Supporting Online Material for

Toll-Like Receptor Triggering of a Vitamin D–Mediated Human Antimicrobial Response

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Reagents

TLR2/1L is a synthetic 19kDa \textit{M. tuberculosis} derived lipopeptide (EMC Microcollections, Tuebingen, Germany). The specificity of the TLR peptide has been documented in Brightbill \textit{et al} using TLR2 transfectants and an anti-TLR2 blocking monoclonal antibody (S1). Both 1,25D3 and 25D3 were purchased (BioMol, Plymouth Meeting, PA, USA) and resuspended in ethanol at 10^{-2}M in amber tubes and stored at -80^\circ\text{C} in small aliquots. 1,25D3 was added to culture at a concentration of 10^{-7}M to 10^{-9}M, or 10^{-7}M in the absence of a titration. 25D3 was used at 10^{-9}M to 10^{-7}M. VAZ was provided by Dr. Zügel from Schering AG, and used at 10^{-7}M. BCG-GFP was prepared and provided by the laboratory of Dr. Bloom, and the LL-37 monoclonal antibody is a kind gift from Dr. Sorensen. CD68 antibody is commercially available (R&D Systems, Minneapolis, MN, USA). Cathelicidin peptide is synthesized by solid phase biochemistry and purified using reverse phase HPLC (Charite, Berlin, Germany). Purity and quality of the individual batches are controlled by HPLC, mass spectroscopy and Western blotting using antibodies of various specificities. The relevant batch has the expected mass of the cathelicidin peptide determined by mass spectroscopy and is 99.92\% pure by HPLC. The peptide is dissolved in water without detergents or other additives, and was tested functionally by measuring its antimicrobial activity against \textit{Pseudomonas aeruginosa} and \textit{Streptococcus pneumonia}. A portion of the African-American serum was provided by Dr. Hollis. All cells types, including monocytes, macrophages, DCs, and alveolar macrophages as well as experimental replicates, were derived from separate and unrelated donors.

\textit{Mycobacteria tuberculosis} growth and maintenance

\textit{M. Tb} (virulent strain H37Rv) was grown in suspension with constant, gentle rotation in roller bottles (Corning, Cambridge, MA) containing Middlebrook 7H9 broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 1\% glycerol (Roth, Karlsruhe, Germany), 0.05\%
Tween-80 (Sigma, St. Louis, MO, USA) and 10% Middlebrook OADC enrichment (Becton Dickinson). Aliquots from logarithmically growing cultures were frozen in PBS containing 10% glycerol and representative vials were thawed and enumerated for viable colony forming units (CFU) on Middlebrook 7H11 plates. Bacterial viability was above 90% (BacLight™, Molecular Probes, Leiden, Netherlands).

Infection of macrophages and dendritic cells
Monocyte derived macrophages and immature dendritic cells were generated as previously described (S2). Macrophages and DCs were infected with single cell suspensions of \textit{M. tuberculosis} at a multiplicity of infection (MOI) of two and five respectively. DCs were harvested after four hours and centrifuged three times at 800 rpm for eight minutes to remove extracellular bacteria. After three cycles of centrifugation the majority of extracellular bacteria (>99%) were removed as determined by auramine-rhodamine stain (Merck, Darmstadt, Germany) and plating of the supernatants after the final centrifugation. Since infected macrophages were adherent to the plastic flask, extracellular bacteria were removed by vigorous washing. Adherent cells were detached by treatment with EDTA and plated at a concentration of $5\times10^5$ cells/ml in a 24 well plate. The efficiency of infection, as quantified by auramine rhodamine stain was in a range of 20-35%. Cell viability of infected cells was determined by trypan blue exclusion and was >90% in all experiments.

Quantification of mycobacterial growth
To measure survival of viable bacilli the infected cells were lysed with 0.3% saponin (Sigma) to release intracellular bacteria. At all time points an aliquot of unlysed, infected cells was harvested and counted. This allowed an exact quantification of cells as well as the determination of cellular viability by trypan blue exclusion. Recovery of cells was >70% in all experiments, with cell viability regularly exceeding 90% of total cells. Lysates of infected cells
were resuspended vigorously, transferred into screw caps and sonicated in a preheated (37ºC) water bath sonicator for 10 min. Aliquots of the lysates were diluted ten-fold in 7H9-medium. Four dilutions of each sample were plated in duplicates on 7H11 agar plates and incubated at 37ºC and 5% CO₂ for 21 days. Figure 1A presents a typical result of experiments performed with macrophages derived from 12 non-related Caucasian donors. All 12 donors responded with increased antimicrobial activity to treatment with TLR2/1L. Inter-individual differences were restricted to the absolute numbers of CFU after 96h and the extent of antimicrobial activity.

**DNA microarray data analysis**

The monocyte microarray data were previously described (S3). Dendritic cells were generated by culturing monocytes with GM-CSF (800 U/ml) and IL-4 (1000 U/ml) for 7 days. The cells were then harvested and recultured for 12 hours with TLR2/1L or control. RNA was isolated from the cells using Trizol reagent according to the manufacturers recommended protocol. The UCLA Microarray Core Facility performed probe synthesis and hybridization to Affymetrix U133A Genechip. We used two-way ANOVA (S4-6) to identify the most informative set of differentially expressed genes in the two cell types, monocytes and DCs, upon TLR2/1L treatment. Each gene was ranked by the probability that its expression value was statistically distinct between the two cell types, and calculated the fold change in mean expression of each gene when comparing monocytes vs. DCs and TLR2/1L-treated sample vs. media control and used these values to further sort genes. We focused our attention on genes meeting the following three criteria: a p-value of the differences between cell types < 0.05, >1.5 fold change in expression comparing monocytes to DCs, and >2 fold change in expression comparing TLR2/1L-treated to media-treated samples. This yielded 259 probesets that were significantly upregulated in monocytes and 75 probesets that were significantly upregulated in DCs, and these corresponding genes were cross-referenced against a list of genes known to be associated with antimicrobial activity. The false discovery rate (FDR) from multiple testing was
estimated by the q value my (S7). Cluster diagrams were generated using the Cluster and TreeView software programs from the Eisen Lab at http://rana.lbl.gov/ (S8). The monocyte arrays mentioned above were analyzed for genes related to the VDR pathway that were induced by TLR2/1L. Despite not being discovered in the two-way ANOVA analysis, Cyp27B1 was found to be significantly upregulated at both 12 and 24 hours in monocytes. Two-way ANOVA excluded Cyp27B1 because it was upregulated by TLR activation in some of the DC samples, but this upregulation was not statistically significant. Since two-way ANOVA simultaneously compares gene regulation in both monocytes and DC, the difference in Cyp27B1 expression between the cell types was not significant. However, when additional monocytes, macrophages and DC were analyzed by qPCR, the data revealed upregulation in monocytes and macrophages but not DCs (Figure 1d).

**Quantitative PCR**

We obtained whole blood from healthy donors with informed consent (UCLA Institutional Review Board #92-10-591-31). cDNA was prepared from adherent monocytes and mRNA levels were assessed and calculated with qPCR as previously described (S9). Primer sequences used are as follows: Cyp27B1 5’-ACC CGA CAC GGA GAC CTT C, 3’-ATG GTC AAC AGC GTG GAC AC; VDR 5’-AAG GAC AAC CGA CGC CAC T, 3’-ATC ATG CCG ATG TCC ACA CA; Cathelicidin 5’-TGG GCC TGG TGA TGC CT, 3’-CGA AGG ACA GCT TCC TTG TAG C; and Cyp24 5’-CGC AGC GGC TGG AGA T, 3’-CCG TAG CCT TCT TTG CGG TA. Sequences for DEF4 and h36B4 were previously described (S10).

**Intracellular flow cytometry**

Monocytes were stimulated for 16 hours with 1,25D3 and their cathelicidin levels were assessed using intracellular flow cytometry as previously described (S11). Optimal detection of cathelicidin is achieved through a three step labeling process. First the cells are incubated for
one hour with either an isotype control or a monoclonal cathelicidin antibody, followed by a
biotinylated secondary antibody, then PE-conjugated Strep-avidin, with washes in between each
step.

**Surface enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry**

Stimulated and control monocytes from 3 patients were dissolved in 1 ml of 1 M HCl and 1%
trifluoroacetic acid, sonicated for three minutes on ice, rotated at 4°C overnight and
subsequently centrifuged 10 min at 14,000 rpm. Supernatants were transferred to new tubes,
lyophilized, and dissolved in 100 µl of RIPA buffer containing protease inhibitors (Roche Applied
Science, Indianapolis, IN, USA). Protein chips (RS100 ProteinChip array®, Ciphergen
Biosystems, Fremont, CA, USA) were coated with 4 µl of anti-LL-37 rabbit antibody for 2 h at
RT, followed by blocking with 0.5 M ethanolamine in PBS (pH 8.0). After washing three times
with PBS / 0.5 % Triton X-100, proteinchips were assembled in the Bioprocessor™ (Ciphergen)
reservoir, samples (50µl) were applied in duplicates and incubated 2 h at RT. Protein chips
were washed twice with RIPA buffer, once with PBS / 0.5 % Triton X-100, and three times with
PBS. Proteinchips were then soaked in 10 mM HEPES buffer and spots air dried. 0.5µl of
energy absorbance matrix (50%-saturated alpha-cyano-4-hydroxy cinnamic acid in 50%
acetonitrile, 0.5% trifluoracetic acid) was applied twice, and all spots were again air dried.
Samples were analyzed on a SELDI mass analyzer PBS IIC with a linear time-of-flight mass
spectrometer (Ciphergen) using time-lag focusing. Mass was calibrated with peptide standards
(All-in-1 peptide standard, Ciphergen).

**Cathelicidin direct antimicrobial activity**

The culture conditions for measuring the antimycobacterial activity of cathelicidin were carefully
established to meet the optimal requirements for both the growth of extracellular bacteria and
the biological activity of cathelicidin. Typical conditions used for growing M. tuberculosis (7H9-
Medium) abolished the antimicrobial activity of cathelicidin against Escherichia coli, which is the standard for assessing cathelicidin function. The appropriate medium for cathelicidin (tryptic soy broth) did not permit mycobacterial replication; therefore, we tested several media and found that standard cell culture medium (RPMI 1640 supplemented with NaHCO3 and L-glutamin) diluted 1:4 in distilled water allowed multiplication of M. tuberculosis as efficiently as 7H9 and maintained the activity of cathelicidin against Escherichia coli. Under these conditions the activity of cathelicidin reflected the published results, making it highly unlikely that protein clumping in our culture medium is responsible for the observed effects. We cultured 2x10^6 M. tuberculosis in 1:4 diluted RPMI in 96 well round bottom plates in the presence of cathelicidin peptide in the indicated concentrations in triplicate wells. After three days of culture, bacteria were harvested and sonicated in a preheated water bath. The number of colony forming units (CFUs) were assayed by plating four 10-fold dilutions of the sonicated bacteria on 7H9 Middlebrook agar plates and counting the number of visible colonies after 21 days of incubation. To assess bacterial viability, uracil uptake assay was used as previously described (S12). Both the CFU and uracil data is derived from triplicate wells in three independent experiments using three separate donors.

Serum collection and 25D3 quantification

Serum was collected as previously mentioned and used after informed consent from healthy donors 20-40, both populations containing both males and females and categorized by self-identification or visual determination according to race. Blood was collected in the absence of any anti-coagulants, and allowed to completely clot for two hours, and then broken up and centrifuged for 20 minutes at maximum speed. The serum layer is collected and passed through a 0.22 micron filter. A portion of the serum is frozen at -80°C for future use. Circulating concentrations of 25D3 were determined by radioimmunoassay as previously described (S13).
Reference List


   Ref Type: Computer Program


