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Age-Associated Induction of Cell Membrane CD47 Limits Basal and Temperature-Induced Changes in Cutaneous Blood Flow

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Abstract

Objective—We tested the hypothesis that the matricellular protein thrombospondin-1 (TSP1), through binding to and activation of the cell receptor CD47, inhibits basal and thermal-mediated cutaneous blood flow.

Background Data—Abnormal and decreased cutaneous blood flow in response to temperature changes or vasoactive agents is a feature of cardiovascular disease and aging. The reasons for decreased cutaneous blood flow remain incompletely understood. Further, a role for matricellular proteins in the regulation skin blood flow has never been proposed.

Methods—C57BL/6 wild type, TSP1- and CD47-null 12 and 72 week old male mice underwent analysis of skin blood flow (SkBF) via laser Doppler in response to thermal stress and vasoactive challenge.

Results—Young and aged TSP1- and CD47-null mice displayed enhanced basal and thermal sensitive SkFB changes compared to age matched wild type controls. Nitric oxide-mediated increases in SkBF were also greater in null mice. TSP1 and CD47 were expressed in skin from young wild type mice, and both were significantly upregulated in aged animals. Tissue 3',5'-cyclic guanosine monophosphate (cGMP), a potent vasodilator, was greater in skin samples from null mice compared to wild type regardless of age. Finally, treating wild type animals with a CD47 monoclonal antibody, that inhibits TSP1 activation of CD47, enhanced SkBF in both young and aged animals.

Conclusions—The above results suggest that secreted TSP1, via its cognate receptor CD47, acutely modulates SkBF. These data further support therapeutically targeting CD47 to mitigate age-associated loss of SkBF and maximize wound healing.

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Introduction

Adequate skin blood flow (SkBF) is necessary for wound healing and to modulate core body temperature[1]. The processes that regulate cutaneous blood flow are complex and include input from the neural system[2]. Additionally local factors at the level of blood vessels and vascular cells contribute to regulate cutaneous flow[3,4]. Decreased or abnormal SkBF has been demonstrated in patients with diabetes, peripheral vascular disease[5], scleroderma[6], thromboangiitis obliterans (Buerger's disease)[4], Raynaud's phenomenon[7] and in the elderly[8,9]. Among these patient groups, abnormal and decreased SkBF is a major contributor to the pathogenesis and chronicity of soft tissue wounds, though the reasons for altered SkBF remain incompletely defined. Conversely, the goal of enhancing SkBF to increase wound healing has met with limited experimental and clinical success[10,11].

The biogas nitric oxide (NO) participates in the regulation of SkBF[12,13] and wound healing[14,15]. Loss of NO bioavailability and sensitivity contributes to abnormal SkBF[16–18], whereas therapeutic enhancement of NO signaling increases SkBF in pre-clinical models[19–21]. Studies in human subjects also suggest a role for NO in the regulation of SkBF[22], with local thermally-induced cutaneous vasodilation mediated substantially through NO signaling[23]. Loss of cutaneous NO and decreased cutaneous vasodilatory response[24] are associated with decreased healing capacity in the elderly[25,26]. Yet it is not known what factors account for the age-associated loss of cutaneous NO signaling.

The secreted matricellular protein thrombospondin-1 (TSP1) is unregulated in several disease states that are associated with loss of SkBF and impaired wound healing including diabetes, scleroderma[27] and systemic sclerosis[28], and has recently been postulated to account for the loss of cutaneous blood flow in these individuals[29]. In pre-clinical models of cutaneous wound healing TSP1 antisense oligomers delayed wound healing[30] and overexpression of TSP1 in the skin of mice greatly slowed wound closure and wound-associated angiogenesis[31]. We have reported that TSP1-null and CD47-null mice demonstrated enhanced ischemic wound healing in aged animals compared to wild type controls[21]. Herein then we tested the hypothesis that temperature- and age-associated changes in sKBF are limited by TSP1-activation of the cell receptor CD47. TSP1- and CD47-null mice displayed enhanced basal cutaneous blood flow and a greater dynamic response in flow to both core temperature changes and pharmacologic activation of the NO pathway compared to wild type controls at any age. These findings were associated with enhanced levels of the NO second messenger 3',5'-cyclic guanosine monophosphate (cGMP) in skin from null mice regardless of age. In wild type murine skin TSP1 and CD47 expression increased with age and were paralleled by a concurrent drop in NO signaling and SkBF. Finally, blocking CD47 activation in wild type mice with an antibody that prevents TSP1 binding to CD47 increased cutaneous flow in young and aged mice. Together these data suggest (1) that induction of the TSP1-CD47 signaling axis may account, in part, for age-associated decreases in cutaneous NO signaling and SkBF, and (2) that CD47 targeting enhances skin blood flow and may promote wound healing.

Materials and Methods

Animals

C57BL/6 male wild type, TSP1-null and CD47-null mice (Jackson Lab stock numbers 000664, 006141 and 003173 respectively) were maintained in a pathogen-free environment with ad libitum access to standard rat chow and water. Animal ages at the time of use were 12 or 72 weeks as indicated. Care and handling of animals was in accordance with the Institutional Animal Care and Use Committees of the National Institutes of Health and of the University of Pittsburgh School of Medicine. Hair from the dorsum of the animals was

removed with electric sheers followed by a depilatory lotion (Nair®) prior to laser Doppler flow measurements.

Laser Doppler analysis of skin blood flow

Core temperature was monitored via rectal probe and maintained at 37.5°C by a heating pad and warming lamp. In experiments were core temperature was altered the heating lamp and pad were adjusted to raise or lower core temperature by 0.5°C intervals. Animals were acclimated at new core temperatures for 15m prior to laser Doppler analysis. Anesthesia was obtained with isoflurane (1.5% wild type and TSP1 null mice; 1.2% CD47 null mice) with a 50:50 ration of oxygen to room air via nose cone inhalation. (See below for an explanation concerning the variation in anesthesia dosing between mouse strains.) A MoorLDλ 1–2 laser Doppler scanner (Moor Instruments, Devon, England) was used to acquire real time cutaneous blood flow data with the following parameters: scan area, 1.6× 2.5 cm; scan speed, 4 ms per pixel; scan time, 1 minute 54 sec; override distance, 25 cm. The override distance was 20 cm.

Blood pressure measurement

We have published that CD47-null mice display decreased mean arterial blood pressure (MAP) at rest compared to wild type and TSP1-null animals[32]. For this reason, we placed femoral arterial catheters (Millar Mikro-Tip pressure Catheter) in age matched wild type, TSP1- and CD47-null mice and monitored MAP under inhalation isoflurane anesthesia. Though MAP at a concentration of 1.5% isoflurane trended slightly lower in CD47-null mice, decreasing the concentration of anesthetic to 1.2% brought MAP pressure in CD47-null animals to within 3–5 mm Hg of values recorded in wild type and TSP1-null animals.

Vasoactive challenge experiments

Baseline SkBF data was obtained in animals (n = 6 per strain/treatment group). Animals then received a vasoactive challenge with the primary NO donor (DEA-NO, 100 nmol/g body weight in 100 μl pre-warmed normal saline via rectal installation) or the endothelial nitric oxide synthase (eNOS) activator acetylcholine (ACh, 0.08 μg/gram weight in 100 μl pre-warmed normal saline via i.p. injection) and 60s later cutaneous blood flow was measured via laser Doppler.

Therapeutic blockade of TSP1-CD47 signaling

Age matched male C57BL/6 wild type mice were randomized into one of four groups: no treatment, vehicle (sterile phosphate-buffered saline), CD47 blocking antibody (clone 301), or isotype matched control antibody. Treatments were administered via intraperitoneal injection. Blood flow analysis was performed via laser Doppler 3h later. Antibody dose (0.4 μg/g weight i.p. in 100 μL sterile phosphate-buffered saline) was based on our prior published data showing therapeutic efficacy[33].

Protein expression by western blot

Sections of dorsal skin were homogenized in ice-cold lysis buffer containing NP-40, protease inhibitor cocktail (Sigma), sodium fluoride, sodium orthovanadate and PhosStop (Roche), centrifuged at 12,000 rpm for 20 min at 4°C, supernatants collected and lysates stored at −20°C. Protein was quantified using a Bradford assay (BioRad). Thirty micrograms (30 μg) of total protein was boiled, resolved by SDS-page and transferred onto nitrocellulose (BioRad). In blots for CD47, non-reducing Laemmli buffer was used with 8% SDS-PAGE as previously published[34]. Blots were probed with primary antibody to the respective proteins and were visualized after 1 h incubation in secondary antibody on an Odyssey Imaging System (Licor). The following antibodies were employed - mouse anti-

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thrombospondin-1 (Abcam, 1:500 dilution, Cat.No. ab1823); goat anti-CD47 C-18 (Santa Cruz, 1:500 dilution) and rabbit anti-β-actin (Cell Signaling, dilution 1:5000, Cat. No. 4967). The intensity of the bands was quantified using the Odyssey software or Image J (rsbweb.nih.gov/ij/).

Determination of mRNA transcript

TSP1 and CD47 mRNA levels in skin samples from 12 and 72 week old C57BL/6 wild type mice were determined by qtPCR. Specific Taqman primers and probes for HPRT1 (Mm_01545399_m1), CD47 (Mm_00495005_m1) and TSP1 (Mm_01335418_m1) were obtained from Applied Biosystems (Carlsbad, CA). Total RNA was extracted using Qiagen RNeasy® Mini Kits (Qiagen, Hilden, Germany) and Proteinase K digestion as per the manufacturer instructions. RNA was quantified using the Take3 Gen5 spectrophotometer (BioTek, Winooski, VT). One microgram (1 μg) of RNA was treated with DNase I (amplification grade, Invitrogen, Grand Island, NY) and then reverse-transcribed using the Superscript III First Strand Synthesis Supermix (Invitrogen). cDNA was amplified using Platinum® Quantitative PCR SuperMix-UDG (Invitrogen) in 20μl volumes in triplicate with gene specific primers and probe on the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), according to manufacturer instructions. Thermal cycling conditions were 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed using the ΔΔCt method with expression normalized to the housekeeping gene.

Determination of tissue cGMP

Skin cGMP was measured as we have published[35] with slight modification. Briefly, skin biopsies of equal wet weight were excised, flash frozen in liquid nitrogen and pulverized in a mortar and pestle. Homogenates of the pulverized tissue were prepared lysis buffer chilled to 4°C, centrifuged at 4°C and supernatants used for analysis via immunoassay (Amersham, GE Healthcare) as per the manufacture instructions.

Statistics

Significance was calculated with Student's *t* test and 1-way or 2-way ANOVA as appropriate, with a Bonferroni post test using a soft ware package (GraphPad Prism 5, La Jolla, CA) with $p < 0.05$ taken as significant.

Results

Cutaneous TSP1 and CD47 expression increases with age

In the absence of injury or disease TSP1 expression is minimal[29,36]. However, with acute injury TSP1 expression increases rapidly[37]. In pre-clinical models age-associated induction of TSP1 has been described in the kidney[38] and heart[39]. To date little is know about CD47 expression in health or disease. Analysis of cutaneous biopsies from wild type male mice at 12 weeks demonstrated expression of both TSP1 and CD47 protein and mRNA (Figs. 1A–C). In cutaneous samples from aged 72 week old mice both TSP1 and CD47 protein and CD47 transcript increased significantly (Figs. 1A–C). Interestingly though in aged skin TSP1 mRNA was significantly decreased. These data provide the first evidence of aged-associated induction of tissue CD47.

TSP1 and CD47 limit basal cutaneous blood flow

We have published that TSP1 and CD47 are hypertensive and support blood pressure[32,40]. To control for this in the present work we measured blood pressure in age matched wild type, TSP1- and CD47-null mice and adjusted the concentration of inhalation anesthesia

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accordingly to achieve parity in MAP between strains (see Methods Section). We now show in 12 week old mice, at a constant core temperature of 37.5 °C, that SkBF is significantly greater in both TSP1- and CD47-null mice compared to wild type C57BL/6 controls (Fig. 2A). These findings are important in light of our recent report that physiologically relevant TSP1 signaling occurs through binding to and activation of cell receptor CD47[41].

Temperature-induced changes in blood flow are limited by TSP1 and CD47

Thermal stress alters SkBF and this process is both directly and indirectly mediated via NO[42,43]. We reported that blood vessels from TSP1 and CD47 null mice demonstrate enhanced vasodilatation in *ex vivo* myography bioassays compared to wild type controls[40]. Considering these findings, we tested the hypothesis that SkBF changes to thermal stress are limited by TSP1 and CD47. Beginning with animals at a core temperature of 34°C, we increased core body temperature by 0.5°C to a maximum of 38°C and measured cutaneous blood flow after each thermal adjustment. Interestingly, both TSP1- and CD47-null mice showed increased SkBF under basal conditions that persisted throughout the period of controlled elevation of core body temperature as compared to wild type (Fig. 2B). Likewise, TSP1- and CD47-null mice also showed maintenance of greater cutaneous perfusion during periods of controlled cooling (Fig. 2C). Null mice did experienced a drop in SkBF with cooling, however at the lowest core temperature achieved TSP1- and CD47-null mice still demonstrated approximately 25% greater SkBF compared to wild type animals, suggesting a possible primary deficiency in reflex protection of core temperature in null animals.

Vasoactive alterations in SkBF are regulated by TSP1 and CD47

We treated mice with the primary NO-donor diethylamine NONOate (DEA-NO, t½=30s), to assess endothelial-independent effects on SkBF, and acetylcholine (ACh) (a physiologic activator of endothelial nitric oxide synthase (eNOS) that has been reported to alter SkBF[44]) to test endothelial-dependent blood flow effects. Consistent with enhanced basal cutaneous blood flow, SkBF in TSP1- and CD47-null animals was greater following treatment with DEA-NO (Fig. 3A) and also greater following treatment with ACh in TSP1-null animals (Fig. 3B) compared to controls. To further assess the role of NO in the above results we treated mice with the eNOS inhibitor L-nitro-L-arginine methyl ester (L-NAME). Basal wild type and TSP1- and CD47-null SkBF normalized following L-NAME treatment (data not shown).

Age-associated alterations in SkBF are less prominent in TSP1- and CD47-null mice

Loss of cutaneous blood flow, associated tissue necrosis and poor wound healing are known consequences of age and certain vasculopathies[27,28,45]. We have reported that skeletal muscle blood flow is maintained in old TSP1- and CD47-null mice compared to old wild type animals[21]. These findings suggested that TSP1-CD47 signaling may also limit SkBF in aged animals. We tested this hypothesis in 72 week old wild type and null mice. Both basal and thermal associated changes in SkBF were greater in 72 week old TSP1- and CD47-null mice compared to wild type controls, though statistical significance was only obtained in CD47-null mice (Fig. 4A).

Tissue cGMP is elevated in young and old TSP1- and CD47-null skin samples

Nitric oxide activation of soluble guanylyl cyclase (sGC) results in rapid production of cGMP and subsequent vasodilation[46,47]. Analysis of tissue cGMP levels in freshly harvested skin biopsies demonstrated increased cGMP in samples from 12 week old TSP1- and CD47-null mice compared to wild type (Fig. 4B). Surprisingly, analysis of cGMP levels in skin from 72 week old TSP1- and CD47-null mice confirmed persistent elevation of cutaneous cGMP (Fig. 4C). In contrast cutaneous cGMP levels decreased with age in wild type mice

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(Fig. 4C). These findings demonstrate persistence of NO signaling in null animals independent of the aging process and suggest age-associated loss of NO signaling may be secondary, in part, to induction of activated CD47 axis.

Interruption of CD47 activation enhances SkBF

Loss of adequate skin blood flow contributes to delayed healing and wound chronicity in the elderly[8,48,49]. We have identified several therapeutic agents that effectively block the TSP1-CD47 signaling axis through preventing TSP1 binding to and activation of CD47[33,35,50]. We tested the potential for one of these therapeutic agents, by blocking TSP1-CD47 signaling, to enhance SkBF. A CD47 monoclonal antibody (clone 301) was given to 12 week old wild type mice prior to laser Doppler assessment of SkBF. Interestingly, wild type animals demonstrated enhanced SkBF under basal conditions (Fig. 5A) compared to animals treated with an isotype IgG2α control antibody. Importantly, treating aged 72 week old wild type mice with the CD47 blocking antibody also increased SkBF (Fig. 5B).

Discussion

To our knowledge, this is the first report to demonstrate in pre-clinical studies that matricellular TSP1 is an immediate regulator of cutaneous blood flow via its activation of CD47 signaling. SkBF was greater under basal conditions in TSP1- and CD47-null animals and in response to both vasoactive and thermal challenge, suggesting a global upregulation in flow in the absence of activated CD47. Aged null animals lacking activated CD47 demonstrated more robust SkBF than wild type controls and minimal change in SkBF compared to young CD47 null animals, suggesting a primary role for activated CD47 in age-associated deficiencies in SkBF.

Autonomic pathways are known to control SkBF and we have reported that TSP1-null mice display greater shifts in blood flow after post-ganglionic autonomic blockade with hexamethonium chloride[32]. It is possible that differences in autonomic signaling may play a role SKBF flux measured in null mice and future studies will addresses this.

The acute effects of TSP1 on SkBF identified here are distinct from its previously reported chronic effects on tissue perfusion via its anti-angiogenic activity. Cutaneous TSP1 limits ultraviolet light-mediated angiogenesis in part through limiting VEGF signaling to decrease cutaneous vascularity[51]. We have shown activated CD47 inhibits both NO- and VEGF-driven angiogenesis in endothelial cells[34,52]. Also antibody blockade of TSP1[35] or gene suppression of CD47[53] can increase cutaneous vascularity and subsequent blood flow. However, we have reported comparable vascular density under basal conditions in skin in 12 week old wild type and null mice, though in wound and ischemic models TSP1- and CD47-null cutaneous tissue units show enhanced vascularity at 3 and 7 day intervals[53,54]. Nonetheless young TSP1-and CD47-null mice have more basal cutaneous blood flow and show persistence of this advantage in SkBF after core temperature changes or NO pathway activation supporting a role for activated CD47 in the acute regulation of SkBF. However, it is not clear why SkBF is not more responsive to changes in core temperature alterations in TSP1-and CD47-null mice. Particularly unexpected was our finding that at a core temperature of 32°C TSP1- and CD47-null mice still maintained significantly increased SkBF compared to wild type. Such a result predicts a difficulty in null mice in maintaining core temperature in response to environmental challenge. Though the present work can not address this, these data are consistent with previous findings by our group of limited homeostasis in TSP1- and CD47-null mice to several stresses including anesthetic agents, vasodilators and sympathetic tone blockade[32].

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Interestingly, cutaneous TSP1 and CD47 levels increase with age. It is not clear if age-associated induction represents altered production, degradation or stability of these proteins. Given the identified role of TSP1 to inhibit angiogenesis, the decreased SkBF in older wild type mice may in part be secondary to decreased cutaneous vascular density. However, our data reveal that in aged animals activated CD47 also limits SkBF through an acute regulatory mechanism. Findings of increased expression of ligand TSP1 and cell receptor CD47 in aged skin are in agreement with our recent report of concurrent upregulation of both TSP1 and CD47 protein and mRNA in human and pre-clinical models of pulmonary arterial hypertension [55], suggesting linked gene regulation of these proteins.

Blockade of CD47 activation with a monoclonal antibody recapitulated null levels of cutaneous SkBF in wild type animals. This therapeutic advantage was enjoyed by antibody treated young and aged wild type mice. Increased cutaneous blood flow was detected shortly after a single injection of antibody. Hence, the enhanced SkBF experienced by treated wild type animals represents an acute response from existing cutaneous vascular networks rather than induction of angiogenesis. In addition, the activity of CD47 to inhibit both NO-[56] and VEGF [34]- mediated angiogenesis predicts that therapeutic targeting of CD47 will result in beneficial effects at the level of acute increases in blood flow and through increased angiogenesis. Thus, these data predict that drugs targeting CD47 will be multiply beneficial in enhancing blood flow and wound healing in the elderly.

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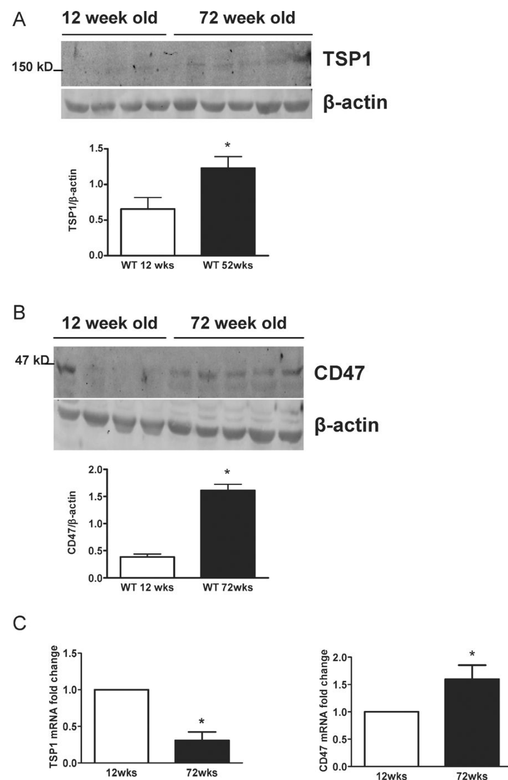

Figure 1. Cutaneous TSP1 and CD47 protein and mRNA are increased with age

Biopsies of skin from the dorsum of 12 and 72 week old male C57BL/6 mice were collected, tissue lysates prepared, protein separated by SDS-PAGE and Western blotted for TSP1 and CD47 (**A**). Densitometry represents the mean ± SD of blots prepared from distinct tissue samples from individual mice (n = 4 12 week old mice and n = 5 72 week old mice). * = p < 0.05 compared to 12 week old animals. (**B**). q-PCR analysis of TSP1 and CD47 mRNA (**C**). Results normalized to HPRT are the mean ± SD of material prepared from animal cohorts described in A. * = p < 0.05 compared to 12 week old animals.

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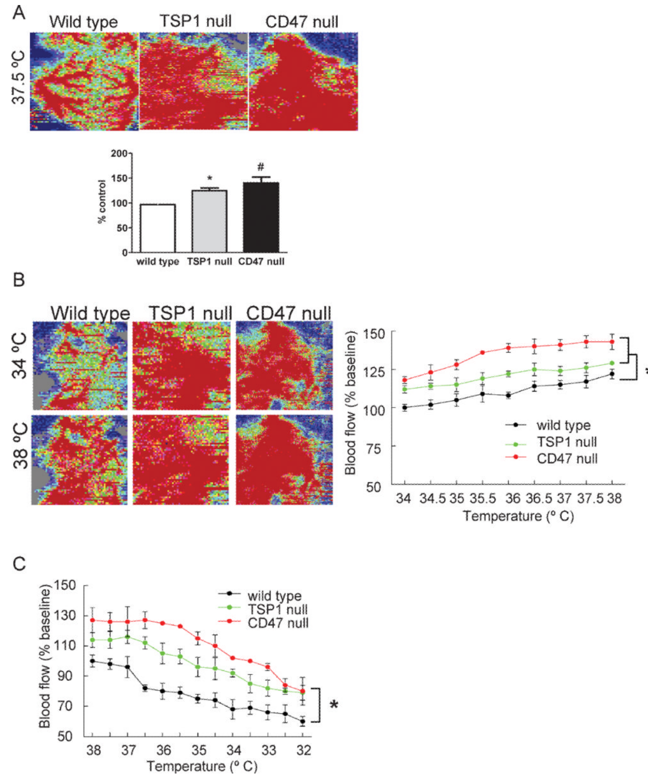

Figure 2. Basal and thermal-mediated SkBF is increased in the absence of TSP1 and CD47

Under general anesthesia 12 week old male wild type, TSP1- and CD47-null mice underwent SkBF analysis with core temperature maintained at 37°C (**A**). Typical images of real time laser Doppler analysis are presented for wild type, TSP1- and CD47-null mice. Results represent the mean ± SD of 6 animals of each strain. * and # = $p < 0.05$ compared to wild type. Under general anesthesia 12 week old male C57BL/6 wild type, TSP1- and CD47-null mice under went SkBF analysis with laser Doppler beginning at 37°C, followed by controlled elevation of core temperature by 0.5°C with SkBF determined at each new core temperature, proceeding to a maximum core temperature of 38°C (**B**). Typical images of real time laser Doppler analysis are presented for wild type, TSP1- and CD47-null mice at the indicated core temperatures. Results represent the mean ± SD of 6 mice of each strain. * = $p < 0.05$ compared to wild type. Under general anesthesia 12 week old male C57BL/6 wild type, TSP1- and CD47-null mice under went SkBF analysis with laser Doppler beginning at 38°C, followed by controlled lowering of core temperature by 0.5°C with SkBF determined at each new core temperature, proceeding to a core temperature of 32°C (**C**). Results represent the mean ± SD of 6 mice of each strain. * = $p < 0.05$ compared to wild type.

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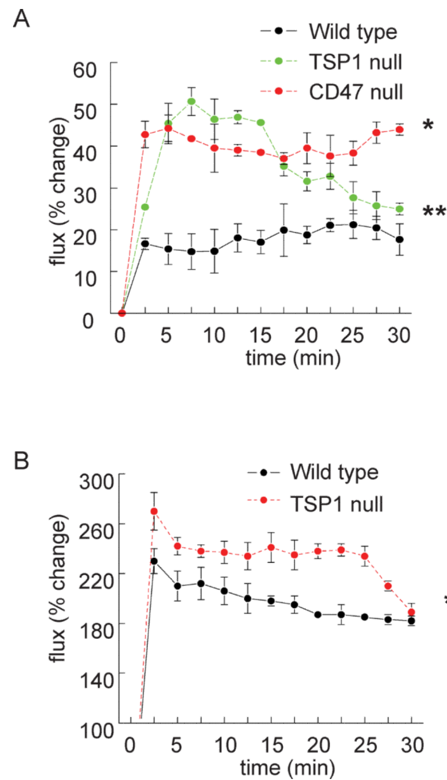

Figure 3. NO-mediated effects on SkBF are limited by TSP1 and CD47

Under general anesthesia 12 week old male C57BL/6 wild type, TSP1- and CD47-null mice underwent SkBF analysis with laser Doppler with core temperature maintained constant at 37°C. After a 30 minute stabilization interval basal SkBF was determined, and animals were challenged with either vehicle (normal saline) or the primary NO donor DEA-NO (100 nmol/g body weight via rectal installation) (**A**), or the eNOS activator acetylcholine (ACh, 0.08 μg/gram weight via intravenous injection; wild type and TSP1-null mice) (**B**) and SkBF determined. Results are the mean ± SD of 6 animals of each strain. * and ** = $p < 0.05$ compared to wild type.

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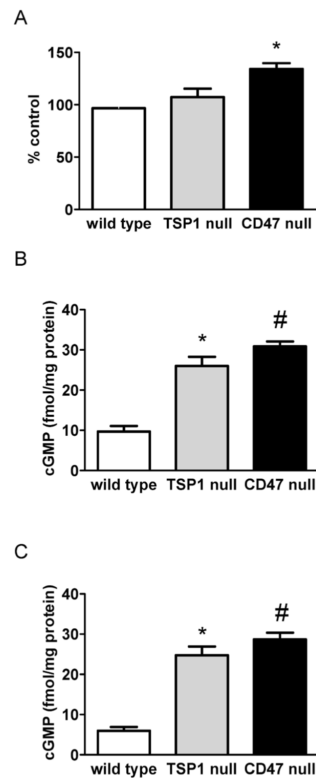

Figure 4. Aged TSP1- and CD47-null mice maintain SkBF and NO signaling

Under general anesthesia 72 week old male wild type, TSP1- and CD47-null mice with core temperature maintained at 37°C underwent SkBF determination via laser Doppler. Results are the mean ± SD of 6 animals of each strain. * = $p < 0.05$ compared to wild type. Dorsal cutaneous skin samples from 12 week (**B**) or 72 week (**C**) old male wild type, TSP1- and CD47-null mice were harvested, snap frozen in liquid nitrogen, pulverized, homogenized in lysis buffer and assayed for tissue cGMP as via ELISA (Amersham, GE Healthcare). Results presented are the mean ± SD of 4 animals of each strain. * and # = $p < 0.05$ compared to wild type.

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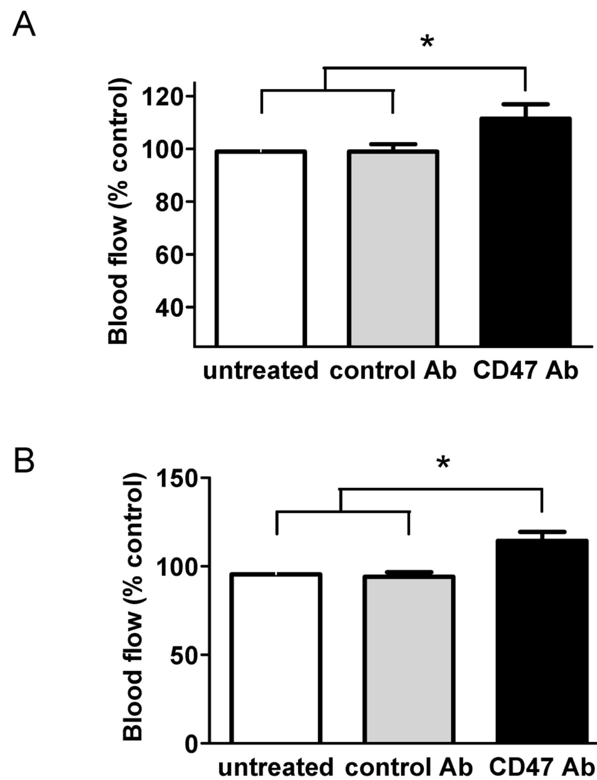

Figure 5. Therapeutic blockade of CD47 activation increases SkBF

12 week (**A**) or 72 week (**B**) old male wild type C57BL/6 mice were treated with a CD47 blocking antibody (clone 301, 40 μg delivered as 10 μL of a 4 mg/mL stock in 100 μL of PBS in injected in the skin) or an isotype IgG2α control antibody as we had previously published[53]. Three hours later animals underwent general anesthesia with their core temperature maintained at 37°C for 30 minutes prior to assessment of SkBF via laser Doppler. Results presented are the mean ± SD of 4 animals in each treatment group. * = $p < 0.05$ compared to isotype control antibody treated and untreated.