Supplementary Data:

Zymosan activates protein kinase A via adenylyl cyclase VII to modulate innate immune responses during inflammation

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Figure S1. Construction and characterization of a BRET sensor for PKA activation, pm-AKARB (*A* *k*inase *a*ctivity *r*eported by *B*RET).

A. Design of the BRET sensors for PKA, AKARB and pm-AKARB. The FRET sensor for PKA, AKAR2, was converted to a BRET sensor by flanking the core of the sensor, FHA1 and its substrate, with YFP (citrine) and *Renilla* luciferase. The first 10 amino acids from Lyn kinase that contains the myristoylation and palmitoylation signals was added to AKARB to make the plasma membrane anchored sensor, pm-AKARB. The localization of this sensor to the plasma membrane was confirmed by expressing the sensor in HEK 293 cells and examining the expression pattern of YFP.

B. Comparison of the dynamic responses of the AKARB and pm-AKARB sensors. RAW 264.7 cells expressing AKARB or pm-AKARB were treated with 2 mM 8-bromo-cAMP at time 0 to maximally activate PKA and thus determine the maximal signal change of the sensors. Compared to the basal signal, addition of 8-bromo cAMP caused a 45% change in the BRET ration of the pm-AKARB sensor while the change in the AKARB sensor was less than 10%.

C. The pm-AKARB sensor is reversible. RAW 264.7 cells expressing pm-AKARB were treated with 50 nM isoproterenol (ISO) at time 0. Upon ligand addition, the BRET signal increased rapidly and robustly. Addition of propranolol (PRO), an antagonist of β-adrenergic receptors, at 120s, caused the BRET signal to return to the basal level within 2 to 3 minutes.

D. The pm-AKARB signal can be blocked by H-89. RAW 264.7 cells expressing pm-AKARB responded to stimulation of prostaglandin E_2 with a rise of BRET signal. If the cells were treated with 100 μM H-89, a PKA inhibitor, for 30 minutes prior to ligand addition, the response was abolished. The basal BRET signal also appeared to be reduced by 6%.

Figure S2. Generation of AC7 conditional knockout (cKO) mice

A. Design of the targeting vector for generation of conditional knockout allele of the Adcy7 gene. The pPGKneo.F2L2.DTA vector was used as the targeting vector backbone (38). Exon 6 of the Adcy7 gene was amplified by PCR and cloned into the XmaI site of the vector between the loxP and FRT site. About 4 kb of genomic sequence encompassing exon 2 to exon 5 was amplified by PCR and cloned into the NotI site as the 5' recombination arm. About 5.5 kb of genomic sequence encompassing exon 7 to exon 11 was amplified by PCR and cloned into SalI and EcoRV site as the 3' recombination arm. The final construct was verified by sequencing and restriction enzyme digestion.

B. Selection of ES cells for integration of the targeting vector at the *Adcy7* locus. The purified linearized targeting construct was delivered into mouse ES cells (SM-1) via electroporation at the transgenic core facility at UT Southwestern Medical Center. A PCR screen of ES cell clones was carried out for positive amplification with primer pairs 554/597 (5') and 598/573 (3') and for negative amplification with primer pair 19/595. Potential targeted ES cell clones were expanded and genomic DNAs were prepared from 6 clones. The purified genomic DNAs were digested with BamHI or EcoRI and hybridized with 5'- or 3'- probes on southern blot. The expected sizes of a correctly targeted allele are 10 kb and 11 kb for the 5'- and 3'- probes, respectively. Clones 2F8, 5A11, and 5F12 displayed banding pattern expected from correct targeting. Probes and hybridization of southern blots were performed using the DIG-high prime DNA labeling and detection kit from Roche according to manufacturer's protocol.

C. Detection of exon 6 deletion. After establishment of germline transmission, the mice were crossed to the FLPe strain (39), which expresses the FLP enzyme that acts on the FRT sites and leads to excision of PGKneo portion of the vector sequence. Deletion of PGKneo was verified by PCR. The mice were then crossed with C57BL/6J-LysMcre line to generate the conditional knockout of AC7 in the myeloid lineage. Deletion of exon 6 was detected in BMDMs by PCR using primer pair 613/703 in two strains, 2F8 and 5F12; while deletion of exon 6 was not detected in strain 5A11. Deletion of exon 6 was further confirmed by RT-PCR using primer pair 513/596. Multiple preparations of BMDMs and peritoneal macrophages (Pmac) showed a consistent absence of AC7 expression as determined by qPCR. qPCR was performed using the RT^2 SYBR Green master mix (SABiosciences) and Biorad CFX real-time PCR machine. During further backcrosses the wild type and mutant AC7 alleles were genotyped using primers 675/765 (wt, 280bp) and 765/795(mutant, 550bp).

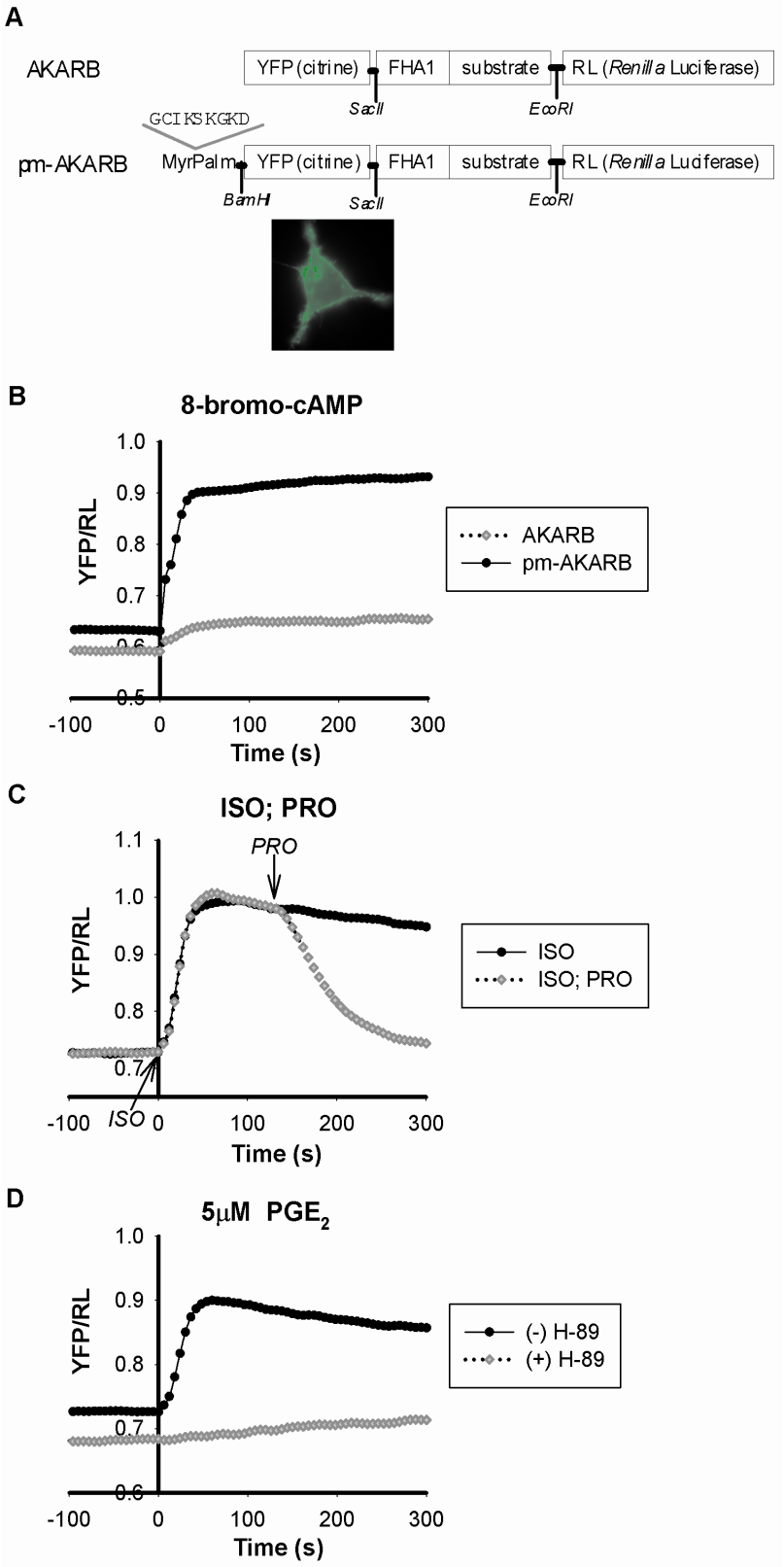
D. Primers used for detection of targeted AC7 allele:

Primer	Sequence (5'-3')
19	TAATACGACTCACTATAGGGAG
513	GGAAGCTTCGTGTGGAGAAG
554	GTGTTTGGAGCTGTGACAAGAG
573	CTGTGACCTGTACGTCTTGCG
595	CCCTACACAGTGAGGCACAGTA
596	GCCTGACACGCAGTAGTAACAG
597	CCATCTGCACGAGACTAGTGAG
598	ATTGCATCGCATTGTCTGAG
613	GGTAGGATCTCTCGTGAGGTG
703	AGCGTGGCCGAATTAGAGTA
675	CACTGAGCCGTGTGTCTGG
765	TGAACACCAGCATTTGCTTTAC
795	GACCAGATTGCTAAGGTGAGCC

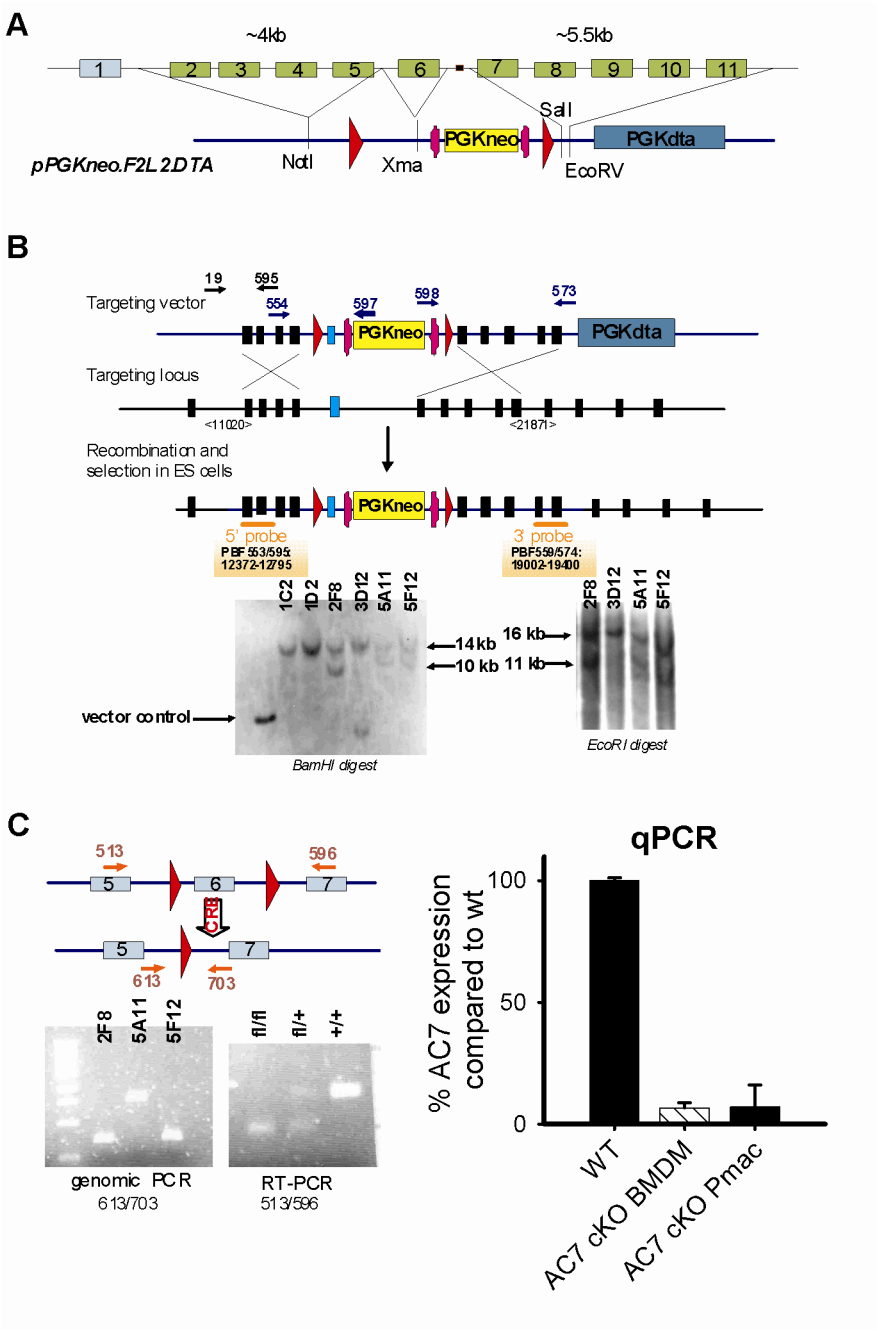
Figure S3. Different lots of zymosan induced cAMP and PKA responses.

BMDMs were isolated from 6-10 week old wild type mice and infected with retrovirus carrying the BRET sensor for cAMP, CAMYEL (A), or the BRET sensor for PKA, pm-AKARB (B). Intracellular cAMP and PKA activity were measured using BRET assays. At time 0, cells were treated with two different lots of zymosan at the indicated concentrations. Lot A is Lot #092K1240 and Lot B is Lot #BCBG6429V. Each trace is the average of two to three independent experiments and error bars represent the standard deviation. (C). BMDMs were stimulated with 0.5 mg/ml zymosan from the two different lots, 1 μM P3C, or 10 ng/ml LPS + 25 pM LBP as indicated for 5 minutes. Cell lysates were harvested and analyzed by western blot. Phosphorylation of PKA substrates was detected using antibodies specific for phospho-PKA substrates with the RRXS*/T* motif. Arrows indicate unique bands present only in zymosan treated cell lysates.

Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3