* Lectin.

respond to external stimuli due to increased Bak-dependent apoptosis, and, additionally, to indirect mechanisms caused by vWF “bridging” collagen to GpIb. In addition, the study from Hechler *et al.*<sup>32</sup> found a significant loss of glycoprotein V (GPV) after Amotosalen/UVA treatment, and GPV was found to participate in platelet response to collagen.<sup>33</sup> Therefore, we may speculate that IBS-induced GPV shedding could also be responsible for the reduced adhesion and aggregation in response to collagen. Increased loss of GpIb is associated with the typical PSL in untreated platelets<sup>28,34</sup> due to the activation of TACE; our results support the hypothesis of an accelerated lesion induced by the IBS which seems to be independent from the PSL, since it is observed from day 1 of storage. It has

also been shown that cold storage of platelets induces GpIb desialylation, which primes the receptor for TACE-dependent shedding.<sup>35</sup> Our results extend this observation to the IBS treatment, since we detected increased platelet desialylation in the treated samples as compared to the untreated platelets (Figure 3E) as well as a significantly increased neuraminidase activity in the supernatant from IBS samples, suggesting release of the enzyme from platelets after the PI; these observations suggest that this is an effect of the IBS (and perhaps of all PI technologies in general) as well as of storage over time. Interestingly, this is in line with our earlier structural and functional observations that deglycosylation of GpIb results in a collapse of GpIb on the membrane and a loss of platelet-vWF inter-



**Figure 6. Neuraminidase inhibitor DANA partially rescues platelet survival *in vivo*.** (A) control (◇), IBS (■) and DANA (●) platelets were injected *in vivo* into NOD/SCID mice and their survival analyzed over 5 hours. DANA-treated platelets were protected from clearance at the early time point (2 hours), at which time the clearance matched the non-IBS controls, but not at the later stage (5 hours). (B) GpIbα cleavage in DANA-treated samples was comparable to IBS samples at all days of storage; \* $P=0.03$  IBS/DANA vs. non-IBS. (C) Desialylation was abrogated in the presence of DANA up to 7 days of storage; \* $P=0.03$  IBS vs. non-IBS. (D) Immunofluorescence staining and (E) WB analysis of Bak and cleaved caspase-3 (F) revealed no difference between non-IBS, IBS and DANA samples;  $n=5$ ,  $P>0.05$ . IBS: Intercept Blood System; plt: platelet; hu: human; MFI: mean fluorescence intensity; ECL: *Erythrina Cristagalli Lectin*; DANA: 2,3-dehydro-2-deoxy-N-acetylneuraminic acid.



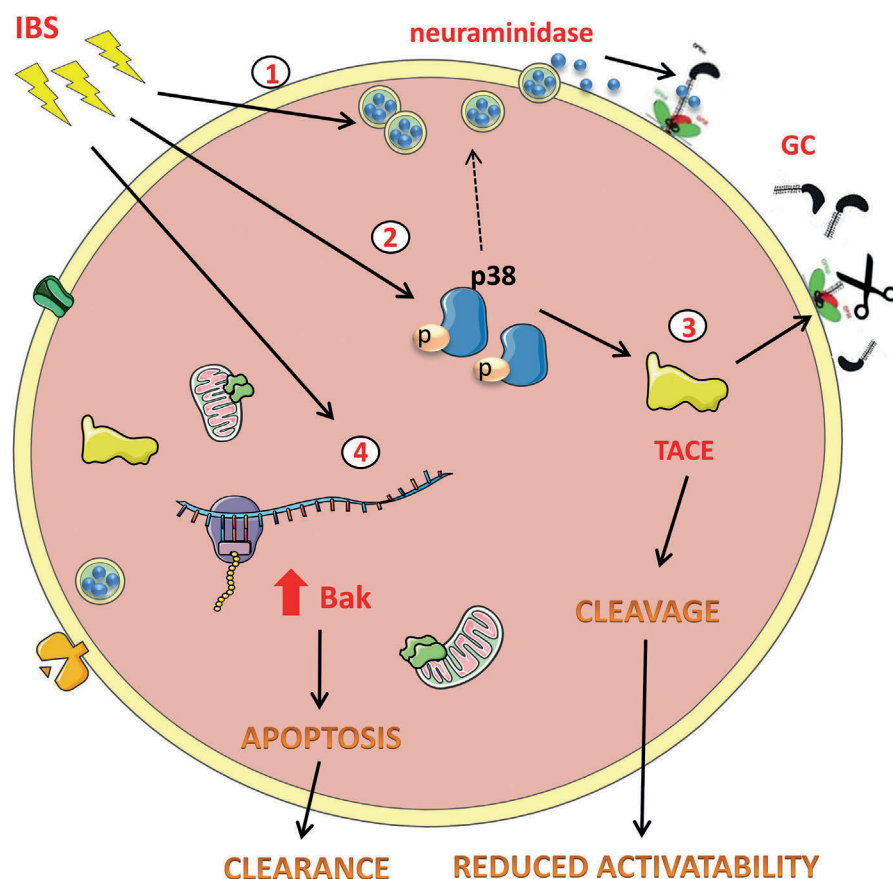
action.<sup>36</sup> Thus, a dual effect of the IBS on GpIb (cleavage and desialylation) may lead to platelet clearance. Indeed, *in vivo*, we found a significant correlation of the increased clearance of IBS platelets in the spleen of NOD/SCID mice; platelet clearance correlated with GpIb levels, confirming the important role of this receptor for platelet survival, as also demonstrated by other groups.<sup>35,37,38</sup> The sialidase inhibitor DANA seems to reduce, in part, the loss and clearance of IBS-treated platelets *in vivo* in our platelet survival model in NOD-SCID mice (Figure 5A-C), albeit only at the early time point. DANA pre-treated samples did not show an increase in Bak or in cleaved caspase-3 observed after IBS treatment. Interestingly, and perhaps surprisingly, we could not detect platelet clearance in the liver of these mice, in spite of the important role played by the Ashwell-Morell receptor on hepatocytes in the removal of desialylated platelets<sup>26,39</sup> (*data not shown*); however, the extensive shedding of GpIb $\alpha$  after Amotosalen/UVA could be responsible for blocking the recognition and hepatic clearance of desialylated platelets.

At the intracellular level, we found that the IBS was linked to an increased phosphorylation of the signalling molecule p38 (Figure 2D), in agreement with previous reports of storage of untreated platelets.<sup>21,34</sup> Interestingly, p38 is a known TACE activator,<sup>40</sup> thus its increased phosphorylation is directly linked to the increment in GpIb cleavage observed in IBS platelets in this study (Figure 2A,B). Pre-incubation with a specific p38 inhibitor (SB203580) reduced GpIb shedding and desialylation upon

IBS treatment but did not improve platelet survival in mice (Figure 5A,B), suggesting that restoring GpIb levels is not sufficient to reduce platelet removal and that other mechanisms play a role in the accelerated clearance of IBS platelets, possibly through the induction of apoptosis, which was worsened by the p38 inhibitor as shown by cleaved caspase-3 levels (Figure 5D).

Other than cleavage, the induction of apoptosis could represent an important mechanism of platelet clearance, as has been shown for the riboflavin/UV light-based (Mirasol) PI.<sup>17,22</sup> Expression of the pro-apoptotic protein Bak and cleavage of caspase-3 were significantly increased in IBS samples compared to non-IBS, confirming induction of platelet apoptosis as a mechanism of the reduced platelet function, and accelerated clearance after PI (Figure 2E-G). In contrast to previous studies,<sup>19,32,41</sup> we did not detect an increased activation of the fibrinogen receptor GpIIb/IIIa (*Online Supplementary Figure S1C*); this could be partly explained by the different protocol used for platelet collection, which was shown to affect platelet activation.<sup>42,43</sup>

Schripchenko *et al.* recently reported that p38 or sialidase inhibition could not block PSL caused by 4°C storage, in agreement with our results.<sup>44</sup> However, this is in contrast to the results of other groups, which show amelioration of platelet function after p38 inhibition or GpIb shedding blockade during storage.<sup>21,34,45</sup> The reason for these contrasting results remains unclear at this point. An intriguing hypothesis is that p38 activation in response to the stress associated with PI may have a protective



**Figure 7. Schematic image representing the mechanisms found in our study.** (1) IBS leads to phosphorylation of p38 (2), which in turn causes TNF- $\alpha$  converting enzyme (TACE) activation and GpIb shedding (3). The latter is also up-regulated by the release of neuraminidase from intracellular stores, which cleaves sialic acid residues on GpIb $\alpha$ , priming the receptor for cleavage. IBS also induces (likely by UV) upregulation of the pro-apoptotic Bak (4), which results in the induction of apoptosis through a caspase-dependent pathway. Both processes lead to a reduced response of platelets to agonists and an accelerated clearance *in vivo*. GC: glycocalicin.

role, which leads to an increased apoptosis when inhibited, as reported by Rukoyatkina *et al.*<sup>46</sup> An interesting observation of our study is that the IBS induces expression of the pro-apoptotic protein Bak, and this is replicated when freshly isolated platelets are irradiated with UVA without the addition of Amotosalen (Figure 3E,F and Figure 4B). We were also able to show that this occurs through an increased mRNA translation following its association with the protein eIF4E, considering that the protein synthesis inhibitor cycloheximide was able to block the increase in Bak after UV (Figure 4A,C). Since platelets contain mRNA and all the necessary machinery to enable them to translate into proteins,<sup>29,47</sup> our results suggest that PI might trigger translation of specific mRNA inducing apoptosis, similar to the way in which it alters mRNA and microRNA expression.<sup>18,48</sup> The development of PI represents a major cornerstone in transfusion medicine by reducing the risk of transfusion transmitted diseases in patients receiving blood products. The downside of this technology is the observation that PI exacerbates the PSL and has an impact on platelet function, although one study reported no change in platelet aggregation when washed platelets were used, the significance of which is not clear since the number of platelet concentrates analyzed was low.<sup>7,17,22,32,49,50</sup> The study herein clearly demonstrates that platelet treatment with Amotosalen/UVA causes an alteration of platelet function. However, we also observed a detrimental effect with UVA treatment alone, and a negative impact on

platelet function has been reported for  $\gamma$ -irradiation.<sup>51</sup> Therefore, whether the IBS has a different or greater effect on platelets remains unclear. Importantly, we provided a mechanistic insight into the pathways involved in the negative effects of the Amotosalen/UVA treatment on platelets.

Although a large number of clinical studies did not demonstrate an inferior clinical efficacy of IBS-treated platelets, further research on the clinical outcomes of IBS-treated platelet transfusion, focusing on bleeding, are necessary. The implementation of the IBS in more than 40 countries worldwide shows the necessity of technologies capable of reducing the risk associated with blood products transfusion, in spite of the alterations in platelet function caused by the procedure. Nevertheless, our observations indicate the importance of developing strategies that can be implemented to PI methods (such as new platelet additive solutions) in order to preserve platelet function and thus provide patients with safer, qualitatively optimal transfusion products.<sup>52-54</sup>

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