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Supplementary Materials for

Coincident signals from GPCRs and receptor tyrosine kinases are uniquely transduced by PI3Kβ in myeloid cells

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Methods

Generation of p110β Gβγ-insensitive knock-in mice

The *Pik3cb*^{KK526,527DD} targeting construct was generated through recombineering methodology, as follows. First, 200 ng of high-purity BAC DNA of C57BL/6J origin [clone number RP23-13L19 (Geneservice)] encompassing the entire *Pik3Ccb*locus was transferred into recombination-competent EL350 bacteria by electroporation. Recombinants were selected with chloramphenicol conferred by the presence of the BAC DNA. To retrieve the portion of DNA for gene targeting, we cloned 500-bp 5' and 3' mini-homology arms in pBluescript SK (+) (pBS-SK+). Both mini-homology arms were amplified from BAC DNA as a template, with the 5' homology arm flanked by the restriction sites Kpn I and Eco RI, and the 3' arm flanked by Eco RI and Bam HI. The resulting pBS-SK+ construct was linearized with Eco RI before it was used to electroporate EL350 bacteria carrying the BAC DNA. A total of 200 ng of DNA was used for the electroporation. Recombinants carrying the retrieved, gap-repaired *Pik3cb* portion in pBS-SK+ were selected with ampicillin conferred by the presence of the pBS-SK+ backbone. Correct recombination was confirmed by restriction digestion analysis of the isolated plasmid. The mini-targeting vector PL452 tACE-Cre PGK EM7 Neo^{R} was constructed by insertion of the tACE-Cre module from pACN [obtained from A. Plagge, Babraham Institute, (*66*)] into the Nhe I site of PL452 (*70*). In the PL452 plasmid, the *neo* gene is expressed both from the prokaryotic *em7* promoter and the eukaryotic *pgk1* promoter. Thus, kanamycin can be used to select for recombinants containing the NeoR cassette, whereas electroporated embryonic stem (ES) cell clones can be selected for resistance to neomycin. In the PL452 tACE-Cre PGK EM7 NeoR construct, the tACE-Cre PGK EM7 NeoR module (hereafter referred to as the Neo^{R} cassette) is flanked by loxP sites. The tACE promoter drives expression of the Cre recombinase in the testes, which enables deletion of the Neo^{R} cassette in the male germline. To enable insertion of the Neo^{R} cassette into the retrieved *Pik3cb* fragment, we inserted 5' and 3' mini-homology arms into the Kpn I/Sal I and Not I/Sac II restriction sites flanking the Neo^{R} cassette of the mini-targeting vector on the 5' and 3' ends, respectively. The 520-bp 5' mini-homology arm was amplified from BAC DNA as a template. The 3' mini-homology arm carrying the KK526,527DD mutation in exon 3 of the *Pik3cb* gene was synthesized by DNA 2.0. We used the codons CAG CAG to encode aspartic acid residues, resulting in the introduction of a Bcg I restriction site. We used this restriction site to track the presence of the KK526,527DD mutation. To generate the *Pik3cb*^{KK526,527DD} final targeting construct, we digested the *Pik3cb*^{KK526,527DD} mini-targeting construct with Kpn I and Sac II, resulting in 5.2- and 2.9-kb fragments. The 5.2-kb fragment, encompassing the NeoR cassette flanked by the 5' and 3' mini-homology arms, was used to electroporate EL350 bacteria carrying the plasmid containing the retrieved, gap-repaired *Pik3cb*. Recombinants were selected with kanamycin, and correct recombination was confirmed by restriction digestion analysis of the isolated plasmid. The final targeting construct was linearized with Kpn I-HF and used to electroporate Bruce4 C57BL/6 ES cells by the Gene Targeting Facility at The Babraham Institute. A total of 480 clones were screened for homologous recombination with a 5' Southern blot as the primary screen [the probe was 5' to the homology arm boundary; a Bgl II digest yielded a shift from 8.7 to 12.6 kb in the targeted locus (fig. S2A)]. Positive clones were subsequently screened by a 3' Southern blot [the probe was 3' to the homology arm boundary; a Bci VI digest yielded a shift from 9.1 to 7.2 kb in the targeted locus (fig. S2A)]. In parallel, positive clones were also screened for single insertion of the Neo^{R} cassette [the probe was internal to the NeoR cassette; an Nsi I digest yielded a 12.7-kb fragment and an EcoR V digest yielded an 11.5-kb fragment]. Two clones were carried forward for

injection into C57/Bl6Tyr$^{-/-}$ blastocysts by the Gene Targeting Facility at the MRC Laboratory of Molecular Biology. Chimeric males were bred with 129/Sv females to generate heterozygous animals that were on a mixed C57BL/6J, 129/Sv genetic background. Separate *Pik3cb*^{KK526,527DD} mouse colonies were generated from each of the two original targeted ES cell lines. Initial experiments were performed on both derived mouse lines. These revealed identical results, and so all further experiments were performed in a single generated line. Cre/loxP-mediated deletion of the Neo^{R} cassette was predicted to leave 140 bp of foreign DNA in the intron between exons 12 and 13. This was confirmed by PCR analysis in progeny derived from the two independent ES cell clones. A forward primer (5'-TGAGTTGGCTCAGGAGCGATAGTG-3') and a reverse primer (5'-GTACTAGGTTCACTAACGTGGCTCC-3') were used to amplify a WT 402-bp fragment and a mutant 542-bp fragment encompassing the sole remaining loxP site in intronic region 12 to 13. To confirm the presence of the KK526,527DD mutation, a forward primer (5'-CTCACTAGCCTGATCTACAGAGCAAG-3') and a reverse primer (5'-GTCCAGTCCTCCTGTCTCAGCCTT-3') were used to amplify a 730-bp fragment encompassing exon 13. Cleavage with Bcg I was predicted to leave the WT fragment intact and yield 384- and 347-bp fragments in the presence of the KK526,527DD mutation (fig. S2B).

Western blotting analysis

BMNs (1×10^6 cells) or BMDMs (20 μg protein) isolated from at least two pooled mice were solubilized in 1× SDS-loading buffer by probe sonication, and then heated at 95°C for 5 min. Samples were subjected to SDS-PAGE, transferred to PVDF membranes, and analyzed by Western blotting to detect p110β with a 1:1000 dilution of rabbit polyclonal antibody (Cellular Signalling, C33D4), in PBS/5% FAF-BSA/0.1% Tween, and HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, sc-2054). Signals were detected by ECL (GE Healthcare). The PVDF membranes were washed and analyzed for β-COP as a loading control with a monoclonal hybridoma (at a 1:200 dilution, a kind gift from N. Ktistakis, Babraham Institute). The intensities of bands were quantitated by AIDA 2D densitometry software (version 3.27), and the intensity of the band corresponding to p110β in each sample was normalized to that of the β-COP band from the same sample, which was used as a loading control.

Measurement of ROS production

WT BMNs were pretreated with either the BLT1 antagonist CP105-696 (0.3 μM; a kind gift from Pfizer), 40 nM TGX221, or DMSO (vehicle control), as indicated in the figure legends, for 10 min at 37°C in the presence of HRP:luminol prior to their addition to wells of a 96-well plate precoated with either immobilized immune complexes (IgG-BSA) or PolyRGD+ or containing 10 μM fMLP, 300 nM PMA, or IgG-opsonized SRBCs prepared as described earlier. The rate kinetics of ROS production was measured by chemiluminescence with a luminol-based assay in polystyrene 96-well plates (Berthold Technologies), as described earlier. Data output was in relative light units (RLUs) per second or total RLUs integrated over the indicated measured periods of time.

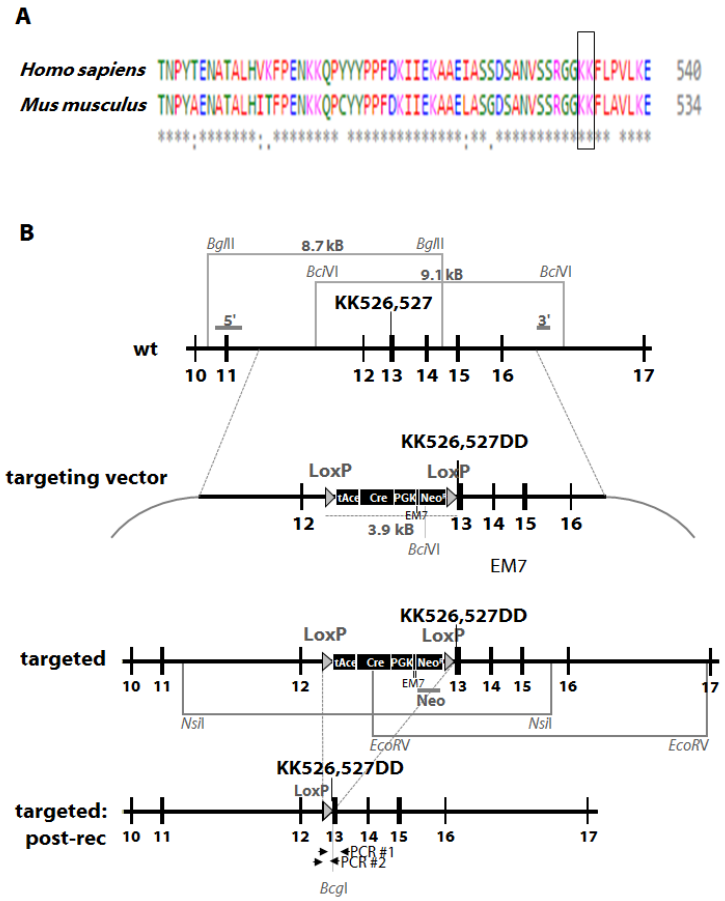

Fig. S1. Targeting of *Pik3cb*^{KK526,527DD}. (**A**) Color-coded amino acid sequence alignment of the N termini of human and mouse p110β, with the box highlighting homologous residues for mutation in the mouse sequence. (**B**) Schematic representation of the *Pik3cb*^{KK526,527DD} gene targeting vector and strategy (for full details see Methods). Top: The wild-type (WT) *Pik3Ccb*locus and targeting vector. The ends of the 5' and 3' homology arms are indicated by dashed gray lines. Restriction enzyme sites and resulting fragments for Southern blotting are demarcated by gray boxed regions. The 5', 3', and Neo Southern probes are labelled and indicated by a horizontal gray line. Bottom: The targeted allele before and after Cre-mediated recombination between the loxP sites is shown. We used a tAce-Cre-PGK-EM7-NeoR selection cassette, which is deleted after Cre-mediated recombination between loxP sites in the testes. Exons are represented by vertical black bars and are numbered. The position of the K526 and K527 residues in the WT allele and of the mutated KK526,527DD residues in the targeted allele are shown. Schematics are drawn to scale, except for the sizes of the loxP sites and genotyping PCR primers.

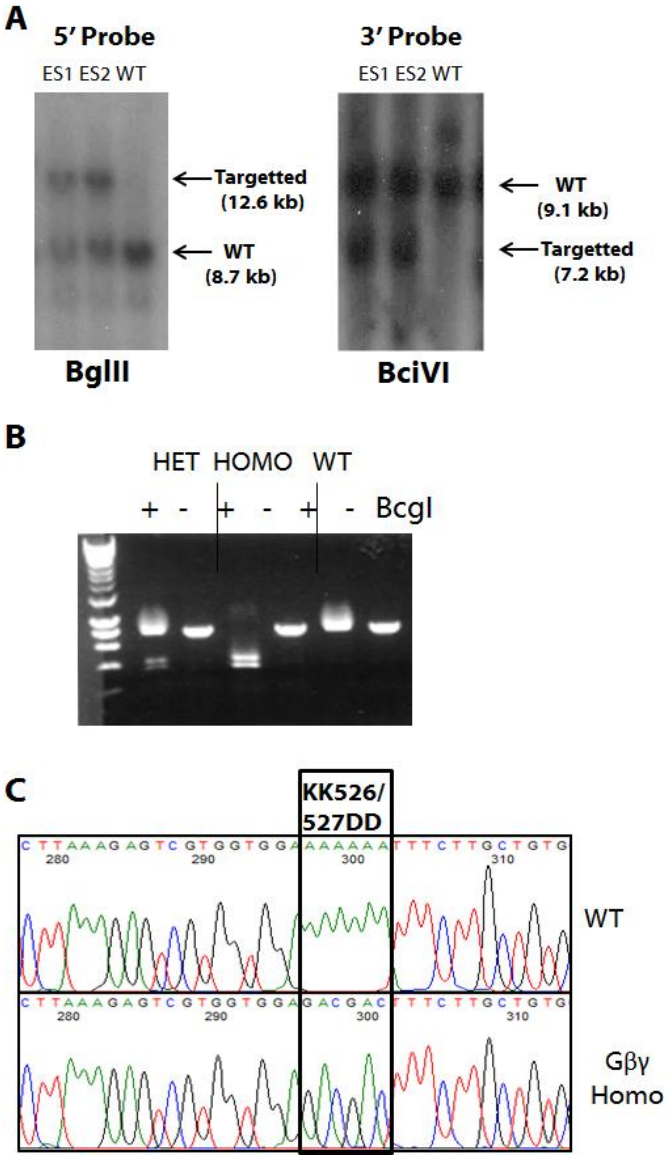

Fig. S2. Confirmation of the correct targeting of *Pik3cb*^{KK526,527DD}. (**A**) Correct targeting of *Pik3cb*^{KK526,527DD} was confirmed by Southern blotting analysis of ES cell DNA digested with either Bgl II or Bci VI and probed with [^{32}P]-5' and 3' oligonucleotide probes, respectively, as described in Methods. Shown are Southern blots of two correctly targeted (ES1 and ES2) and one WT ES cell genomic DNA. (**B**) The presence of the correct mutation was confirmed by PCR analysis and sequencing of genomic DNA isolated from generated mouse tissue. The cDNAs extracted from biopsies of *Pik3cb*$^{KK526,527DD/KK526,527DD}$ (HOMO), *Pik3cb*$^{KK526,527DD/+}$ (HET), and *Pik3cb*$^{+/+}$ (WT) mice were amplified by PCR with primers flanking exon 13 (see fig. S1B for primer positions) to generate a 730-bp fragment, which was cleaved to generate 384- and 347-bp fragments by restriction digestion with Bcg I, through a restriction site introduced by the KK526,527DD mutation, as described in Methods. (**C**) DNA from identified β-RBD homozygous and WT mice was isolated, amplified by PCR as described in (B), and sequenced using standard techniques. Shown is the sequence surrounding the mutation, with the KK526/527DD mutation sequence boxed.

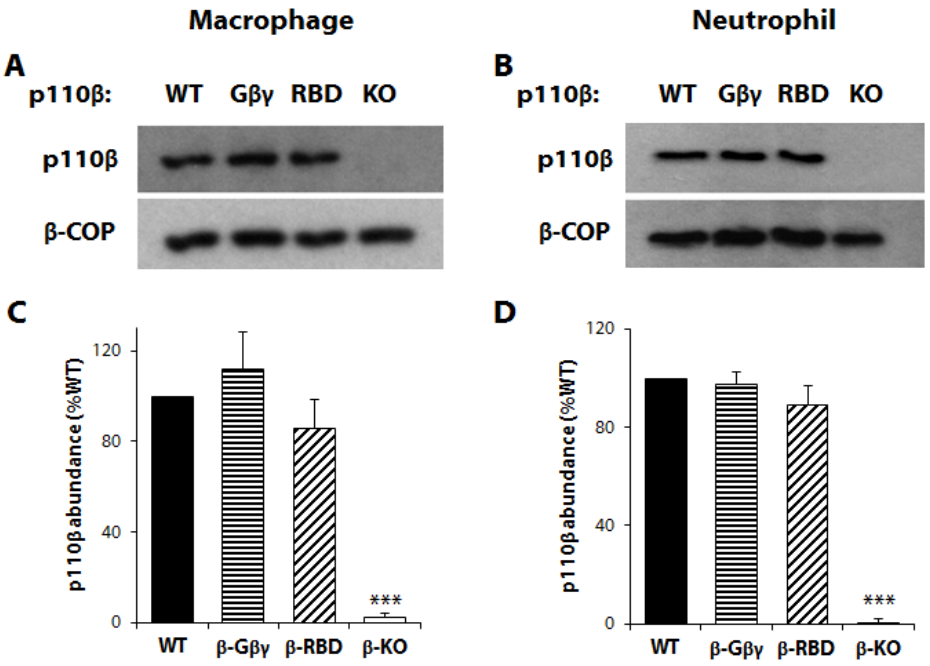

Fig. S3. p110β quantities in BMDMs and BMNs are unaffected by the introduction of the RBD- and Gβγ-insensitive mutations. (**A** to **D**) Analysis of the abundances of p110β and β-COP (loading control) proteins in BMDMs (A and C) and BMNs (B and D) generated from WT, p110β Gβγ-insensitive (Gβγ), p110β RBD-insensitive (RBD), and p110β knockout (KO) mice. Western blotting analysis was performed on BMDM lysates (20 μg) (A) or 1×10^6 BMNs (B) from mice of the indicated genotypes, with each sample prepared from two pooled mice. Western blots for each of the indicated cell types are representative of three independent experiments. (C and D) The abundance of p110β protein in samples from mice of each genotype was quantitated by densitometry with β-COP used as the loading control. Data are means ± SEM of three independent experiments and are expressed as a percentage of the p110β band density of WT samples on each blot normalized to its loading control. ***$P < 0.005$ by one-sample *t* test with Holm-Sidak correction for multiple comparisons on non-normalized data, with comparisons made to WT samples.

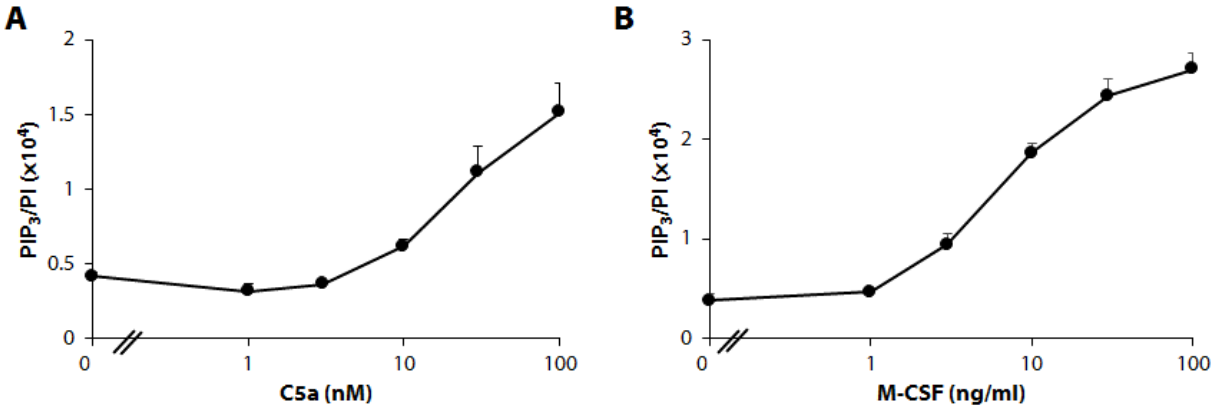

Fig. S4. Dose responses of GPCR (C5a) and RTK (M-CSF) agonists on PIP_3 production by BMDMs. (**A** and **B**) WT BMDMs (1.2×10^6) were starved overnight before being stimulated for 30 s with the indicated concentrations of C5a (A) or M-CSF (B). The reactions were quenched, and the lipids extracted and quantitated by MS as described in Materials and Methods. Data are means ± SEM of three independent experiments, each performed in duplicate, and are expressed as the ratio of the abundance of PIP_3 to that of PI (PIP_3/PI) to account for any variation in cell input.

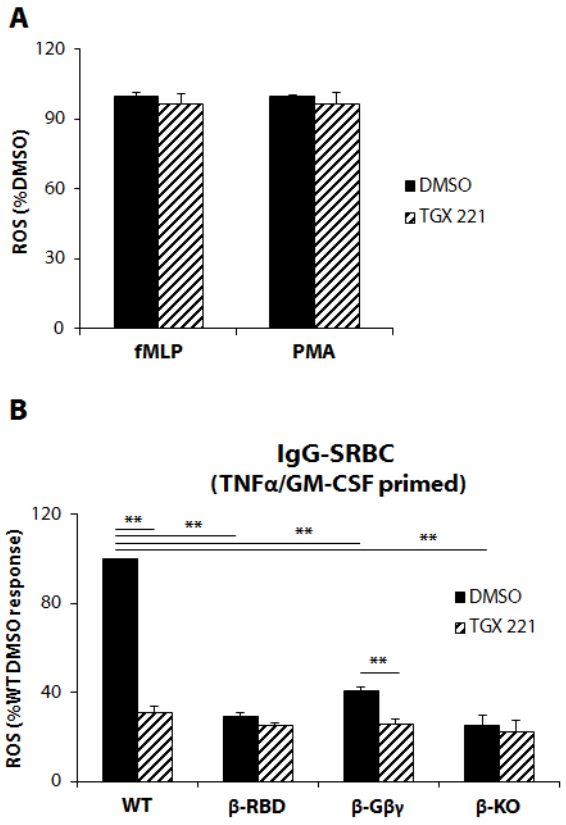

Fig. S5. ROS generation in BMNs in response to the phagocytosis of IgG-SRBCs, but not soluble GPCR agonists, requires p110β activity. (**A** and **B**) BMNs (0.5×10^6) from WT mice (A) or from WT, β-RBD, β-Gβγ, and β-KO mice (B) were preincubated with HRP/luminol in the presence of 40 nM TGX221 (hatched bars) or 0.05% DMSO (closed bars) before being added to a 96-well plate containing 10 μM fMLP or 300 nM PMA (A) or containing IgG-opsonized SRBCs (B). ROS responses were measured by chemiluminesence and at least duplicate measurements for each condition were performed. Data are means ± SEM of the accumulated light emission over 5 min (A, fMLP), 15 min (A, PMA), or 20 min (B) from a combination of at least three experiments, and are expressed as a percentage of the response in DMSO-treated BMNs. $^{**}P < 0.01$ by *t* test with Holm-Sidak correction for multiple comparisons.

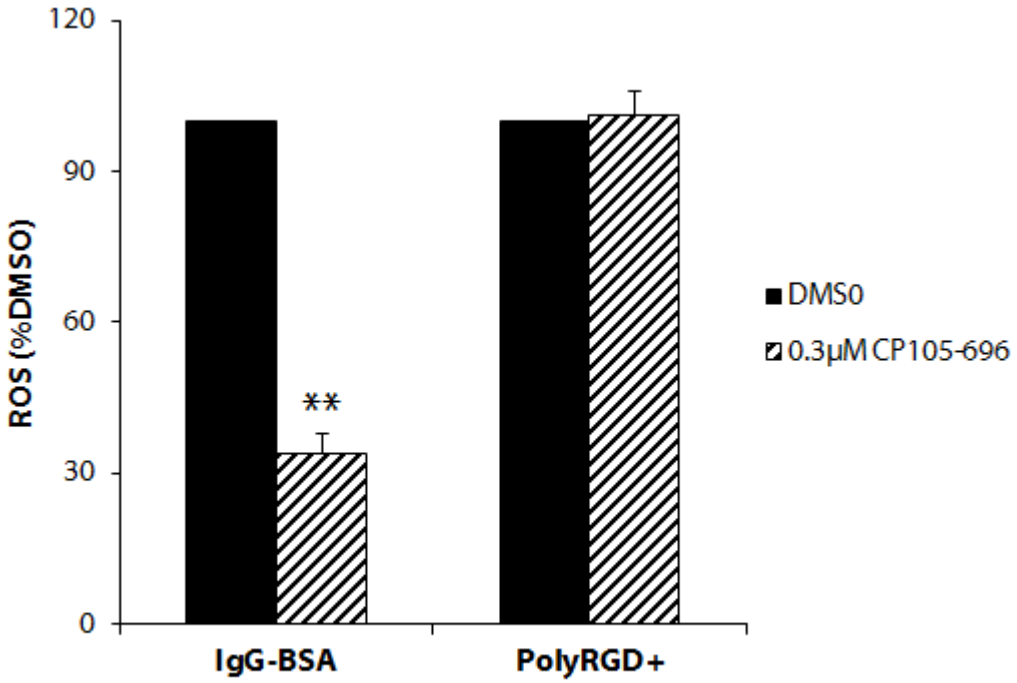

Fig. S6. ROS generation in BMNs in response to adhesion to poly-RGD+, but not immune complexes, is independent of BLT1 activation. BMNs (0.5×10^6) from WT mice were preincubated with HRP/luminol in the presence of the BLT1 antagonist CP105-696 (0.3 µM) or DMSO for 10 min at 37°C before being added to a 96-well plate precoated with IgG-BSA or polyRGD+. ROS responses were measured by chemiluminesence and at least duplicate measurements for each condition were performed. Data are means ± SEM of the accumulated light emission over 20 min from a combination of at least three experiments, and are expressed as a percentage of the response in vehicle-treated BMNs. $^{**}P < 0.01$ by paired *t* test with Holm-Sidak correction for multiple comparisons compared to DMSO controls (on non-normalized data).