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## Thrombopoietic Effect of VPAC1 Inhibition during Megakaryopoiesis



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## Thrombopoietic Effect of VPAC1 Inhibition during Megakaryopoiesis

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**Running Title:** stimulation of platelet production by VPAC1 inhibition

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## Summary

Megakaryocytes and platelets express the stimulatory G protein (Gs)-coupled VPAC1 receptor, for which the pituitary adenylyl cyclase-activating peptide (PACAP) and vasoactive intestinal peptide (VIP) are agonists. We found that the neuropeptide PACAP and VPAC1 negatively regulate megakaryopoiesis. We demonstrated that inhibition of their physiological pathway has a thrombopoietic effect, in conditions where megakaryopoiesis and thrombopoiesis are impaired, such as chemotherapy-induced thrombocytopenia and congenital thrombocytopenia. In the present study, we explore the thrombopoietic effect of VPAC1 inhibition in a murine model of syngeneic bone marrow (BM) transplantation and in passive immune thrombocytopenia. We demonstrate that the treatment of donor mice with a neutralizing anti-VPAC1 antibody (Ab) stimulates the initial, most critical recovery of the platelets in irradiated mice. In the passive immune thrombocytopenia model, we observe a thrombopoietic effect, resulting in a less severe platelet drop after induction of their removal in the spleen by an anti-platelet Ab. We conclude that inhibition of the physiological PACAP/VPAC1 pathway can stimulate *in vivo* megakaryopoiesis. This inhibition can be applied to attenuate thrombocytopenia in conditions, in which platelets are destroyed as the major pathogenetic mechanism, e.g. immune thrombocytopenia purpura (ITP) or need to be produced *de novo*, e.g. after irradiation and BM transplantation.

## Keywords

VPAC1, thrombocytopenia, megakaryopoiesis, platelet, mouse

## Introduction

Megakaryopoiesis and thrombopoiesis are complex processes that involve many thrombopoietic cytokines, including interleukin-3 (IL-3), stem cell factor (SCF), IL-6, IL-11, Fms-related tyrosine kinase 3 ligand (FLT-3 ligand), thrombopoietin (THPO) and many others (Broudy, *et al* 1995, Kaushansky 1995, Kaushansky 2005, Peeters, *et al* 2008, Schulze and Shivdasani 2005, Weich, *et al* 2000). In the 1990s, significant advances were made in understanding the transcriptional basis of hematopoietic cell differentiation (Shivdasani and Orkin 1996). The particular importance of erythromegakaryocyte transcriptional regulators, for example GATA1 and FOG1, in certain stages of megakaryocyte and platelet differentiation, has been described (Chang, *et al* 2002, Orkin, *et al* 1998, Vyas, *et al* 1999). Other transcription factors which play a role during megakaryopoiesis include Fli1, NF-E2, Ets1, Tel and Runx1 (Szalai, *et al* 2006). Defects in regulators of megakaryopoiesis, for example GATA1 and MPL, the receptor for THPO, result in congenital thrombocytopenia. Patients with nonsense or missense mutations that severely reduce, or obliterate the activity of MPL suffer from congenital amegakaryocytic thrombocytopenia (Ballmaier, *et al* 2001). The D218Y mutation in GATA1 results in a severe congenital macrothrombocytopenia (Freson, *et al* 2002). Acquired forms of thrombocytopenia include chemotherapy-induced thrombocytopenia and ITP.

Recently, new strategies were discovered to treat thrombocytopenia in a less obvious way, not by stimulating THPO signaling, but by neutralizing physiological inhibitors of megakaryopoiesis, for example platelet factor 4 (Lambert, *et al* 2007) and PACAP (ADCYAP1) and VPAC1 (VIPR1) (Freson, *et al* 2008). The Gs-coupled VPAC1 receptor is expressed on megakaryocytes and platelets and has 2 agonists: PACAP and VIP, which belong to the glucagon hormone superfamily. Effects of these pleiotropic neuropeptides have been described in many tissues, such as the digestive tract, cardiovascular system, airways, immune system, brain, reproductive system and endocrine glands (Vaudry, *et al* 2009). We previously found an important role for PACAP and its receptor VPAC1 in megakaryopoiesis and platelet function (Freson, *et al* 2004). Upon binding to its Gs-coupled receptor, PACAP increases intracellular cyclic adenosine monophosphate levels and inhibits platelet function and megakaryopoiesis. By

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3 using a VPAC1-neutralizing Ab we were able to achieve the reverse effect, i.e. the  
4 stimulation of megakaryopoiesis (Freson, *et al* 2008). We demonstrated such an effect in  
5 myelosuppressed mice and rabbits and in a mouse model of GATA1 deficiency  
6 characterized by congenital thrombocytopenia (Freson, *et al* 2002, Orkin, *et al* 1998,  
7 Vyas, *et al* 1999). These models of thrombocytopenia represent impaired  
8 megakaryopoiesis and thrombopoiesis.  
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14 In this study we have further explored the thrombopoietic effect of VPAC1 inhibition,  
15 which we previously described (Freson, *et al* 2008), in other thrombocytopenia models.  
16 To this end, the thrombopoietic activity of anti-VPAC1 treatment was studied in mice,  
17 after total body irradiation (TBI), followed by syngeneic bone marrow (BM)  
18 transplantation. Platelets are the last blood cells to arise in the peripheral blood (PB), after  
19 TBI and BM transplantation, resulting in serious bleeding risks, e.g. in leukemia patients.  
20 In addition a murine model of passive immune thrombocytopenia was applied and  
21 platelet production was monitored in anti-VPAC1 treated mice.  
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## 30 **Material and Methods**

### 31 *Antibodies*

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33 The monoclonal IgG1 Ab (clone 23A11) (Freson, *et al* 2008) was obtained in our  
34 laboratory from BALB/c mice, immunized with human recombinant VPAC1, coupled to  
35 glutathione S-transferase (GST). This recombinant fusion protein was expressed in  
36 *Escherichia coli* and purified by affinity chromatography on immobilized glutathione  
37 (Amersham Biosciences, Freiburg, Germany). The primary Ab was purified on protein A  
38 Sepharose beads (Amersham Biosciences) and controlled for their reactivity toward  
39 VPAC1 by enzyme-linked immunosorbent assay (ELISA). We will refer to this anti-  
40 VPAC1 antibody as 23A11. We used an isotype-matched control mouse IgG1 (clone  
41 1C8) (Fischer, *et al* 2007) to which we will refer as 1C8.  
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### 51 *In vitro differentiation of CD34+ into megakaryocytes*

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53 Human CD34+ hematopoietic stem cells (HSCs) were isolated from buffy coat bags from  
54 peripheral blood (PB) from healthy volunteers by magnetic cell sorting (Miltenyi Biotec,  
55 Auburn, CA). Isolated CD34+ cells were cultured in StemSpan SFEM medium (Stem  
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3 cell technologies, Vancouver, Canada), which was supplemented with 20 ng/ml **THPO**,  
4 10 ng/ml SCF, 10 ng/ml IL-6 and 10 ng/ml FLT-3 (all from Peprotech, London, United  
5 Kingdom). On day 6 of culture, cytokines were again added, i.e. 10 ng/ml **THPO**, 10  
6 ng/ml IL-6 and 10 ng/ml IL-1 $\beta$ . To determine megakaryocyte ploidy, in vitro  
7 differentiated megakaryocytes (day 13) were costained with FITC-conjugated CD61 (VI-  
8 PL2) (BD Biosciences Pharmingen, Heidelberg, Germany) and propidium iodide (Sigma-  
9 Aldrich), and the DNA content was determined by 2-color flow cytometry (Mathur, *et al*  
10 2004). We used Cell Quest software for 2-color immunofluorescence acquisition on a  
11 FACSCalibur flow cytometer (both from BD Biosciences Pharmingen) and for data  
12 analysis.

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21 Anti-VPAC1 Ab (23A11) (Freson, *et al* 2008) or control Ab (1C8) (Fischer, *et al* 2007)  
22 (100  $\mu$ g/ml) was added on day 0, 6 and 10.  
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#### 25 26 27 *Electron microscopy (EM) of megakaryocytes*

28 Differentiated megakaryocytes were fixed in 2.5% glutaraldehyde and 0.1 M phosphate  
29 buffer at 4°C overnight. At 1 hour after fixation in 1% osmium tetroxide and 0.1 M  
30 phosphate buffer at 4°C, samples were dehydrated in graded series of alcohol and  
31 embedded in epoxy resins. Ultrathin sections were cut, stained with uranyl acetate-lead  
32 citrate, and examined using a Zeiss electron microscope (Jena, Germany).  
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#### 39 40 41 *Hematological investigations*

42 Peripheral citrate-anticoagulated blood samples were obtained from the retro-orbital sinus  
43 from mice. A diluted aliquot of whole blood was analyzed on an automated blood cell  
44 counter (Cell-Dyn 1300; Abbott Laboratories, Abbott Park, IL) to determine leukocyte,  
45 erythrocyte and platelet counts, hematocrit and hemoglobin levels.  
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#### 49 50 51 *Thpo measurement in plasma*

52 **Thpo** levels were determined in mouse citrated plasma according to the manufacturer's  
53 guidelines (**Thpo** Elisa kit, R&D Systems, Minneapolis, MN).  
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#### 56 57 58 59 60 *Syngeneic BM transplantation after TBI*

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3 Female C57/Bl6 mice were given 9 Gy TBI on day -1 and were transplanted via the tail  
4 vein with 250,000 whole BM cells derived from female C57/Bl6 mice on day 0. BM was  
5 harvested by flushing femur and tibia with D-PBS supplemented with 5% fetal bovine  
6 serum (FBS). Mice from whom BM was collected (donor mice, D mice) received either 1  
7 mg/kg 23A11 (D+) subcutaneously or vehicle (D-), 3 times a week for 2 weeks.  
8 Recipient mice (R) respectively received 1 mg/kg 23A11 (R+) subcutaneously or vehicle  
9 (R-), 3 times a week, starting 2 weeks prior to TBI for the entire length of the experiment.  
10 As such, 4 groups of animals (D-R-, D+R-, D-R+, D+R+) (**n=7 for each group**) were  
11 created. PB was taken on days -15, -1, 11, 15, 19, 23, 27 and 32.  
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### 20 21 *Induction of passive immune thrombocytopenia*

22 ITP was induced in female Balb/c mice (15 animals per group) using the anti-platelet Ab  
23 MWReg30 (rat anti-mouse integrin  $\alpha_{IIb}$  monoclonal Ab, anti-GPIIB, IgG1, BD  
24 Pharmingen), adapted from (Deng and Balthasar 2007). Briefly, MWReg30 was  
25 continuously administered using an osmotic pump (Alzet micro osmotic pump, model  
26 1007D; Alzet, Palo Alto, CA) implanted subcutaneously dorsally in the animal. The  
27 osmotic pumps were filled with 100  $\mu$ l MWReg30 (82.5  $\mu$ g/ml) and human serum  
28 albumin (1.5 mg/ml) to prevent absorption of proteins to the device, in sterile saline and  
29 then surgically subcutaneously inserted in the mice. Following insertion, the pumps  
30 released the MWReg30 solution at a rate of 0.5  $\mu$ l/h for a duration of 7 days. Mice  
31 received 1 mg/kg anti-VPAC1 Ab (**n=15 for anti-VPAC1**) or control Ab 1C8 (**n=15 for**  
32 **control Ab**) subcutaneously, 3x/week, starting from day -15 onwards until day 12.  
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### 45 *Statistics*

46 We used GraphPad Prism4 software for statistical analysis. Continuous variables with  
47 little to mild skewness were summarized as means plus minus SEM and compared by  
48 means of the 1-way anova of variance (ANOVA) test, complemented with the Bonferroni  
49 multiple comparison t test to identify statistically differences at each individual time  
50 point. Data were considered significant in all cases when the P value was less than .05.  
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### 56 **Results**



### *Stimulating in vitro megakaryopoiesis by inhibiting VPAC1 signaling*

Human CD34+ HSCs, isolated from buffy coat bags of PB were differentiated into megakaryocytes. From day 10 onwards, proplatelet extrusions were visible under a light microscope (see figure 1A). On day 13, formation of fully mature megakaryocytes and platelets was confirmed by EM (see figure 1B and 1C).

To demonstrate the in vitro thrombopoietic effect of inhibiting VPAC1 signaling during megakaryopoiesis, cells were cultured in the presence of control Ab 1C8 and the VPAC1 inhibiting Ab 23A11. By adding the anti-VPAC1 Ab, the autocrine inhibitory effect of PACAP and VIP (Nam, *et al* 2009), the VPAC1 agonists, is blocked. **The early stage as well as the late stage of megakaryopoiesis were stimulated by inhibiting VPAC1 signaling since more CD61+ megakaryocytes were generated that showed a higher degree of maturation, i.e. more ploid nuclei.** More CD61+ (77.7±3.0% for incubation with 23A11 vs. 60.3±3.3% for incubation with control Ab 1C8, P<0.05) positive cells were generated by inhibiting VPAC1 (see figure 1D for a representative plot). In addition, these cells showed a further stage of maturation since they have higher polyploidy nuclei (see figure 1E for a representative plot). Highly polyploid megakaryocytes responsible for the release of platelets (Mattia, *et al* 2002), i.e. 8N, 16N and 32N megakaryocytes were more abundantly present in cultures where the neutralizing anti-VPAC1 Ab was added vs. control Ab 1C8 (see figure 1F).

### *Anti-VPAC1 stimulates megakaryopoiesis in a model of syngeneic BM transplantation*

We questioned whether a thrombopoietic effect would be discernible in vivo, for the anti-VPAC1 treatment during the platelet recovery phase following TBI and BM transplantation. Platelets are the last blood cells to recover in these settings, resulting in a serious bleeding risk. A previous study showed that besides megakaryocytes and platelets (Park, *et al* 1996), also HSCs (Kawakami, *et al* 2004) express the VPAC1 receptor. VIP was also found to inhibit the proliferation of BM progenitors through VPAC1 (Rameshwar, *et al* 2002). We previously showed that more megakaryocyte colonies are generated from murine BM-derived Sca1+ cells when 23A11 is added versus a control Ab (Freson, *et al* 2008). Mice were irradiated with 9 Gy TBI and consequently transplanted with whole BM derived from D+ or D- donor mice, i.e. the donor mice being

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3 treated or not with the neutralizing anti-VPAC1 Ab. The same applies to the recipient  
4 mice who were either R+ or R-, i.e. treated or not by 23A11.

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7 From our data, we can deduce that the platelet recovery after syngeneic BM  
8 transplantation is enhanced, if the BM that was given to the irradiated mouse was derived  
9 from an anti-VPAC1 treated animal (see figure 2). Administration of the anti-VPAC1 Ab  
10 (23A11) to the recipient mice themselves didn't have any additional effect regarding the  
11 platelet recovery. More precisely, the platelet nadir at day 11 post transplantation was  
12 attenuated in D-R+ vs. D+R+ mice ( $18\pm 2$  vs.  $30\pm 11$   $10^9$  platelets/L,  $P<0.05$ ) and D-R-  
13 vs. D+R+ ( $18\pm 3$  vs.  $30\pm 11$   $10^9$  platelets/L,  $P<0.05$ ) mice (see figure 2B). The initial,  
14 most critical platelet recovery at day 15 however was enhanced in all D+ mice, that is D-  
15 R- vs. D+R- ( $58\pm 5$  vs.  $135\pm 28$   $10^9$  platelets/L,  $P<0.05$ ), D-R+ vs. D+R+ ( $79\pm 13$  vs.  
16  $179\pm 31$   $10^9$  platelets/L,  $P<0.05$ ) and D-R- vs. D+R+ mice ( $58\pm 5$  vs.  $179\pm 31$   $10^9$   
17 platelets/L,  $P<0.01$ ) (see figure 2C). This suggests that the megakaryopoiesis of the  
18 transplanted donor BM cells, engrafting in the irradiated recipient mice, needs to be  
19 stimulated in order to have an effect on the platelet recovery in the R mice. This finding  
20 is comparable to **Thpo**: only when D mice are treated, an effect can be observed in the  
21 platelet recovery (Fibbe, *et al* 1995, Schuster, *et al* 2002, Wolff, *et al* 2001). **Red and**  
22 **white blood cell recovery did not differ between the groups (figure 2D and 2E).**

### 33 34 35 36 37 *Anti-VPAC1 stimulates megakaryopoiesis in a murine model of ITP*

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39 Currently, **THPO** mimetics are approved for the treatment of chronic ITP in humans  
40 (Bussel, *et al* 2007, Bussel, *et al* 2006, Kuter, *et al* 2008). We already demonstrated an  
41 effect of VPAC1 inhibition on platelet recovery after myelosuppressive and  
42 myeloablative therapy. We next choose to verify this effect in a totally different  
43 mechanism of thrombocytopenia, i.e. ITP where the main cause is an increased  
44 destruction of platelets and not mainly from a defective megakaryocyte and platelet  
45 formation. To induce ITP in mice, we used the MWReg30 anti-platelet Ab. As previously  
46 described (Teeling, *et al* 2001), the platelets reach about 25% of their initial level after 3  
47 to 4 days, using this protocol. When megakaryopoiesis is stimulated by giving the anti-  
48 VPAC1 23A11 vs. control Ab to the animals, the platelet drop is less severe, indicative of  
49 a thrombopoietic effect (see figure 3). At day 3, platelet counts were  $131\pm 10$   $10^9$ /L for  
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control Ab vs.  $197 \pm 22 \times 10^9/L$  for 23A11 ( $P < 0.05$ ) and at day 4 they were  $170 \pm 17 \times 10^9/L$  for control Ab vs.  $250 \pm 25 \times 10^9/L$  for 23A11 ( $P < 0.05$ ). The thrombopoietic effect remained noticeable when platelet counts had already been stabilized to normal levels: on day 8, platelet counts were  $888 \pm 71 \times 10^9/L$  for control Ab vs.  $1167 \pm 73 \times 10^9/L$  for 23A11 ( $P < 0.05$ ). On day 10 was this respectively  $1013 \pm 76 \times 10^9/L$  and  $1293 \pm 50 \times 10^9/L$  for 23A11 ( $P < 0.01$ ). Animals treated with 23A11 vs. vehicle showed no differences in plasma **Thpo** levels ( $283 \pm 11$  pg/ml for vehicle vs.  $307 \pm 20$  pg/ml for 23A11 on day 6) and 23A11 does not affect binding of MWReg30 to murine platelets (data not shown).

## Discussion

We found that inhibiting VPAC1 signaling using a neutralizing Ab stimulates normal megakaryopoiesis, in vitro, via increased megakaryocyte generation and production of megakaryocytes with a higher polyploidy.

As such, we demonstrate that the early stage of megakaryopoiesis, i.e. the generation and proliferation of megakaryocytes, as well as the late stage of megakaryopoiesis, i.e. the maturation of megakaryocytes can be stimulated by inhibiting VPAC1 signaling. We believe that this is the reason why the anti-VPAC1 treatment could be effective in different pathologies of thrombocytopenia.

The main regulator of thrombopoiesis is **THPO**. The use of **THPO** mimetics is well established in ITP. **THPO** mimetics, peptide (Nplate®, Amgen) (Wang, *et al* 2004) or non-peptide (Promacta®, GSK) (Bussel, *et al* 2007) have quite recently been approved by the FDA, for the treatment of chronic ITP. We, therefore, also questioned whether it is possible to treat ITP by interfering with a less pronounced and negative player of megakaryopoiesis, i.e. the PACAP/VPAC1 pathway, for which we had shown its efficacy in other models of platelet formation (Freson, *et al* 2008). In our previous studies we have ruled out non specific effects of using 23A11 since we also showed the thrombopoietic effect by using an anti-PACAP Abs (Freson, *et al* 2008).

In the present study, we have used 2 different murine models of thrombocytopenia to investigate the thrombopoietic effect of inhibiting VPAC1 signaling during megakaryopoiesis. In the past, we already demonstrated the therapeutic potential of interfering with this physiological pathway in chemotherapy-induced thrombocytopenia

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3 and in GATA1 deficiency, a congenital form of thrombocytopenia (Freson, *et al* 2008).  
4 Both models of thrombocytopenia represent impaired megakaryopoiesis: platelets are not  
5 formed in normal quantities. We continued exploring the thrombopoietic effect of this  
6 strategy in another model of thrombocytopenia as a result of a totally different  
7 mechanism; i.e. with a proper platelet formation, but undergoing an increased destruction  
8 after release in the PB. To achieve this goal, we used a murine model of passive immune  
9 thrombocytopenia. The mouse model of ITP used in this study is a passive model of  
10 sustained thrombocytopenia, adapted from (Deng and Balthasar 2007), where MWReg30,  
11 a rat monoclonal Ab directed against murine GPIIb/IIIa is administered via a constant-  
12 rate, continuous subcutaneous infusion. We observed an attenuated drop of platelet levels  
13 at the time points were the platelets were the lowest, that is at day 3 & 4 after starting the  
14 MWReg30 administration. The thrombopoietic effects were still obvious, even when  
15 platelet levels had risen again to normal levels. As such, we show that by targeting the  
16 PACAP/VPAC1 pathway, a physiological inhibitor of megakaryopoiesis, a  
17 thrombopoietic effect can be established in settings in which THPO, the main key  
18 regulator of thrombopoiesis, is effective as well.

19  
20 We subsequently validated this thrombopoietic effect of anti-VPAC1 in mice receiving  
21 BM after TBI. The time to achieve platelets' complete reconstitution imposes a serious  
22 problem in leukemia patients who receive TBI prior to a BM transplant. Platelets are the  
23 last blood cells to recover, thereby causing real bleeding episodes. We questioned  
24 whether we could ameliorate the platelet recovery in a mouse model, mimicking this  
25 condition, i.e. in mice receiving TBI and a whole BM transplant. In summary, we can  
26 state that BM derived from anti-VPAC1 treated mice gives rise to an enhanced initial  
27 platelet recovery in irradiated recipient mice. VPAC1 is expressed on HSCs (Kawakami,  
28 *et al* 2004) and VIP was found to inhibit the proliferation of BM progenitors through  
29 VPAC1 (Rameshwar, *et al* 2002). We previously found that by adding 23A11 to in vitro  
30 cultures, more megakaryocyte colonies are being generated from Sca1+ (Freson, *et al*  
31 2008), similar to the generation of more CD61+ megakaryocytes in human cultures,  
32 suggesting the stimulatory effect of VPAC1 inhibition on stem cells and the early stage of  
33 megakaryopoiesis. We hypothesize that it is for this reason that in the murine BM  
34 transplantation model, we see an effect only when the donor BM is treated. Although the  
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3 effect seems discrete, the initial recovery from low platelet numbers is most critical and a  
4 small improvement can indeed make a difference with regard to the bleeding risk. The  
5 finding that in this mouse model, the donor mice needed to be treated in order to see an  
6 effect on the platelets counts in the recipient mice is in concordance with similar  
7 observations made for **Thpo** in a mouse model (Fibbe, *et al* 1995). The PACAP/VPAC1  
8 axis acts independently from **THPO**; whether their thrombopoietic effects, by inhibition  
9 and stimulation respectively, are additive remains elusive.

10  
11 In summary, our results demonstrate that by inhibiting the VPAC1 signaling,  
12 megakaryopoiesis can be stimulated. This effect can prevent immune thrombocytopenia  
13 in mice and increases the initial platelet recovery in mice receiving BM from treated  
14 donor mice. There is no association between anti-VPAC1 treatment and stimulation of  
15 the **Thpo** pathway (Freson, *et al* 2008). Inhibition of VPAC1 interferes **with the**  
16 **generation and proliferation of megakaryocytes, i.e. the early stage of megakaryopoiesis**  
17 **as well as** the maturation and differentiation, i.e. the later stage of megakaryopoiesis,  
18 while **THPO** preferentially stimulates the megakaryocyte proliferation. As such, the anti-  
19 VPAC1 treatment and **THPO** may be complementary to each other. We here show that  
20 anti-VPAC1 treatment is effective in models of thrombocytopenia where **THPO** is  
21 effective as well, such as ITP and chemotherapy induced thrombocytopenia (Freson, *et al*  
22 2008, Kuter and Begley 2002). In addition, anti-VPAC1-treatment is effective in models  
23 of thrombocytopenia where **THPO** is not clinically effective, i.e. myeloablation induced  
24 thrombocytopenia (Freson, *et al* 2008, Kuter and Begley 2002) and GATA1 deficiency, a  
25 congenital thrombocytopenia. Thus, we show that anti-VPAC1 treatment results in broad  
26 spectrum thrombopoietic effects, broader than those by **THPO**.

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28 As such, this work illustrates the potential for a less obvious player of megakaryopoiesis  
29 to be targeted in the prevention of thrombocytopenia of different causes and this even in  
30 pathologies in which **THPO** is unsuccessful as treatment.

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10  
11 clinical-fundamental research mandate of the Fund for Scientific Research-Flanders  
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For Peer Review



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## Figure legends

### Figure 1: In vitro thrombopoietic effect of VPAC1 signaling inhibition.

Human buffy coat PB derived CD34<sup>+</sup> were cultured in StemSpan liquid medium supplemented with cytokines. From day 10 onwards large proplatelet extrusion were visible using a light microscope. (A) Light microscopy on day 13 shows the presence of large proplatelet extrusions arising from the megakaryocytes. This confirms the complete maturation of the CD34<sup>+</sup> cells into the megakaryocytic lineage. Magnification: 200x. (B) EM analysis of the cultures reveals fully matured megakaryocytes. Demarcation membranes are fully developed as shown by arrows in this picture. Alpha- and dense granules are indicated by triangles. (C) Release of platelets from these megakaryocytes was confirmed by EM.

Inhibiting VPAC1 signaling during megakaryopoiesis shows a thrombopoietic effect since more megakaryocytes were generated which also show a higher degree of maturation. (D) After 13 days of culture, more CD61<sup>+</sup> megakaryocytes were formed in the presence of 23A11 vs. 1C8. Panel D shows a representative FACS plot. (E) In addition, CD61<sup>+</sup> megakaryocytes showed more polyploid nuclei when the anti-VPAC1 Ab was added to the culture as seen in this representative FACS histogram. (F) After 13 days of culture, more highly polyploid CD61<sup>+</sup> megakaryocytes were generated in the presence of the neutralizing anti-VPAC1 Ab 23A11 (black) vs. a control Ab 1C8 (white). This effect is here shown as the expansion factor of each n-ploid megakaryocyte population normalized to the initial amount of CD34<sup>+</sup> HSCs. Abs were added (100 µg/ml) on days 0, 6 and 10 of the CD34<sup>+</sup> cultures. Each point represents the mean expansion factor of CD61<sup>+</sup> megakaryocytes of each ploidy level plus or minus SEM of 3 experiments. \*\*P<0.01, \*\*\*P<0.001.

### Figure 2: Anti-VPAC1 stimulates megakaryopoiesis in a model of syngeneic BM transplantation.

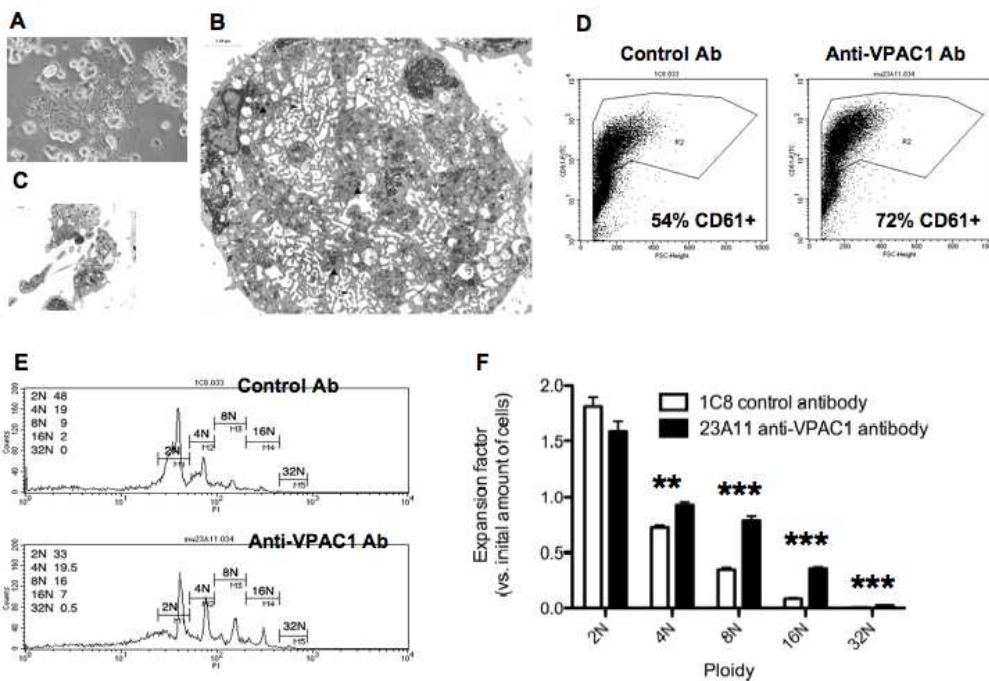
(A) Panel A shows the overall platelet recovery of all animals, i.e. D-R-, D+R-, D-R+ and D+R+ mice (n=7 for each group). Mice from whom BM was collected (donor mice, D mice) received either 23A11 (D+) or vehicle (D-) prior to collection. Recipient mice (R) respectively received 23A11 (R+) or vehicle (R-), starting 2 weeks prior to TBI for

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3 the entire length of the experiment. (B) platelet nadir at day 11 post transplantation.  
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5 D+R+ mice show an attenuated platelet nadir compared to D-R+ and D-R- mice. (C)  
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7 Initial platelet recovery at day 15 post transplantation. When donor mice are treated with  
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9 the anti-VPAC1 Ab, they show an increased platelet recovery. \*: P<0.05; \*\*: P<0.01. (D)  
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11 Red blood cell recovery, shown as hematocrit (HCT), as well as white blood cell  
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13 recovery (E) were unaffected by the anti-VPAC1 treatment.  
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16 **Figure 3: Anti-VPAC1 stimulates megakaryopoiesis in a murine model of ITP.**

17 Time represents hours after insertion of the osmotic pump filled with the anti-platelet Ab.  
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19 Full line represents platelet numbers of mice injected with the control Ab, dashed line  
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21 represents anti-VPAC1 Ab (n=15 for each group). Mice receiving the anti-VPAC1  
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23 treatment showed a less severe drop in platelets at day 3 and 4 and significant higher  
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25 platelet counts at day 8 and 10, when platelets count were already back to normal. \*:  
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27 P<0.05; \*\*: P<0.01.  
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Figure 1

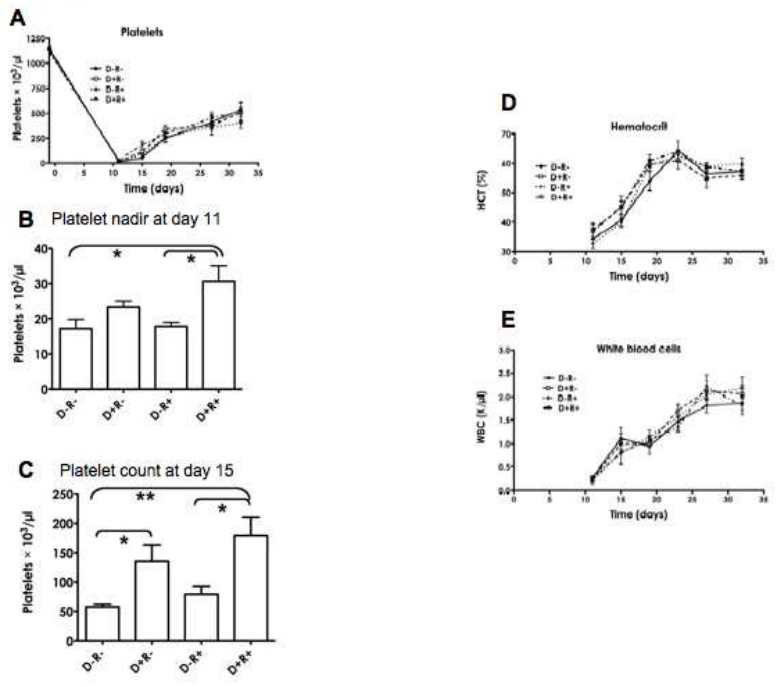


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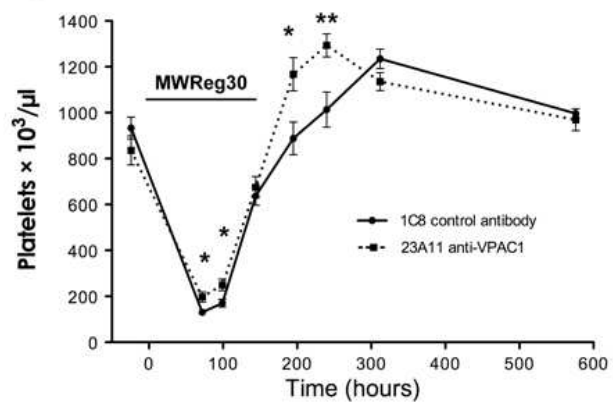
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Figure 2



Review

Figure 3



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