**Supplemental Data**
Cell Host & Microbe, *Volume 5*

# TLR2-Induced Calpain Cleavage of Epithelial Junctional Proteins Facilitates Leukocyte Transmigration

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## *Transepithelial Resistance and Dextran Permeability Assays*

Polarized 16HBE cells grown in 12 mm Transwell-Clear filters (Corning-Costar) were stimulated apically with $10^8$ CFU heat killed PAO1. Transepithelial resistance readings were taken using a Millipore resistance reader which applies a 20 μA square wave alternating current across the monolayers at 12.5 Hz. The resistance of Transwell inserts without cells was used as a baseline control. After 5 h of stimulation, Alexa Fluor 488 labeled dextran (10,000 MW) was added apically for 1 h and fluorescence in the basal compartment monitored at ex 485, em 535 with a SpectraFluor Plus fluorimeter (Tecan). As a positive control, cells were treated apically and basolaterally for 1 h with 0.02 % EGTA, an extracellular $Ca^{2+}$ chelator which increases paracellular permeability by disrupting cadherin mediated cell-cell adhesion.

## *Bacterial Transmigration Assay*

Polarized 16HBE cells were pretreated with media alone, heat killed PAO1 ($10^8$ CFU), P3C (15 ug/ml), or thapsigargin (0.1 μM) for 4 h. Live PAO1 ($2 \times 10^7$ CFU) was added apically for 1 h and media from the basal compartment collected and plated on LB plates. Bacterial transmigration across monolayers pretreated with 0.02% EGTA was used as a positive control.

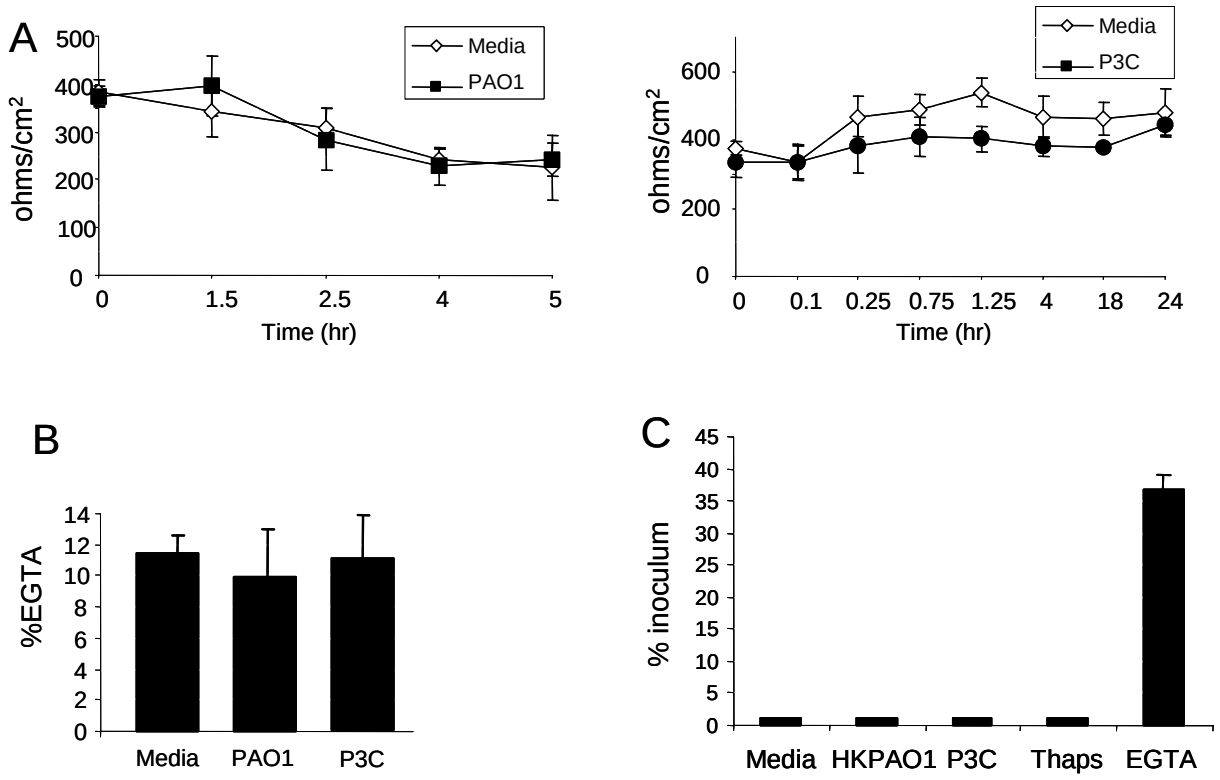
*Real time PCR of Calpains and Calpastatin*

1HAEo- cells were grown in 6 well plates to confluence. After incubation with media alone, heat killed PAO1 or P3C, cells were lysed and RNA was isolated using the Qiagen RNeasy Mini Kit. cDNA was made from 1 μg of RNA using an iScript synthesis kit (Bio-Rad). For quantitative real-time PCR of calpain 1, calpain 2 and calpastatin, cDNA amplification was performed in a Light Cycler using the DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. Primers used for calpain 1 amplification were 5'- TGCGAGAGGTCAGCACCCGC -3' and 5'-CAGGTCAAACTTCCGGAAGATGG -3'. The primers used for calpain 2 amplification were 5'- ATCTGCCAAGGAGCCCTAGG -3' and 5'- TAGTGTTCCAGCTTGGGCAG -3'. The primers used for calpastatin were 5'-AAAGATGGAAAACCACTATTGCCAGAGC-3' and 5'-GACCTCTTCTAATCTATAATCAGGAGG -3'. For calpain 1, calpain 2 and calpastatin quantification, 35 cycles were run with denaturation at 95°C for 8 s, amplification at 50°C for 15 s, and extension at 72°C for 12 s. Amplification of human actin was used as a control for standardization. The primers used for human actin amplification were 5'-TCCTCCCTGGAGAAGAGCTAC-3' and 5'-TAAAGCCATGCCAATCTCATC -3', and 35 cycles were run with denaturation at 95°C for 8 s, amplification at 63°C for 10 s, and extension at 72°C for 12 s.

*PMN migration assay with live PAO1*

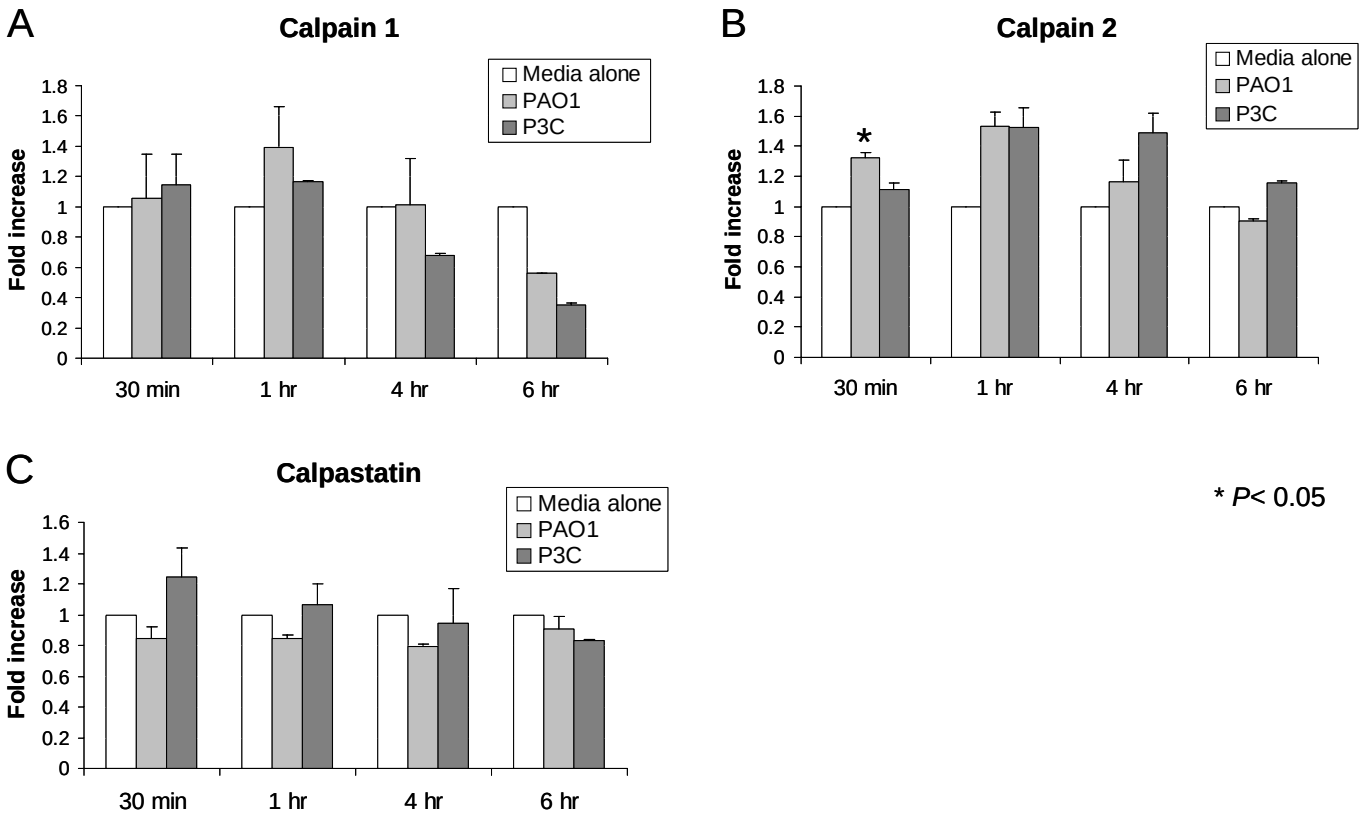
The migration of PMN across polarized 16HBE monolayers in response to live PAO1 was tested as previously described (Hurley et al 2004). Briefly, inverted 16HBE

monolayers were apically treated with various concentrations of PAO1 or media alone. After 2 h, DMEM was added to the apical chamber and $10^6$ PMNs in DMEM added to the basolateral chamber and incubated at 37º for 2 h. PMNs that migrated into the apical chamber were quantified by myeloperoxidase assay.



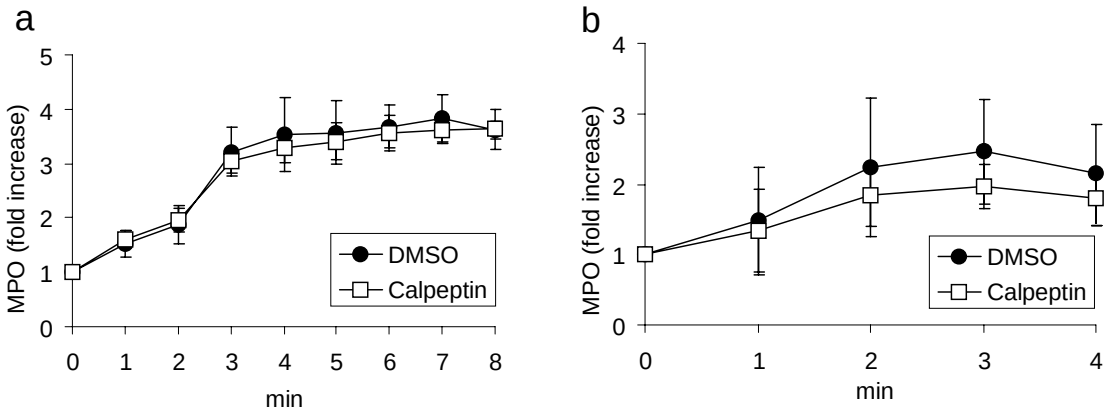

## Supplemental Figure 1

Supplementry Figure 1- Epithelial barrier function is unchanged in response to heat killed PAO1. (A) Transepithelial resistance was assessed at the indicated times following bacterial exposure. (B) Paracellular permeability was measured after 5 h stimulation with heat killed PAO1 or P3C by adding Alexa Fluor 488 dextran (10,000 MW) apically for 1 h and monitoring basolateral fluorescence at ex 485, em 535. Data is represented as the percentage of fluorescence compared with cells treated with EGTA which disrupts the junctions completely. (C) Bacterial transmigration across the monolayer was quantified by adding 2 X $10^7$ CFU live PAO1 apically for 1 h after pretreatment for 4 h with heat killed PAO1, P3C and thapsigargin or 1 h treatment with EGTA and monitoring PAO1 that reach the basal compartment of the Transwell. Data is represented as a percentage of inoculum and represents the mean ± s.d. of quadruplicate samples of one representative of at least three separate experiments.



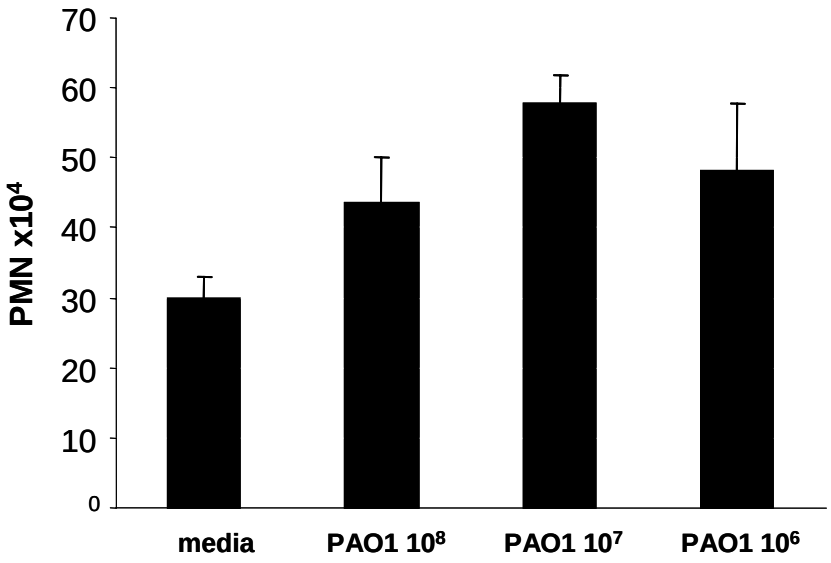

## Supplemental Figure 2

Supplementry Figure 2- Epithelial calpain and calpastatin expression. mRNA levels of (A) calpain 1, (B) calpain 2, (C) and calpastatin were determined in 1HAEo- cells stimulated with PAO1 or P3C by real-time PCR. Values were normalized to actin and are shown as the fold change in expression relative to the endogenous level in media alone treated cells. Data are mean ± s.d. of duplicate samples of one representative from three independent experiments. (* $P$<0.05 compared with media alone controls; Student's t-test).




## Supplemental Figure 3

Supplemental Figure 3- Calpeptin does not inhibit PMN myeloperoxidase activity. (**a**) Myeloperoxidase activity of 0.0125 μg/ml recombinant human myeloperoxidase was not inhibited by 20 μM calpeptin. (**b**) Similarly, myloperoxidase activity of $10^4$ PMNs was not inhibited when incubated with 20 μM calpeptin. Results are presented as fold increase over baseline. Data represents mean ± s.d. of sextuplicate samples.




## Supplemental Figure 4

Supplemental Figure 4- PMN migration in response to live PAO1. Migration of PMN across polarized 16HBE monolayers treated with various concentrations of live PAO1. Result is represented as the number of PMNs multiplied by $10^4$. Data represents mean ± s.d. of sextuplicate samples.