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

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In innate immune responses, activation of Toll-like receptors (TLRs) triggers direct antimicrobial activity against intracellular bacteria, which in murine, but not human, monocytes and macrophages is mediated principally by nitric oxide. We report here that TLR-activation of human macrophages up-regulated expression of the vitamin D receptor and the vitamin D_1–hydroxylase genes, leading to induction of the antimicrobial peptide cathelicidin and killing of intracellular *Mycobacterium tuberculosis.* We also observed that sera from African-American individuals, known to have increased susceptibility to tuberculosis, had low 25-hydroxyvitamin D and were inefficient in supporting cathelicidin messenger RNA induction. These data support a link between TLRs and vitamin D–mediated innate immunity and suggest that differences in ability of human populations to produce vitamin D may contribute to susceptibility to microbial infection.

The innate immune system provides a rapid host mechanism for defense against microbial pathogens. In *Drosophila*, innate immunity is mediated in part by the Toll family of pattern recognition receptors, whose activation induces expression of a series of antimicrobial peptides (*1*). The mammalian Toll-like receptor (TLRs) homologs, including the TLR2 and TLR1 heterodimer (*2*), similarly recognize a variety of microbial-derived ligands, including bacterial lipopeptides. Activation of TLRs results in a direct antimicrobial response in monocytes and macrophages in vitro. In mice, this activity is mediated principally through generation of nitric oxide (*3*). However, we found that TLR2/1-induced antimicrobial activity in human macrophages is not affected by inhibitors of nitric oxide or reactive oxygen intermediates (*3*), and the mechanism of human microbicidal activity remains unresolved.

In studies of resistance to *M. tuberculosis*, we observed that activation of TLR2/1 reduced the viability of intracellular *M. tuberculosis* in human monocytes and macrophages but not in monocyte-derived dendritic cells (DCs) [Fig. 1A and (*3*, *4*)]. Consequently, we examined gene expression profiles of TLR2/1L-stimulated or media-treated monocytes and DCs using DNA microarrays (*4*). A two-way ANOVA was applied to the array data to identify genes differentially expressed in the two cell types upon TLR2/1L treatment (*4*). Genes up-regulated in monocytes, but not in DCs with significant p-values ($p<0.05$, FDR = 0.09) were cross-referenced against a list of genes associated with known antimicrobial function, yielding two candidates: vitamin D receptor (VDR), and S100A12, a calcium-binding pro-inflammatory molecule (*5*) (Fig. 1B). While TLR2/1 stimulation of DCs up-regulated specific genes characteristic of activation (Fig. 1B), the selective up-regulation of the VDR gene in monocytes prompted us to examine further selected VDR related genes.

From these analyses Cyp27B1, the enzyme that catalyzes the conversion of inactive pro-vitamin D_3 hormone (25-hydroxyvitamin D_3; 25D3) into the active form (1,25-dihydroxyvitamin D_3; 1,25D3), was observed to be significantly up-regulated at 12 and 24 hours (Fig. 1C). However, there was no evidence for mRNA up-regulation of other VDR downstream target genes, including CAMP (cathelicidin precursor), beta defensin-4 (DEFB4) and the 1,25D3 regulated VDR-specific Cyp24 hydroxylase gene (Fig. 1C) (*6*). Quantitative PCR (qPCR), confirmed the microarray data, demonstrating up-regulation of mRNAs for both VDR and Cyp27B1 in monocytes and macrophages, but not DC (Fig. 1D) (*4*). However, again no observable up-regulation of cathelicidin, DEFB4, and Cyp24 mRNAs was seen in TLR2/1L treated monocytes (Fig. 1E). Together, these data suggest that TLR-induces up-regulation of the VDR and Cyp27B1 gene expression in monocytes and macrophages, but raised the question of its functional significance.

To determine the functional status of the VDR, 1,25D3 was added to primary human monocytes and downstream target gene induction was assessed. Treatment led to the dose-dependent up-regulation of both cathelicidin and Cyp24 mRNA by qPCR, although the level of DEFB4 remained unchanged (Fig. 2A). The induction of cathelicidin was also evident by intracellular flow cytometry and was found to be processed to its active peptide form (LL-37) by surface-enhanced laser desorption ionization - time of flight (SELDI-TOF) mass spectrometry (Fig. 2, B and C) (*4*). In monocytes infected with *Mycobacterium bovis* BCG expressing green fluorescent protein (BCG-GFP), cathelicidin was observed to colocalize with the bacteria-containing vacuoles with 1,25D3 treatment, but not in untreated samples (Fig. 2D) (*4*). A direct antimicrobial effect of the cathelicidin peptide on *M. tuberculosis* could be demonstrated by both ^{3}H-uracil uptake and CFU assay (Fig. 2E) (*4*). Addition of 1,25D3 to primary human macrophages infected with virulent *M. tuberculosis* reduced the number of viable bacilli (Fig. 2F), consistent with earlier studies performed with monocytic cell lines (*7*, *8*). Taken together, these data indicate that the VDR is functional in primary human monocytes, and that its activation triggers induction of at least one known antimicrobial peptide, cathelicidin, capable of mediating antimicrobial activity.

Since the VDR is functional upon addition of exogenous 1,25D3, we hypothesized that the TLR2/1 induction of Cyp27B1 and the conversion of 25D3 to 1,25D3 could represent key aspects of the TLR pathway. Monocytes were activated by TLR2/1L in medium containing fetal calf serum (FCS) in the presence and absence of 25D3. Although addition of either 25D3 or TLR2/1L alone had no effect, their simultaneous addition up-regulated cathelicidin and Cyp24 mRNA (Fig. 3A). In the viability assays, where exogenous 25D3 was not required for killing, the experiments were performed in human serum to facilitate uptake of bacteria. Only in the presence of human serum did the TLR2/1 stimulation up-regulate cathelicidin and Cyp24 mRNA without addition of exogenous 25D3 (Fig. 3B). We attribute this to the finding that the 25D3 levels in human serum were five-fold greater than in FCS, (Fig. 3C), suggesting that culture of human cells in human serum may be critical for study of innate immune responses in humans (*4*).

Specific inhibition of Cyp27B1 blocked TLR2/1L activation of cathelicidin mRNA by 80% (Fig. 4A). Addition of a VDR antagonist inhibited both induction of cathelicidin mRNA by greater than 80% (Fig. 4B) and the antimicrobial activity was reduced approximately 70% (Fig. 4C). Taken together these data demonstrate that activation of TLRs on human monocytes triggers a microbicidal pathway that is dependent on both the endogenous production and action of 1,25D3 through the VDR.

These findings prompted us to address a problem that has long challenged the field, namely differences in susceptibility of different ethnic populations to the disease. Because of skin melanin content and subsequent diminished UV dependent cutaneous vitamin D_3 synthetic capacity, African Americans have significantly decreased serum 25D3 levels (*9*) and are known to have increased susceptibility to *M. tuberculosis* infection (*10*), and more rapid and more severe course of disease (*11*). We observed that serum levels of 25D3 in African Americans were found to be significantly lower than in a Caucasian cohort (Fig. 4D). Strikingly, when these serum samples were used to support TLR2/1 activation, the induction of cathelicidin mRNA was significantly lower in the presence of serum from African American than the Caucasian individuals [Fig. 4E (*11a*)] (correlation coefficient 25D3 vs. cathelicidin mRNA = 0.63; $p<0.001$). Finally, supplementation of the African American serum with 25D3 to a physiologic range restored TLR induction of cathelicidin mRNA (Fig. 4F).

Although the anti-microbial effects of vitamin D have been previously documented and reduced vitamin D status is known to be associated with susceptibility to *M. tuberculosis* infection, our work describes a potential mechanism by which this might influence the innate immune response. By demonstrating that TLR stimulation of human macrophages induces: (i) the enzyme that catalyzes conversion of 25D3 to active 1,25D3; (ii) the expression of the vitamin D receptor (VDR); and, (iii) relevant downstream targets of VDR (including cathelicidin), the present results provide an explanation for the action of vitamin D as a key link between TLR activation and antibacterial responses in innate immunity. We do not imply that this is the only antimicrobial mechanism available to human macrophages. This innate immune pathway is likely complemented by T cell-dependent

adaptive immune mechanisms, which include macrophage activation by cytokines and release of granulysin (*12*).

We believe these findings may explain a number of puzzling problems: the increased 1,25D3 levels at the site of disease in a localized form of tuberculosis (*13*), TLR-induced induction of antimicrobial peptides in epithelial cells (*14*); and the evolution of divergent antimicrobial pathways in mice (nocturnal animals that utilize nitric oxide) versus humans (daytime creatures that synthesize vitamin D_3 in the skin upon exposure to UV light). These findings also provide new insight into the history of tuberculosis treatment, including the importance of sunlight in the sanatorium movement created by Brehmer and Trudeau, and the award of 1903 Nobel Prize for Medicine to Niels Ryberg Finsen for demonstrating that UV light was beneficial to patients with lupus vulgaris, tuberculosis of the skin, consistent with the importance of vitamin D in all forms of tuberculosis. The harmful effects of sunlight are well documented, but there is also epidemiologic evidence that vitamin D sufficiency has a positive association with lower incidences of colorectal and prostate cancers (*15*). The findings reported here are consistent with the possibility that variation in the ability to synthesize vitamin D, including polymorphisms in the VDR pathway (*16*), may be a contributing factor to increased tuberculosis susceptibility. Consequently, consideration might be given to clinical trials of inexpensive vitamin D supplementation at appropriate doses to enhance innate immunity to microbial infections and possibly neoplastic disease in African or Asian populations.

References and Notes

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11a. All volunteers in the study were categorized by self-identification or visual determination as African-Americans or Caucasians (non-Hispanic White). There were 19 African Americans and 14 Caucasians studied in Fig. 4, D and E. Although the studies were performed using samples from African Americans, we believe that the implications are likely to be relevant for all individuals of African descent and possibly of Asian decent. Future studies will be directed towards comparing responses according to Fitzpatrick skin types that classify individuals according to skin pigmentation.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1123933/DC1
Materials and Methods
References

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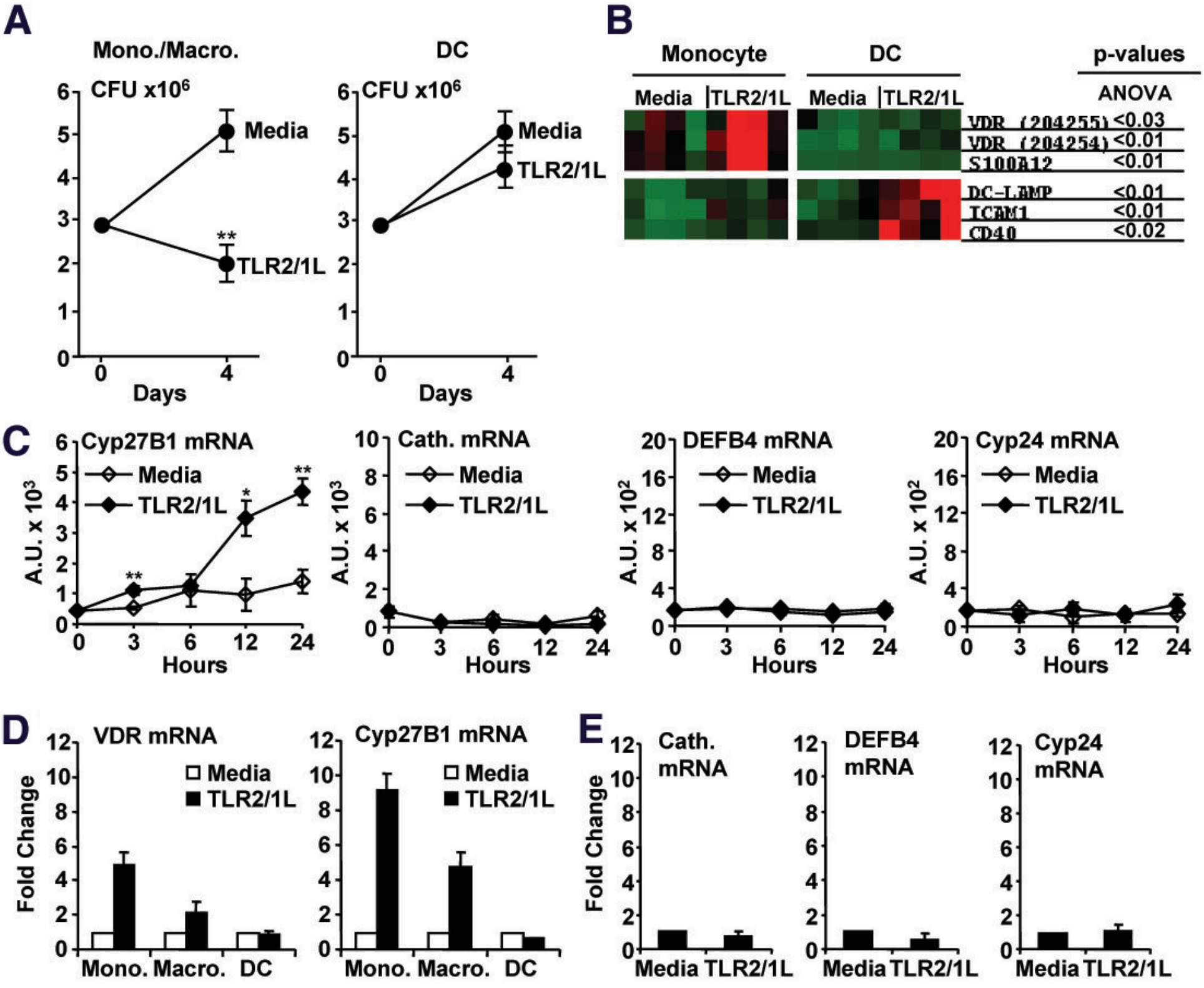
Fig. 1. Comparison of gene expression profiles in human primary monocytes/macrophages and DC. (**A**) Antimicrobial activity of monocyte/macrophage and DC against intracellular *M. tuberculosis.* Infected cells were stimulated with a TLR2/1 ligand and viable *M. tuberculosis* assessed by CFU assay. Data shown is representative of 12 individual donors for both cell types. (**B**) Gene expression profile in TLR2/1 activated monocytes and DCs. Cells were obtained from four donors, stimulated with TLR2/1L for 12 hours and their gene expression was profiled using gene microarrays. A two-way ANOVA was applied, and genes induced in monocytes and significantly different ($p<0.05$) compared to DCs were cross-referenced against genes with known antimicrobial functions. The profile of genes known to be characteristic of DC function, DC-LAMP, ICAM1 and CD40 were examined. (**C**) Time course of Cyp27B1, cathelicidin, DEFB4 and Cyp24 mRNA expression in TLR2/1L activated monocytes. Cells from four donors were stimulated with either media (◇) or TLR2/1L (◆) for zero, three, six, 12, and 24 hours, and the gene expression was profiled using microarray. Regulation of VDR and Cyp27B1 mRNA (**D**) and cathelicidin, DEFB4 and Cyp24 mRNA (**E**) in monocyte, macrophage and DC. Cells were treated with TLR2/1L at 24hrs and gene expression assessed using qPCR. (mean fold change ± SEM, $n = 4$). *$p \leq 0.05$, **$p \leq 0.01$.

Fig. 2. Monocyte response to 1,25D3. (**A**) Regulation of VDR downstream genes cathelicidin, Cyp24, and DEFB4 mRNA upon stimulation with 1,25D3. (mean fold change ± SEM, $n = 4$) (**B**) Detection of cathelicidin in monocytes by intracellular flow cytometry. Monocytes were stimulated with either control (solid line) or 1,25D3 (grey shaded area).

Isotype control is represented by the dashed line. Data shown is representative of four separate experiments. (**C**) Detection of cathelicidin derived peptides in monocytes by SELDI-TOF. In three separate experiments, SELDI-TOF analysis of monocyte cell pellets detected a peak around 4.5kDa consistently, as indicated by the arrow, corresponding to the cathelicidin peptide (LL-37). (**D**) Colocalization of mycobacteria and cathelicidin. Monocytes were infected with BCG-GFP (green) then stimulated with 1,25D3 and labeled using a monoclonal antibody specific for cathelicidin (red). CD68 is used as a marker for monocytes (blue). Data shown is representative of three separate experiments. (**E**) *M. tuberculosis* viability following incubation with recombinant cathelicidin peptide. Experiments were performed at various concentrations of cathelicidin peptide incubated with bacteria for three days. The bacterial viability was measured by both [^{3}H]uracil uptake (mean CPM ± SEM, $n = 3$) and CFU assay (mean CFU ± SEM, $n = 3$). (**F**) Antimicrobial activity of alveolar macrophages following 1,25D3 stimulation. Cells were infected with *M. tuberculosis* and stimulated with TLR2/1L. The number of viable *M. tuberculosis* bacteria were assessed by CFU assay. Data shown is representative of four experiments. **$p \leq 0.01$.

Fig. 3. Effect of serum on vitamin D receptor activation. (**A**) Effects of 25D3 supplementation to TLR2/1 stimulated monocytes. (mean fold change ± SEM, $n = 3$) (**B**) Effect of FCS and HS on TLR-induced VDR downstream genes. The ability of monocytes cultured in either FCS or HS to up-regulate cathelicidin and Cyp24 mRNA upon TLR2/1L stimulation measured by qPCR. (mean fold change ± SEM, $n = 4$) (**C**) 25D3 serum levels in FCS and HS as measured by radioimmunoassay. FCS data represents the same lot used throughout this study, and HS is the mean concentration (nM) ± SEM of four individual donors.

Fig. 4. Role of the vitamin D pathway in induction of cathelicidin mRNA and antimicrobial activity. Role of VDR (**A**) and Cyp27B1 (**B**) in induction of cathelicidin mRNA. Monocytes were cultured in HS and pretreated or with the Cyp27B1 antagonist ITRA (**A**) or the VDR antagonist VAZ (**B**) then induced with TLR2/1L and cathelicidin mRNA levels determined by qPCR. (mean fold change ± SEM, $n = 4$) (**C**) The role of the VDR in TLR2/1L induced antimicrobial activity. Alveolar macrophages were infected with *M. tuberculosis* and stimulated with media, TLR2/1L, and TLR2/1L with VAZ. Viable bacteria were quantified after four days of growth by CFU assay. Data shown is representative of eight experiments. (**D**) Serum concentration (nM) of 25D3 in African American and Caucasian donors as measured using radioimmunoassay. The mean of the values is represented by the line. (**E**) Effect of serum from African Americans or Caucasians on TLR2/1L induced cathelicidin mRNA levels. Monocytes were cultured in either African American or Caucasian serum, activated with TLR2/1L and cathelicidin mRNA levels determined using qPCR. Points are fold change of each individual donor tested, with the mean value indicated by the line. (**F**) Effect of supplementation of African American sera with 25D3 on TLR2/1L induced cathelicidin mRNA levels. Monocytes were cultured in African American sera supplemented with exogenous 25D3 at physiologic concentrations ranging 1-100 nM to optimize the signal to noise ratio due to monocyte variability, then stimulated with the TLR2/1L. Cathelicidin levels were determined using qPCR and averaged (mean fold change ± SEM, $n = 3$). *$p \leq 0.05$, **$p \leq 0.01$.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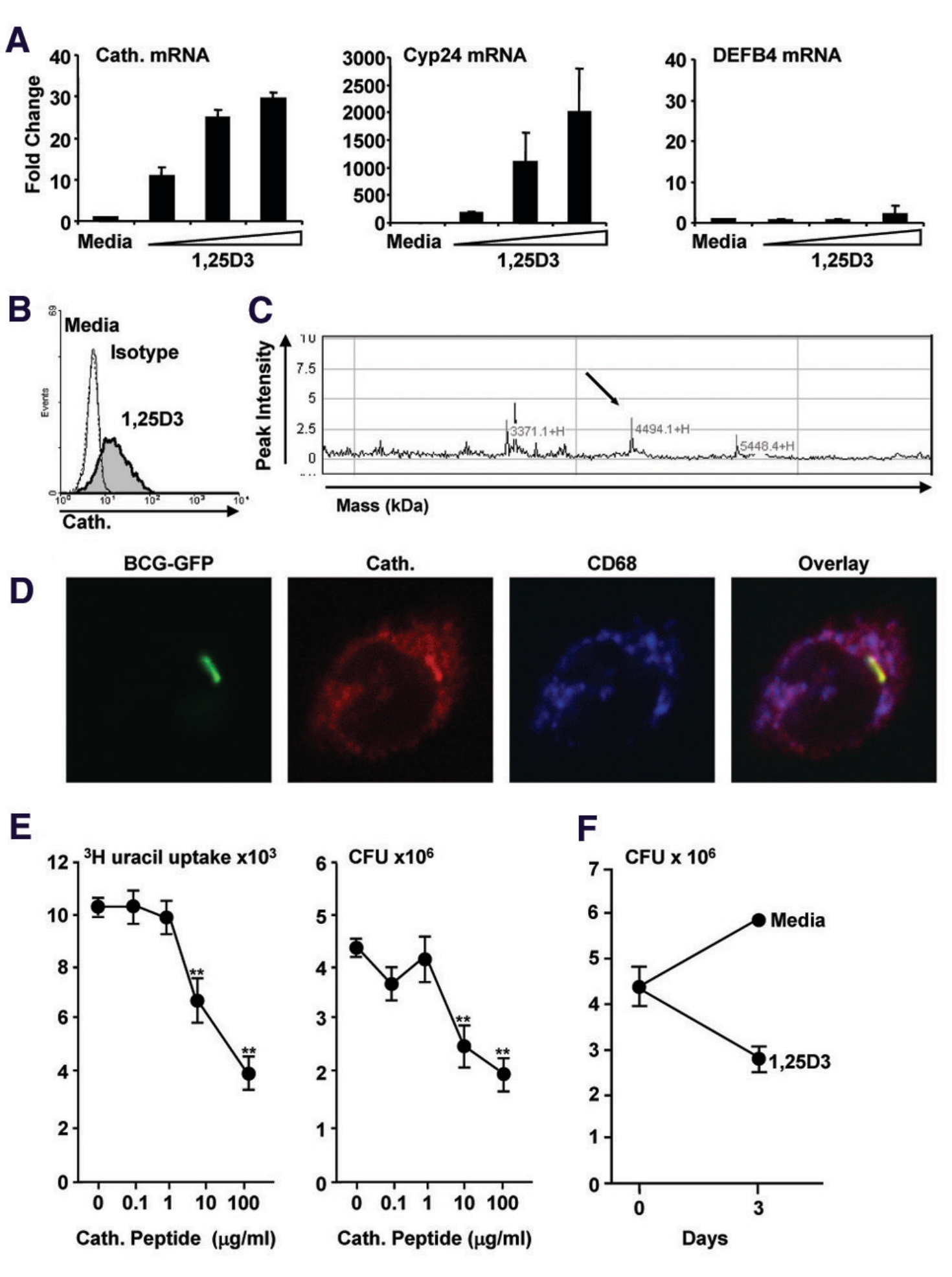
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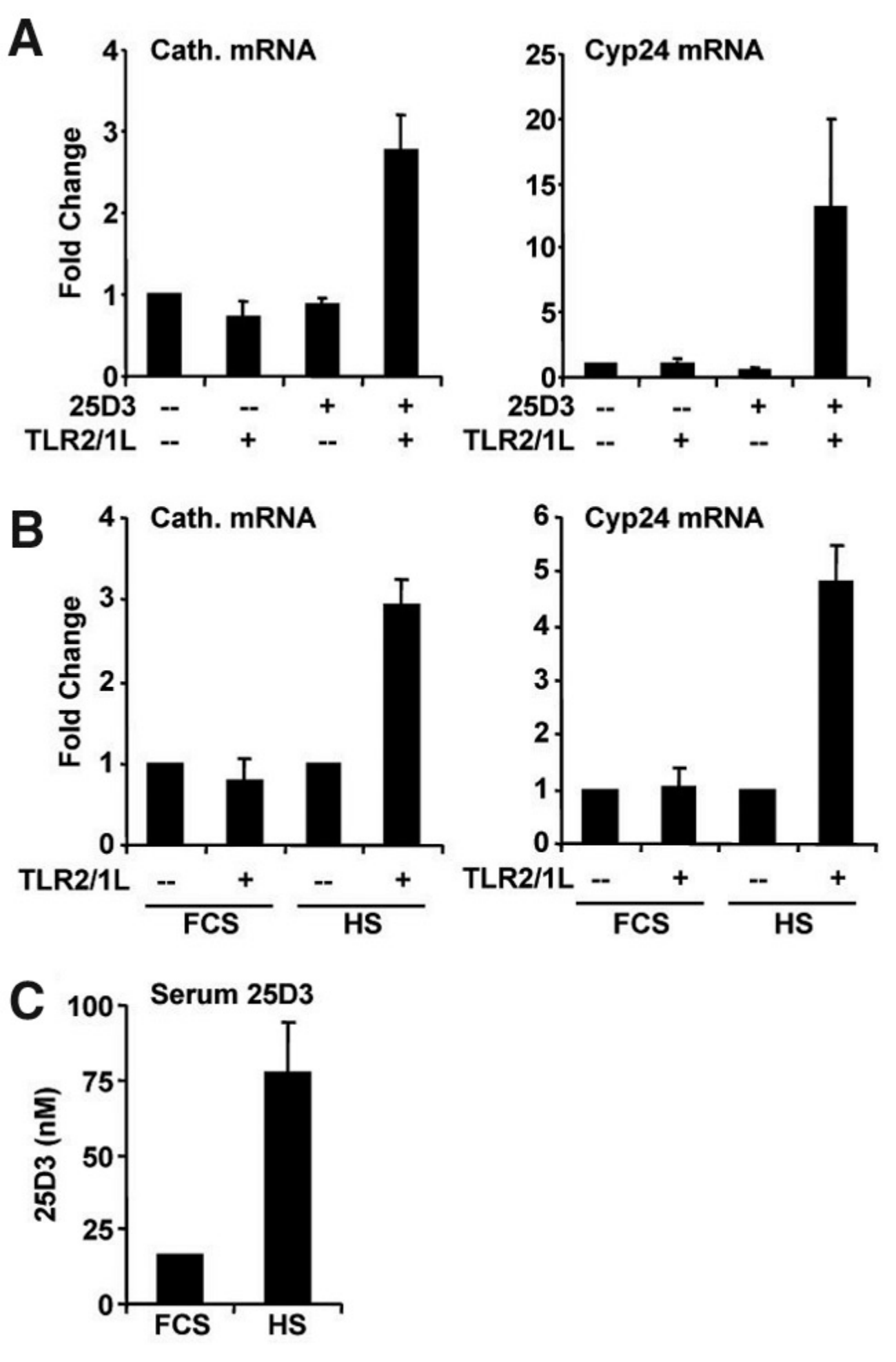
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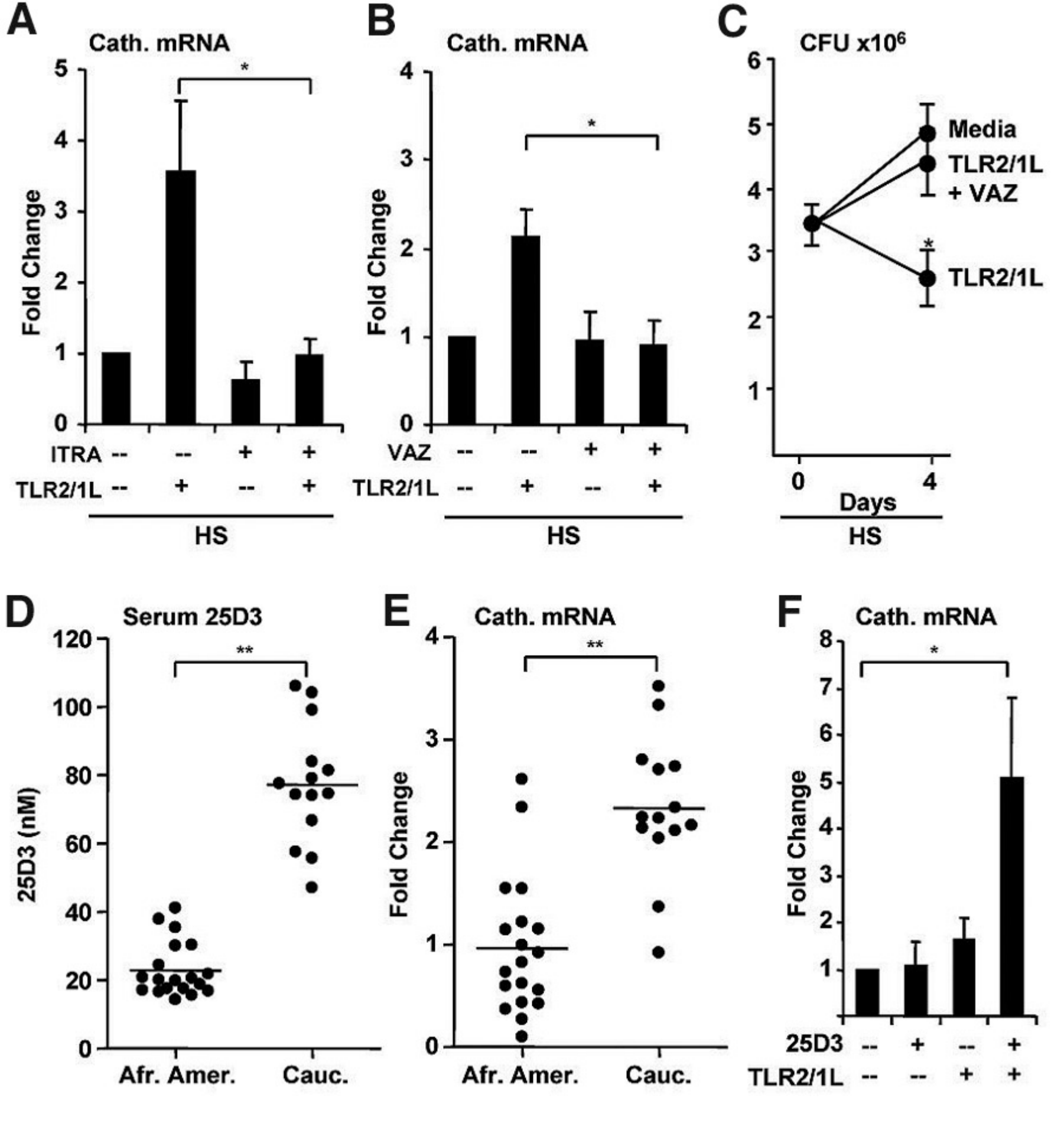
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