

PTEN Limits Alveolar Macrophage Function Against *Pseudomonas aeruginosa* Following Bone Marrow

Transplantation

Online Supplement

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Detailed Materials and Methods:

Animals

Wild type (WT) C57BL/6 (B6), conditional PTEN KO ($PTEN^{loxP/loxP}$), and myeloid-specific Cre mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Myeloid-specific PTEN KO mice were generated by breeding as previously described (1). For all experiments involving myeloid-specific PTEN KO mice, $PTEN^{wt/wt}; Cre^{+/-}$ mice were used as WT bone marrow donors and $PTEN^{loxP/loxP}; Cre^{+/-}$ mice were used as PTEN conditional knockout (CKO) bone marrow donors. Mice were housed under specific pathogen-free conditions and monitored daily by veterinary staff. All mice were euthanized by CO₂ asphyxiation. The University of Michigan Committee on Use and Care of Animals approved these experiments.

Bone marrow transplantation

Recipient mice received 13 Gy of total body irradiation (TBI; orthovoltage x-ray source) split in two fractions, three hours apart. Bone marrow cells were harvested from donor mice and resuspended in serum-free media (SFM; DMEM, 0.1% BSA, 1% penicillin-streptomycin, 1% L-glutamine, 0.1% amphotericin B). 5×10^6 bone marrow cells were administered by tail vein injection into TBI recipient mice. All experiments with BMT mice were performed 5-6 weeks post-BMT when mice were fully donor-cell reconstituted (2, 3). Spleen cells were over 94% donor-derived, and AMs were over 80% donor-derived at this time point (3).

P. aeruginosa PAO1 preparation and i.t infection

P. aeruginosa PAO1 stock was grown overnight in tryptic soy broth (Difco; BD, Sparks, MD), and the following day, an inoculum was prepared for i.t. injection by measuring the culture concentration via absorbance measurements as previously described (2). For i.t. injection of *P. aeruginosa*, mice were anesthetized and i.t. injected with 50 μ L of inoculum to provide an estimated sublethal dose of 5×10^5 CFU as previously described (2, 4). Actual dosage was determined by CFU analysis of the inoculum.

Immune serum preparation and opsonization

P. aeruginosa-specific immune serum was prepared from Wistar rats immunized with *P. aeruginosa* as previously described (5). Immune serum was aliquoted and stored at -80°C until use. For serum opsonization of FITC-*P. aeruginosa*, 1×10^8 FITC-*P. aeruginosa* were resuspended in 5% immune serum in PBS and incubated on a rotator for 1h at 37°C . For serum opsonization of live *P. aeruginosa*, 1×10^8 *P. aeruginosa* was resuspended in 5% immune serum in PBS and incubated on a rotator for 15 mins at 37°C . No complement heat-inactivation of immune serum was performed prior to opsonization of heat-killed or live bacteria.

Quantification of bacterial burden in lung and blood

Mice were euthanized 24h following i.t. infection with *P. aeruginosa*. As previously described (4), whole lung and blood samples were collected from each mouse and bacterial burden of each specimen was assessed by performing a CFU assay.

AM isolation and adherence purification

AMs were harvested by bronchoalveolar lavage (BAL), counted, and adherence purified as previously described (2). Following adherence purification, AMs were cultured overnight in complete media (CM) or in the presence or absence of 5 μ M indomethacin (Sigma) in SFM.

Neutrophil recruitment to lung and isolation

As previously described, neutrophils were recruited to the lung via i.t. injection of a 25 µg dose of *P. aeruginosa*-derived LPS (Sigma) and isolated by BAL 24 hours later (4). Neutrophils comprised approximately 80% of all BAL cells at this time point in all groups.

IgG-sheep red blood cell FcγR stimulation assay

AMs cultured overnight in CM were washed once with PBS and incubated at 37°C in SFM for 20 mins. Culture media in wells was then replaced with indomethacin (5 µM, 2h) or PGE₂ (100 nM, 15 min) in SFM. Freshly IgG-opsonized or non-opsonized sheep red blood cells (SRBCs; MP Biomedicals, Solon, OH) were prepared as described previously (6, 7) and incubated with AMs (1:10 ratio) for 15 min at 37°C. Wells were then washed once with PBS on ice, and whole-cell lysates were prepared for Western blot analysis as described below.

Differential cell analysis of total lung leukocytes and BAL cells

Differential cell analysis was performed for BAL cells or total lung leukocytes isolated from collagenase-digested whole lung samples as previously described (8). For each sample, total viable cell number was counted on a hemacytometer by trypan blue exclusion. 5 x 10⁴ cells per sample were cytopun onto slides and stained with modified Wright-Giemsa to determine the percentage of neutrophils, monocytes/macrophages, lymphocytes and eosinophils in each sample.

In vitro phagocytosis assay

AMs isolated by BAL were plated at 2 x 10⁵ cells/well and cultured overnight in CM on a 96-well, flat-bottomed, half-area tissue culture plates (Costar, Corning, NY). The following day, wells were aspirated and adherent AMs were pretreated with or without 100 nM bpV(pic) in SFM for 15 minutes (EMD Chemicals, Gibbstown, NJ) to inhibit PTEN activity. AMs were then incubated with either serum or non-serum opsonized FITC-labeled heat-killed *P. aeruginosa* at 300:1 MOI for 2 hours. For FITC-labeling, *P. aeruginosa* was heat-killed and incubated with 0.2 mg/mL FITC (Sigma, St. Louis, MO) in DMSO as previously described (8).

Phagocytosis of FITC-labeled bacteria was measured after quenching of non-ingested bacteria with trypan blue as previously described (8) and expressed as relative fluorescence intensity units.

Bacterial killing tetrazolium dye reduction assay

AMs or recruited lung neutrophils were isolated by BAL and plated at 2×10^5 cells/well in replicate on two 96-well tissue culture plates (one control and one experimental plate). AMs were cultured overnight in CM, and the following day, AM killing of serum opsonized *P. aeruginosa* was quantified using a tetrazolium dye reduction assay as described previously (9). Briefly, AMs on the control plate were allowed only to phagocytose bacteria, while AMs on the experimental plate were permitted to both phagocytose and kill ingested bacteria. After 5 hours incubation, the amount of surviving ingested bacteria was quantified using an MTT assay according to manufacturer's instructions (Sigma). Results were expressed as % surviving ingested bacteria = $(A_{595} \text{ control} / A_{595} \text{ experimental}) / \times 100\%$ to normalize the amount of bacterial killed to the amount of bacteria phagocytosed.

Western blot analysis

Whole-cell lysates of AMs were obtained by treating cells with RIPA buffer, protease inhibitors, and phosphatase inhibitors as previously described (10). The protein concentration of each sample was determined using the DC Protein Assay kit (Bio-Rad, Hercules, CA). 15 μg protein samples were electrophoresed as previously described (8). For PTEN immunoprecipitation experiments, PTEN was immunoprecipitated as described (7) from 15 μg of protein sample, and the immunoprecipitate was electrophoresed following 3 washes with lysis buffer. Proteins were then transferred to a PVDF membrane. Membranes were blocked for 1 hour at room temperature and probed according to manufacturer's instructions with one of the following primary antibodies: mouse anti-phospho-Akt (Ser⁴⁷³), rabbit anti-PTEN (Cell Signaling, Danvers, MA), rabbit anti-phosphotyrosine (pY; Millipore, Temecula, CA) or rabbit anti-IRAK-M (Stressgen Bioreagents, Ann Arbor, MI). Primary antibody incubations were followed by goat anti-rabbit or anti-mouse IgG-HRP (Pierce) secondary antibody incubations. To assess the amount of phosphorylated protein relative to total protein,

membranes probed for pAKT or pY were stripped and reblocked as previously described (7), and probed according to manufacturer's instructions with anti-AKT or anti-PTEN antibodies (Cell Signaling) respectively. Bands were visualized using chemiluminescence (SuperSignal West Pico Substrate; Pierce). Band intensity was quantified using Image J Software available for download at <http://rsbweb.nih.gov/ij/download.html>.

PTEN phosphatase activity assay

PTEN was immunoprecipitated from control and BMT AM whole-cell lysates to assess *in vitro* lipid phosphatase activity as previously described (7, 11). Briefly, 200 μ M D-*myo*PIP₃ (Echelon Biosciences) was incubated with PTEN immunoprecipitates for 30 min to allow for dephosphorylation of PIP₃. The amount of PIP₃-derived free phosphate released in each sample was determined by adding BIOMOL Green reagent to each sample and measuring the absorbance using a colorimetric plate reader at 630 nm. PTEN lipid phosphatase activity was quantified in each sample as relative absorbance to control samples.

ELISA/enzyme-linked immunoassay (EIA)

AMs were cultured overnight at 2 x 10⁶ cells/mL in a 96-well tissue culture plate, and supernatants were collected the following day for EIA. Lung homogenates were prepared for EIA as previously described (4). TNF- α production was measured using a DuoSet ELISA kit (R&D Systems, Minneapolis, MN), and PGE₂ production was measured using a PGE₂ EIA kit (Cayman Chemical, Ann Arbor, MI), according to manufacturer's instructions.

Statistical analysis

All experiments were repeated at least 2 times with similar results, and in most experiments, data are pooled from both experiments. Statistical significance was analyzed using the Prism 5.01 statistical program (GraphPad Software, San Diego, CA). Comparisons among three or more experimental groups were performed with ANOVA and a post hoc Bonferroni test. A value of $p < 0.05$ was considered statistically significant.

Supplemental Figures

FIGURE S1. Overproduction of PGE₂ reduces PTEN tyrosine phosphorylation in BMT AMs.

AMs were isolated by BAL as described in *Materials and Methods* from BMT and non-transplanted control mice. PTEN protein was immunoprecipitated from whole-cell lysates of AMs cultured at 5×10^5 per well in the presence or absence of 5 μ M indomethacin overnight. Immunoprecipitates were washed, electrophoresed, and immunoblotted with rabbit anti-phosphotyrosine (pY) antibodies to determine the level of PTEN tyrosine phosphorylation (upper panel). Blots were then stripped and reprobbed for total PTEN protein expression (lower panel). Relative band densitometry data are indicated under each lane (n=1).

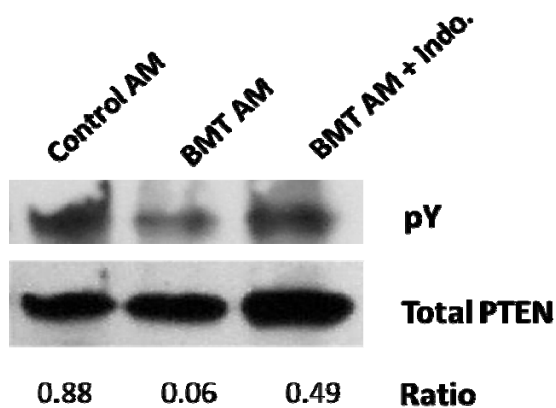
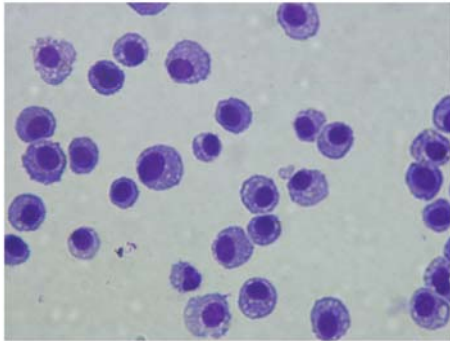
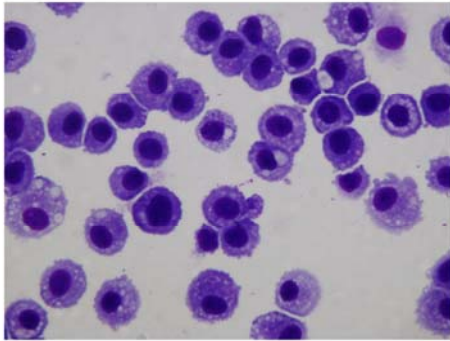


FIGURE S2. Differential analysis of BAL cells from control, WT BMT, and PTEN CKO BMT mice.

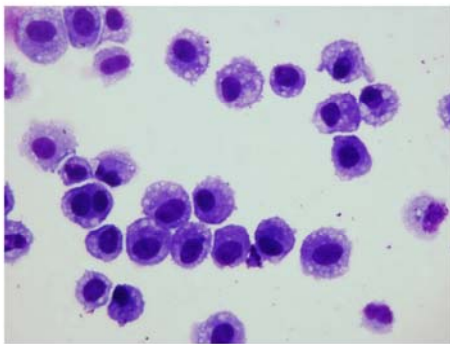
BAL cells were harvested from control, WT BMT (WT>WT), and PTEN CKO BMT (PTEN CKO BMT > WT) mice. BAL cells were cytopun and stained with modified Wright-Geimsa stain. The average percentage of BAL cells with the characteristic morphology of AMs was 97% or greater in each group. Data are representative of 2 individual BAL samples per group.



Control



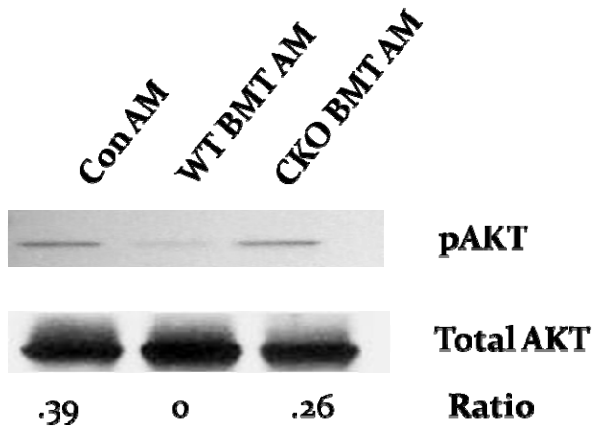
WT BMT



PTEN CKO
BMT

FIGURE S3. Diminished pAKT levels in BMT AMs are improved in the absence of PTEN expression.

Whole-cell lysates were prepared from control, WT BMT, and PTEN CKO BMT AMs following 1h SFM adherence. pAKT expression levels were assessed by Western blot using mouse anti-pAKT antibodies (upper panel). Blots were then stripped and reprobbed with rabbit anti-AKT antibodies to assess total AKT protein expression (lower panel). Relative band densitometry data are indicated under each lane (n=1).



References

1. Zhu D, Hattori H, Jo H, Jia Y, Subramanian KK, Loison F, You J, Le Y, Honczarenko M, Silberstein L, et al. Deactivation of phosphatidylinositol 3,4,5-trisphosphate/AKT signaling mediates neutrophil spontaneous death. *Proc Natl Acad Sci U S A* 2006;103(40):14836-14841.
2. Ballinger MN, Hubbard LL, McMillan TR, Toews GB, Peters-Golden M, Paine R, 3rd, Moore BB. Paradoxical role of alveolar macrophage-derived granulocyte-macrophage colony-stimulating factor in pulmonary host defense post-bone marrow transplantation. *Am J Physiol Lung Cell Mol Physiol* 2008;295(1):L114-122.
3. Hubbard LL, Ballinger MN, Wilke CA, Moore BB. Comparison of conditioning regimens for alveolar macrophage reconstitution and innate immune function post bone marrow transplant. *Exp Lung Res* 2008;34(5):263-275.
4. Ballinger MN, Aronoff DM, McMillan TR, Cooke KR, Okiewicz K, Toews GB, Peters-Golden M, Moore BB. Critical role of prostaglandin E₂ overproduction in impaired pulmonary host response following bone marrow transplantation. *J Immunol* 2006;177:5499-5508.
5. Mancuso P, Standiford TJ, Marshall T, Peters-Golden M. 5-lipoxygenase reaction products modulate alveolar macrophage phagocytosis of *Klebsiella pneumoniae*. *Infect Immun* 1998;66(11):5140-5146.
6. Araki N, Johnson MT, Swanson JA. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J Cell Biol* 1996;135(5):1249-1260.
7. Canetti C, Serezani CH, Atrasz RG, White ES, Aronoff DM, Peters-Golden M. Activation of phosphatase and tensin homolog on chromosome 10 mediates the inhibition of FcγR phagocytosis by prostaglandin E₂ in alveolar macrophages. *J Immunol* 2007;179(12):8350-8356.
8. Hubbard LL, Ballinger MN, Thomas PE, Wilke CA, Standiford TJ, Kobayashi KS, Flavell RA, Moore BB. A role for IL-1 receptor-associated kinase-M in prostaglandin E₂-induced immunosuppression post-bone marrow transplantation. *J Immunol*.
9. Serezani CH, Aronoff DM, Jancar S, Mancuso P, Peters-Golden M. Leukotrienes enhance the bactericidal activity of alveolar macrophages against *Klebsiella pneumoniae* through the activation of NADPH oxidase. *Blood* 2005;106(3):1067-1075.
10. Deng JC, Cheng G, Newstead MW, Zeng X, Kobayashi K, Flavell RA, Standiford TJ. Sepsis-induced suppression of lung innate immunity is mediated by IRAK-M. *J Clin Invest* 2006;116(9):2532-2542. .
11. White ES, Thannickal VJ, Carskadon SL, Dickie EG, Livant DL, Markwart S, Toews GB, Arenberg DA. Integrin alpha4beta1 regulates migration across basement membranes by lung fibroblasts: A role for phosphatase and tensin homologue deleted on chromosome 10. *Am J Respir Crit Care Med* 2003;168(4):436-442.