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A mouse model for vitamin D-induced human cathelicidin antimicrobial peptide gene expression

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Abstract

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AUTHOR CONTRIBUTIONS

M.B. Lowry and A.F. Gombart designed the research with important contributions from H.P. Koeffler, R.L. Gallo, A.K. Indra, G. Ganguli-Indra and J. Xie. M.B. Lowry, C. Guo, Y. Zhang, M.L. Fantacone, Y. Campbell, W. Zhang, I.E. Logan, M. Le, A.K. Indra, G. Ganguli-Indra, and A.F. Gombart performed the research and analyzed data. R.L. Gallo contributed the *Camp* KO mouse line. M.B. Lowry and A.F. Gombart wrote the manuscript with contributions from all the authors and all authors reviewed the submitted manuscript.

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In humans and other primates, 1,25(OH)$_2$vitamin D3 regulates the expression of the cathelicidin antimicrobial peptide (*CAMP*) gene via toll-like receptor (TLR) signaling that activates the vitamin D pathway. Mice and other mammals lack the vitamin D response element (VDRE) in their *CAMP* promoters. To elucidate the biological importance of this pathway, we generated transgenic mice that carry a genomic DNA fragment encompassing the entire human *CAMP* gene and crossed them with *Camp* knockout (KO) mice. We observed expression of the human transgene in various tissues and innate immune cells. However, in mouse *CAMP* transgenic macrophages, TLR activation in the presence of 25(OH)D_3 did not induce expression of either *CAMP* or *CYP27B1* as would normally occur in human macrophages, reinforcing important species differences in the actions of vitamin D. Transgenic mice did show increased resistance to colonization by *Salmonella typhimurium* in the gut. Furthermore, the human *CAMP* gene restored wound healing in the skin of *Camp* KO mice. Topical application of 1,25(OH)$_2$vitamin D_3 to the skin of *CAMP* transgenic mice induced *CAMP* expression and increased killing of *Staphylococcus aureus* in a wound infection model. Our model can help elucidate the biological importance of the vitamin D-cathelicidin pathway in both pathogenic and non-pathogenic states.

Keywords

cathelicidin; macrophage; innate immunity; vitamin D; TLR; Cyp27b1

1. INTRODUCTION

Vitamin D regulates expression of the cathelicidin antimicrobial peptide (*CAMP*) gene in both immune and epithelial barrier cells (1–3). We demonstrated conservation of regulation of *CAMP* by vitamin D in humans and non-human primates, but not other mammals including mice because their cathelicidin genes lacked a vitamin D receptor response element (VDRE) in their promoters (2, 4). The VDRE is located in a primate-specific AluSx short interspersed nuclear element (SINE) (2, 4).

Cathelicidins are synthesized as a prepropeptide consisting of an N-terminal signal peptide, a conserved prosequence (cathelin domain) and a highly variable C-terminal cationic AMP (5). In neutrophils, proteolytic cleavage generates the mature AMP called LL-37 (6, 7). While some mammals express numerous cathelicidins, humans and mice possess a single cathelicidin gene called *CAMP* or *Camp*, respectively (8, 9). The protein hCAP18 in humans and CRAMP in mice is synthesized and secreted in significant amounts by those tissues exposed to environmental microbes (10) as well as other tissues (11–14) and fluids (11, 12, 15–17) and is expressed by a wide array of immune cells (18–20). Subsequently, investigators demonstrated in vitro that TLR2/1-signaling by a synthetic 19-kD Mycobacterium tuberculosis–derived lipopeptide enhances the antimicrobial capacity of human monocytes via a vitamin D_3-dependent pathway through induction of the CAMP gene and its protein (21–23). Other lines of evidence similarly indicate that hCAP18/CRAMP has important functions in host defense and wound healing in the skin (24–26). Mice deficient in CRAMP are more susceptible to skin infection than WT mice (27), and bacteria induce hCAP18/CRAMP secretion that protects the murine urinary tract against invasive bacterial infection (28). Decreased expression of CAMP in particular human

diseases is linked to enhanced susceptibility to infection (29–33). Collectively, these data strongly implicate a key role for *CAMP* in maintaining adequate host defenses.

In the current model, when a human macrophage or epithelial barrier cell detects a pathogen via TLRs, the signal induces expression of the 1-α-hydroxylase (CYP27B1) gene. This increases the 1-α-hydroxylation of $25(OH)D_3$ that is taken up from the circulation and increases the local levels of $1,25(OH)_2D_3$ which binds to the VDR. This induces transcription of the CAMP gene, and the resulting protein participates in host defense (21, 34, 35). This mechanism could explain the ability of vitamin D to boost the innate immune response and protect against infection.

In this study, we developed a transgenic C57BL/6J mouse that carries a human genomic DNA fragment containing the *CAMP* gene to elucidate the biological importance of this pathway in the immune response. We subsequently crossed the transgenic with a *Camp* KO mouse to generate mice that possesses the human *CAMP* gene but lack the mouse homolog. We characterized expression of the transgene in various tissues including immune cells. Human *CAMP* transgenic mice showed increased resistance to colonization by *Salmonella typhimurium* in the intestinal tract, and the human gene also restored wound healing in the *Camp* KO mouse. The human *CAMP* gene was responsive to induction with $1,25(OH)_2D_3$ both *in vitro* in bone marrow-derived macrophages and *in vivo* in the skin of transgenic mice. In a wound infection model, topical skin treatment of the *CAMP* transgenic mouse with $1,25(OH)_2D_3$ lowered the burden of *Staphylococcus aureus* infection, suggesting a functional role for vitamin D induction of *CAMP* in skin immune defense.

Interestingly, comparing expression of the human *CAMP* gene in human and transgenic mouse macrophages substantiated important evolutionary differences in how mice utilize the vitamin D_3 pathway in response to immune stimulation via TLR activation. Transgenic mouse macrophages did not induce *CAMP* following TLR stimulation in the presence of $25(OH)D_3$, due to a lack of *Cyp27b1* expression and induction. We could bypass this problem in transgenic mouse macrophages by inducing *CAMP* with $1,25(OH)_2D_3$ directly. This novel animal model for an innate immune mechanism specific to humans will help elucidate the biological importance of the vitamin D_3-cathelicidin pathway in both pathogenic and non-pathogenic states when using bioactive forms of vitamin D such as $1,25(OH)_2D_3$ and its analogs.

2. MATERIALS AND METHODS

2.1. Generation of human CAMP transgenic mice.

Transgenic mice carrying the human *CAMP* gene were generated by microinjection of donor eggs from the F2 of C57BL/6J x DBA/2 mice with a 6.02-kb KpnI – EcoRI restriction enzyme fragment (chr3:48221417 – 48227439, UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly). This fragment was isolated from a human genomic λDASH library (36) and contained the gene with 2.09 kb of upstream and 2.02 kb of downstream sequence (Fig. 1). The UCLA/JCC ES Cells & Transgenic Mice Shared Resource (Los Angeles, CA) performed the microinjection of mouse eggs and obtained two founder lines. The two lines were backcrossed nine times with C57BL/6J (The Jackson

Laboratory, Bar Harbor, ME). To obtain mice that lacked the murine *Camp* gene, the human transgenic (Tg^+) mice were crossed with *Camp*-deficient (KO) mice on the C57BL/6J background (27). Mice were screened by standard endpoint and quantitative PCR (protocols available upon request) to identify dizygous human *CAMP* (Tg^+/Tg^+) – *Camp* KO/KO mice. Studies were performed with WT, Tg^+/Tg^+: WT/WT, Tg+/Tg+: KO/KO and KO/KO mice. In this report, we refer to the dizygous transgenic mice as Tg/WT for *CAMP* on the WT background, and Tg/KO for *CAMP* on the *Camp* KO background. The integration site of the transgene of the line used in this study was mapped by TLA sequencing to mouse chromosome 4: site 140,451,533–140,456,007 resulting in a 4.5kb deletion of genomic DNA at the insertion site (37) (Cergentis, Utrecht, Netherlands). No coding region was impacted directly by the insertion of about 8 Tg-Tg concatemers. All mice used in this research were bred and maintained in the same temperature (23±1°C), humidity (60–70%), and lighting (6:00 am to 6:00 pm) controlled rooms under specific pathogen-free conditions in an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility at Oregon State University (OSU). Animals were housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the OSU Institutional Animal Care and Use Committee.

2.2 Analysis of gene expression by qRT-PCR.

Murine tissues were preserved in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was isolated using Trizol (Thermo Fisher Scientific) or Direct™-zol RNA kits (Zymo Research Corp, Irvine, CA, USA) according to the manufacturer's protocol. Tissues were homogenized using nuclease-free 1.6 mm stainless steel beads in a Bullet Blender (Next Advance, Inc., Averill Park, NY, USA). RNA (0.25–1 μg) was converted to cDNA using iScript reverse transcriptase and random hexamer primers (Bio-Rad Laboratories, Hercules, CA USA) according to the manufacturer's recommendations. PCR reactions were performed with SsoAdvanced Universal Probes Supermix on a Bio-Rad iCycler iQ5 or CFX-96 QPCR system (Bio-Rad Laboratories). All the threshold cycle (Ct) numbers were normalized to 18S rRNA, -actin or Ywhaz. The probes and primers for the human CAMP and RN18S1 genes were described previously (38). Primers 5'-gtacatggctggggtgtt-3' 5'-ttctacaatgagctgcgt gt-3' and probe dCal Gold 540 5'-aggtctcaaacatgatctgggtcatct-3' BHQ-2 for β-actin were purchased (Thermo Fisher Scientific and Biosearch Technologies, Novato, CA, respectively). Primer-probe mixes for *Cyp24A1*, *Cyp27B1*, and *Ywhaz* were purchased (Integrated DNA Technologies, Coralville, IA, USA).

2.3 Western Blot analysis, ELISA, immunohistochemical staining and immunofluorescence staining.

The primary rabbit polyclonal anti-hCAP18 antibody was described previously (39). Murine monoclonal anti-LL-37 antibody was a kind gift from Dr. Birgitta Agerberth (Karolinska Institute, Stockholm, Sweden). Immunofluorescent staining and flow cytometry were performed as described previously (2, 38, 40). Antibodies used against lineage markers were purchased (Thermo Fisher Scientific). The levels of hCAP18 (ng/ml) in each tissue sample were determined using a non-commercial ELISA with a detection limit of 0.084 ng/ml and an intra- and inter-assay coefficient of variation of ≤6.3% (39). Western blots were performed as described previously (2). Briefly, tissue samples were flash frozen in liquid

nitrogen, and were then homogenized in ice-cold RIPA buffer containing cOmplete™ protease inhibitor cocktail (Millipore Sigma, St. Louis, MO USA) and nuclease/protease-free 2.4 mm stainless-steel beads (OMNI International, Kennesaw, GA USA) using a Precellys 24 homogenizer (3 × 30 s cycles). Immunofluorescence staining and microscopy were performed as described previously (41, 42).

2.4 Tissue culture.

For *ex vivo* experiments, fresh tissues were cultured in DMEM (Mediatech Inc., Manassas, VA, USA) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% Pen/Strep (Invitrogen Corporation, Carlsbad, CA, USA) at 37°C in a humidified 5% CO2 incubator. Mouse BM-derived macrophages (BMDM) and human PBMC-derived macrophages cells were cultured as described previously (2, 43). Briefly, mouse bone marrow was differentiated with MCSF at 25ng/ml for 7 days, while human PBMC were cultured for 7 days with GM-CSF at 25ng/ml prior to experiments. Whole blood from healthy volunteers was collected under approved IRB guidelines. The stock solution of $1,25(OH)_2D_3$ (1 mM) was prepared in 100% ethanol and subsequently diluted in culture media to a concentration as described in the figure legends.

2.5 Phagocytosis and Superoxide assays.

BMDM were mixed with fluorescently labeled inactivated *Escherichia coli* bioparticles and processed as described by the manufacturer (pHrodo *E. coli*, Thermo Fisher Scientific). The samples were analyzed on a BD Facscalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The pHrodo bioparticles increase fluorescence when internalized into acidic phagosomes, thus the percentage of cells internalizing particles and the mean fluorescence intensity were used as measures of phagocytosis.

BMDM or neutrophils were stimulated with PMA (3 μg/ml), incubated at 37°C with a dura-luminol substrate (Thermo Fisher Scientific, Waltham, MA, USA) and monitored for superoxide production using a 96 well plate luminometer (RLU every 2 min for 30 min).

2.6 In vivo gastrointestinal infection with Salmonella typhimurium.

S. typhimurium strain JS135, a generous gift from Dr. James Slauch (University of Illinois, Champaign Urbana, USA), was grown in Luria Bertrani (LB) broth with 25 μg/ml tetracycline overnight (44). The bacteria were pelleted, washed three times in PBS and density adjusted to an OD_{600} of 1.0 (1.2×10^9 CFU/ml). Infectious doses were calculated and re-suspended in a total of 0.2 ml PBS for oral gavage. Prior to infection, mice were fasted 10 to 12 hours. Post infection, food was returned, and mice monitored for symptoms. Mice were euthanized 3 and 5 days post-infection, spleens and ceca were collected, homogenized and serially diluted for bacterial CFU determination on LB agar plates containing 25 g/ml tetracycline. The inoculum used for infection was also plated to enumerate the starting CFU used for infection.

2.7 In vivo treatment of skin with vitamin D, skin wounding and LPS injection.

A dorsal patch of skin near the shoulder blades was shaved one day prior to treatment. For treatment, 1 nmole of $1,25(OH)_2D_3$ was dissolved in vehicle consisting of a 50% glycerin/

ethanol solution. Either 6 l of vehicle or 1,25$(OH)_2D_3$ was applied to the skin and spread to facilitate drying. After 24 to 48 h, mice were sacrificed and skin samples were processed for RNA or protein, respectively (42). Skin wounding and analysis of healing was performed as described previously (42). Mice were IP injected with 10 μg/g body weight LPS in PBS or either 0.5 μg/g body weight LPS or with PBS. After 3h or 24 h, mice were euthanized, and tissues were collected and processed for RNA.

2.8 Staphylococcus aureus wound infection model.

Mice were shaved on a dorsal patch of skin as in 2.6 and then a single 5mm skin punch was made using a circular biopsy punch under anesthesia. Mice were injected subcutaneously with Buprenorphine-SR for pain management post wounding (ZooPharm, Fort Collins, CO). Mice were treated 24h later with vehicle or 1,25$(OH)_2D_3$ as in part 2.6 directly onto the wounded area. The following day 8×10^5 CFU of *S. aureus* Rosenbach strain were applied in a 10μl volume to the wound, and the mice were bandaged with non-stick gauze and Tegaderm™ transparent film dressing (3M™, St. Paul, MN). Bandages were removed 24h post infection and mice were monitored daily. At 2 days post infection, the mice were treated again with either vehicle or 1,25(OH)2D3 at the wound site. At 5 days post infection, mice were euthanized and a 10mm circular biopsy of the skin was taken at the wound site. The skin samples were placed in 1ml PBS and homogenized using steel 2.4mm beads in a Precellys 24 homogenizer. The lysate was then serially diluted and plated on tryptic soy agar plates to enumerate surviving *S. aureus* bacteria.

2.9 Statistical analysis.

Statistical analysis was performed using GraphPad Prism version 7 software (GraphPad Software, La Jolla, CA, USA). T-test and one-way ANOVA with Tukey's multiple comparison post-test were used for determination of significance ($p<0.05$).

3. RESULTS

3.1 Generation of a human *CAMP* transgenic mouse.

Because the human *CAMP* gene is regulated by vitamin D but the murine gene is not, in vivo studies with mice would have limited or uncertain relevance to the situation in humans (2, 4). To study the in vivo role of vitamin D-induced human *CAMP*, we generated a transgenic mouse with a 6.02 kb *KpnI-EcoRI* fragment containing all four exons (~2 kb) and flanking sequence (~2 kb 5'of the first exon and ~2 kb 3' of the last exon). The fragment was isolated from a human placental genomic DNA library (Stratagene, La Jolla, CA) and sub-cloned it into pGEM3Z (Promega Corporation, Madison, WI, USA) (Fig. 1A). This fragment contains the region of highest conservation among all mammals in the current database (Fig. 1A) including the AluSx SINE with the VDRE. The high degree of conservation in the promoter region and 3'-downstream region suggests the presence of important regulatory elements controlling the expression of the gene. Potential transcriptional regulatory elements in the *CAMP* transgene fragment have been described previously (2, 45–47). The human *CAMP* promoter (−693 to +14) contains potential binding sites for the CCAATT displacement protein (CDP), STAT3, C/EBP, PU.1, HIF-1 and VDR. DNase I hypersensitivity peak clusters are found immediately upstream of the start ATG and

within the 5'-half of the AluSX SINE in the region of the VDRE (UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly). In addition, the Genome Browser shows numerous H3K27Ac sites in the 5'-promoter region which are generally associated with increased transcriptional activation (48). Transfection of the 6.02 kb fragment into murine NIH3T3 cells and treatment with $1,25(OH)_2D_3$ induced human *CAMP* gene expression from the genomic DNA as detected by qRT-PCR (data not shown). Two founder transgenic lines were subsequently backcrossed nine generations onto the C57BL6/J mouse strain and crossed with the *Camp* KO mouse on the same background (27). The insertion site of the human *CAMP* gene into the mouse genome of the line used in this study was mapped using TLA sequencing (37). A single insertion site was identified on mouse chromosome 4, position 140,451,533 to 140,456,007. The insertion of the transgene resulted in a 4.5 kb deletion of mouse genomic DNA that does not contain any known coding genes. The transgene inserted as a concatemer of eight copies per chromosome or 16 copies total in the dizygous mice. Experiments were performed with Tg^+/Tg^+: WT/WT mice, which we term Tg/WT mice, as well as mice with the human *CAMP* gene on the mouse *Camp* KO background (Tg^+/Tg^+: KO/KO), which we term Tg/KO mice.

3.2 Tissue specific expression of the human *CAMP* gene in transgenic mice.

We used qRT-PCR to determine human *CAMP* gene expression from various tissues in the transgenic mice (Fig. 1B). As expected for hematologic tissues and immune cells, the gene was highly expressed in the BM and spleen, and in non-hematologic organs we observed the highest levels in the salivary glands, followed by skin, lung, liver, ileum, colon and kidney (Fig. 1B). An ELISA for hCAP18 protein expression in tissues and plasma showed that expression generally paralleled the levels of mRNA expression (Table 1). We detected the highest level of hCAP18 in bone marrow, followed by plasma, salivary gland, skin, spleen and lung. To characterize further the expression of hCAP18 in cells of the immune system, FACS staining was performed with splenocytes, peritoneal lavage, and blood and bone marrow cells. We detected little or no expression in $CD3^+$ T-cells from the spleen (Fig. 2A), but significant expression in $CD19^+$ splenic B-cells (Fig. 2B), limited expression in $CD19^+$ positive peritoneal B-cells and robust expression in $F4/80^+$ peritoneal macrophages (Fig. 2C). As expected, $Gr\text{-}1^+$ neutrophils from the blood and total bone marrow expressed high levels of hCAP18 (Fig. 2D & E).

3.3 Phagocytosis and superoxide production are not altered by human *CAMP* in murine cells.

To determine if the human transgene affected phagocytic function, we tested BMDM from WT, Tg/KO and *Camp* KO macrophages for phagocytosis using labeled *E. coli* and FACS. The percentage of phagocytic macrophages detected was similar among the three genotypes with no statistically significant differences (Fig. 3A). We also tested superoxide production by WT and Tg/WT macrophages that were stimulated with PMA. We found similar production for each genotype with no statistically significant differences in macrophage superoxide production (Fig. 3B). Neutrophils produce much higher levels of superoxide than macrophages and are the most potent immune cell producers of superoxide. Exogenous LL-37 can induce superoxide production by human neutrophils (49), therefore, we tested whether the transgene in murine neutrophils altered their superoxide production. Bone

marrow neutrophil superoxide production was compared between WT and Tg/WT mice, with no statistical differences noted (Fig. 3C). Thus, the human transgene does not alter superoxide production in murine neutrophils.

3.4 The human *CAMP* Tg decreases *S. typhimurium* burden in the murine cecum during infection.

To determine the ability of the human *CAMP* transgene to protect against infection in the gastrointestinal tract, WT, *Camp* KO and *CAMP* Tg/WT were infected by oral gavage with 8×10^8 CFU *S. typhimurium*. Five days post-infection, mice were euthanized and the burden of infection was determined in both their cecum and spleen. The number of CFUs in the cecum of *CAMP* Tg/WT mice was significantly lower than the burden in the WT and the *Camp* KO mice (Fig. 4A, **left panel**). WT and the *Camp* KO mice were not significantly different, but the burden of infection in the *Camp* KO trended higher than in the WT (Fig. 4A, **left panel**). The splenic burden of infection trended downward in the *CAMP* Tg/WT and upward in the *Camp* KO as compared to the WT, but values were not statistically significant (Fig. 4A, **right panel**). In additional experiments comparing the *Camp* KO with the human *CAMP* Tg/KO mice, mice were infected with a 10-fold lower dose (8×10^7 CFU) of *S. typhimurium* and surviving CFU were measured in the cecum and spleen five days post infection. Human *CAMP* Tg/KO mice showed a greater resistance to infection than the *Camp* KO mice as reflected by the statistically significant lower burden in the cecum (Fig. 4B, **left panel**). The transgenic mice showed a non-significant trend toward lower splenic burden than the knockout (Fig. 4B, **right panel**). In summary, the *CAMP* transgene increases protection and lowers CFU burden in the murine cecum, but does not reduce systemic spread once *S. typhimurium* escapes the intestinal tract.

3.4 Induction of the human *CAMP* gene in murine cells and tissues *in vitro*.

CAMP expression is induced in human macrophages following treatment with $1,25(OH)_2D_3$. To test the response in Tg/KO macrophages, BMDM were treated with increasing doses of $1,25(OH)_2D_3$ ranging from 1 to 100 nM for 48 h. A dose-responsive increase in hCAP18 protein expression occurred as determined by FACS (Fig. 5A), showing that mouse Tg macrophages responded like human macrophages. Induction of *CAMP* gene expression was also observed in tissues cultured *in vitro* after treatment with 100 nM $1,25(OH)_2D_3$ for 24 h (data not shown). Strong induction of *CAMP* was observed in the kidney with modest increases in liver, ileum, colon and spleen (data not shown). Variability in the level of induction occurred between mice, with some responding more strongly than others. Overall, the results are consistent with expression of the VDR in most tissues.

3.5 Topical application of $1,25(OH)_2D_3$ on skin of Tg mice induces *CAMP* mRNA and hCAP18 protein.

Humans and mice express cathelicidin in the skin where this protein functions in barrier defense and wound healing (24, 26). In humans, topical application of $1,25(OH)_2D_3$ and an analog calcipotriol to the skin induces both mRNA and hCAP18 protein (3, 50). To test the induction of the *CAMP* transgene in the skin of Tg/KO mice, a dorsal patch of skin was shaved and either vehicle or 1 nmole 1,25(OH)2D3 was applied to the area. After 24 h, a

significant increase in *CAMP* mRNA expression was detected by qRT-PCR in the group of mice receiving $1,25(OH)_2D_3$ (Fig. 5B). Strong induction of another VDR target gene *Cyp24a1* was also measured in the skin of the treated group (Fig. 5C). To determine the induction of hCAP18 protein, the experiment was repeated with two groups of mice and hCAP18 levels were measured by ELISA after a 48 h treatment. A significant induction of hCAP18 protein was observed in the skin of the $1,25(OH)_2D_3$- treated mice as compared to the controls (Fig. 5D).

While the ELISA quantifies the level of hCAP18 protein expression, it does not discriminate between the various processed forms of the protein. In humans, hCAP18 is processed by proteolytic cleavage to produce a 14-kDa fragment containing the cathelin-like pro-domain and a 4-kDa C-terminal fragment known as LL-37. LL-37 possesses antimicrobial activity and promotes wound healing. We characterized processing of the hCAP18 protein into its 14-kDa and 4-kDa forms in the mouse using western blot analysis. Using a rabbit polyclonal antibody (39), the full length hCAP18 protein was detected and the processed 14-kDa form was found in protein extracts from BM and skin of Tg mice (Fig. 5E). The amount of processing was significantly less in the skin than the BM. The full-length, but not the 14-kDa form was detected in the lung and spleen of the Tg mice, suggesting minimal if any processing of the 18-kDa form (Fig. 5E). The 4-kDa LL-37 fragment was not efficiently detected by the rabbit polyclonal, so a mouse monoclonal antibody specific to LL-37 (51) was used to detect both the hCAP18 full length protein as well as a 4-kDa protein in BM cells from Tg mice that co-migrated with a synthetic LL-37 peptide (Fig. 5F). Skin samples from $1,25(OH)_2D_3$-treated animals were included and a low abundance band was identified that co-migrated with the synthetic 4-kDa LL-37 peptide, consistent with a very low level of processing in the skin (Fig. 5F). Specificity of both antibodies was confirmed using the BM and skin of a WT mouse that were negative for both hCAP18 and LL-37 (Fig. 5E & F). These patterns of detection are consistent with previously published work from human neutrophils and semen (7, 26, 52). Our findings suggest that processing of human hCAP18 protein occurs in the mouse producing both the 14-kDa cathelin-like domain and the 4-kDa peptide in bone marrow and possibly at low levels in unperturbed skin.

To determine expression of hCAP18 *in situ* in the skin, immunofluorescence microscopy was performed on skin samples from vehicle and 1,25(OH)2D3-treated mice (Fig. 6). hCAP18 staining was not found in the epidermis of vehicle-treated skin, but was noted to increase in the epidermis in 1,25(OH)2D3-treated skin (Fig. 6, **upper vs. lower panels**). Higher levels of hCAP18 were detected in the dermis as compared with the epidermis, and again in the 1,25(OH)2D3-treated mice an increase in the staining intensity was observed in the dermis with notable expression near the base of hair follicles (Fig. 6, **lower panels**).

3.6 Human *CAMP* transgene restores wound healing in *Camp* knockout mice.

In mice, the *Camp* gene promotes wound healing and closure in the skin (24). Based on the expression data described above, we hypothesized that the presence of the human *CAMP* gene would restore defective wound healing observed in the *Camp* knockout mice. Following a full-thickness wound using a 5 mm skin punch in each flank of the mice, wound closure was monitored over time in Tg/KO, *Camp* KO and WT mice. The wounds in *Camp*

KO mice showed minimal closure and contraction of the wound over nine days after the initial skin punch (Fig. 7A). The defect observed with the *Camp* KO mouse was quite striking, in that at five days post wounding no measurable signs of wound healing or closure had occurred, while the WT and Tg/KO mice showed about 50% wound closure and healing (Fig. 7A). The rate of wound closure in the Tg/KO mice was identical to that observed in WT mice throughout the nine days. The *Camp* KO mice showed additional tissue damage and scab formation that was not observed in the Tg/KO or WT mice (Fig. 7B). Taken together, these data demonstrate that the human *CAMP* gene restores the healing defect observed in the *Camp* KO mice.

3.7 Topical application of $1,25(OH)_2D_3$ on the skin of Tg mice increases killing of *S. aureus* in a wound infection model.

To test whether application of 1,25(OH)2D3 to the skin of transgenic mice could improve resistance to wound infections, we infected mice with *S. aureus* and compared bacterial killing in the skin between vehicle control and 1,25(OH)2D3 treated mice. Tg/KO mice were wounded using a 5mm skin punch on a single dorsal site and 24 h later, matched littermates were treated with either vehicle control or 1nmole 1,25(OH)2D3 directly on the wound site. The next day the wound was infected with 8×10^5 CFU of *S. aureus*, and then 2 days post infection the wound sites were re-treated with either vehicle or 1,25(OH)2D3. Five days post infection, a 10mm biopsy around the wound site was taken and surviving *S. aureus* CFU were measured (Fig 7C). In the group of mice treated with the vehicle control, the mean log CFU of *S. aureus* was 6.2±0.4 (1,840,000 mean CFU) while the $1,25(OH)_2D_3$ treated group of mice had a statistically significant decrease to log 4.8±0.4 CFU (74,400 mean CFU) (Fig 7C). Therefore, in the Tg/KO mice, topical skin treatment with $1,25(OH)_2D_3$ significantly lowered the burden of *S. aureus* in a wound infection model.

3.8 Absence of *Cyp27b1* gene induction upon TLR activation of macrophages in mice compared to humans.

In humans, activation of macrophages with TLR ligands induces CYP27B1 expression and subsequent synthesis of active $1,25(OH)_2D_3$ from 25(OH)D3 which then induces *CAMP* expression (21). We tested whether activation with TLR ligands in the presence of $25(OH)D_3$ would induce *CAMP* expression in Tg mouse bone marrow derived macrophages, but found no induction by LPS, PolyIC, and flagellin (data not shown). One explanation for the lack of *CAMP* induction in mouse macrophages is that TLR activation does not induce *Cyp27b1* gene expression, as has been previously reported for LPS (53, 54). In BMDM from *CAMP* Tg/WT mice, induction of *Cyp27b1* was not observed 24 h post-treatment with either PolyIC, LPS, or flagellin (Fig. 8A). Priming of mouse macrophages with IFN-γ prior to treatment with TLR ligands did not influence the lack of *Cyp27b1* induction following TLR stimulation (data not shown). A second vitamin D target gene, *Cyp24a1*, also showed no induction in Tg/WT macrophages following TLR stimulation (data not shown). On the other hand, basal expression of *CYP27B1* in unstimulated human monocyte PBMC-derived macrophages was 5-log higher in unstimulated cells compared with the basal level in unstimulated mouse macrophages (Fig. 8A). Human *CYP27B1* was significantly induced 24 h post-treatment with LPS (Fig. 8A). Also, very low basal levels of *Cyp27b1* in BM-derived dendritic cells from Tg/WT mice were observed with no significant

induction 24 h post-treatment with TLR ligands PolyIC, LPS, flagellin, or CpG DNA (data not shown). In summary, these results demonstrate significant differences between mouse and human macrophages and myeloid dendritic cells with regards to basal and TLR-induced *Cyp27b1* expression and explains why human *CAMP* is not induced in these mouse cells in the presence of 25(OH)D3 and TLR ligands.

3.9 Expression and induction of *Cyp27b1 in vivo* in mice.

The lack of basal *Cyp27b1* expression and its induction in mouse macrophages during TLR activation raises the question, what is the source of its activity during the mouse immune response? In humans and mice *Cyp27b1* is strongly expressed in the kidney where it serves a primary role in producing $1,25(OH)_2D_3$ that circulates systemically (55–58). In mice, expression of *Cyp27b1* in other tissues and cells is not well characterized, but is much lower than in the kidney (54, 59, 60). We hypothesized that TLR activation may induce *Cyp27b1* expression in cells from other tissues, thus providing a source of 1,25(OH)2D3 during infection and compensating for the lack of synthesis by macrophages. To test this, *CAMP* Tg/WT mice were injected IP with either vehicle control or LPS at 20 g/g body weight and the expression of *Cyp27b1* was measured 24 h post injection in the kidney, salivary gland, skin, lung, ileum and colon using qRT-PCR (Fig. 8B). As expected, we observed high levels of basal *Cyp27b1* expression in the kidney followed by lower levels in the salivary gland and skin (about 1 and 1.5 logs lower, respectively), and even lower expression in the lungs, ileum, and colon (about 2–3 logs lower). LPS did not significantly induce expression of *Cyp27b1* in most organs with the exception of the lungs (approximately 6-fold increase) (Fig. 8B). *Cyp24a1* expression was also measured in this same experiment by qRT-PCR, but no significant induction was found (data not shown).

A second study was performed in WT mice to determine if a lower dose of LPS at an earlier time point might induce *Cyp27b1*. Mice were injected IP with LPS at 0.5 g/g body weight. A significant induction of *Cyp27b1* mRNA occurred in the kidneys at 3 h post-injection (Fig. 8C), associated with a concurrent decrease in *Cyp24a1* expression (Fig. 8D). In all other tissues, no significant increase in *Cyp27b1* expression occurred (data not shown). These findings suggest that the kidney may respond to systemic inflammatory challenges with a transient increase of *Cyp27b1* at early time points (3 h) that returns to basal levels at a later time point (24 h). Because of the lower expression and a lack of strong induction of *Cyp27b1* in most tissues following a systemic inflammatory challenge, the role that extra-renal production of 1,25(OH)2D3 has in the murine immune response is unclear.

4. DISCUSSION

In this report, a human *CAMP* transgenic mouse was generated and characterized. The BM and immune cells express hCAP18 in a pattern similar to that observed in humans, with neutrophils showing the highest expression, followed by macrophages and lower expression in B-cells (43). Mature murine peritoneal macrophages and BM-derived macrophages expressed levels comparable to that observed in human monocytes and in monocyte-derived macrophages (1, 2). Salivary glands from the transgenic mouse also expressed abundant hCAP18 protein, suggesting a protective function in the oral mucosa. Salivary glands and

lingual epithelial cells in mice express CRAMP and in humans, hCAP18/LL-37 is detected in saliva (12). Deficiency of LL-37 in humans due to congenital defects in morbus Kostmann syndrome results in severe periodontal disease, highlighting the importance of LL-37 in protection of the oral cavity (29). We also detected expression of both *CAMP* mRNA and hCAP18 in the skin of transgenic mice that parallels an important site of expression in humans (10, 26). In both humans and mice, cathelicidin expression is increased following wounding and it is important for wound healing (24, 27). Expression of CRAMP in the skin and neutrophils of mice is also important in defending against group A streptococcal skin infections (27). We detected hCAP18 protein by ELISA in the spleen, lungs, intestinal tract, kidney and liver (Table 1). Using two different antibodies against hCAP18 and LL-37, we observed processing of hCAP18 into both the 14-kDa cathelin pro-domain fragment and the 4-kDa peptide in BM cells of the Tg mouse. While this peptide co-migrated with synthetic LL-37 peptide and was detected by the anti-LL37 antibody, additional studies are needed to determine if it is LL-37.

In the intestinal tract, the human transgene increased resistance to colonization by the enteric pathogen *S. typhimurium* in the cecum. The presence of the transgene on the mouse WT background increased resistance to colonization compared with the mouse gene alone, suggesting an additive effect of having both the mouse and human genes together in the Tg/WT mice. When the human transgene was present in the *Camp* KO background, increased resistance to colonization of the cecum occurred as compared to the KO mouse, confirming the functional role of *CAMP* in the intestinal tract. The role of the endogenous mouse *Camp* gene in the intestinal tract was previously defined using the KO mouse strain. These mice had an increased susceptibility to enteric infections by *Citrobacter rodentium* (61).

$1,25(OH)_2D_3$ induced the *CAMP* transgene in a dose-dependent manner *in vitro* in transgenic mouse BMDM. Organs from transgenic mice cultured *in vitro* with $1,25(OH)_2D_3$ also showed induction of *CAMP* mRNA to various degrees depending on tissue type. Topical application of $1,25(OH)_2D_3$ to the skin induced in vivo *CAMP* expression in the mouse. In humans, application of the vitamin D analog calcipotriol induced hCAP18 protein expression in human skin (3). Calcipotriol is used clinically to treat psoriasis and has several immune modulating functions, including decreasing expression of Th17 pathway cytokines IL-17 and IL-23 (62, 63), while the role of hCAP18 in modulating psoriasis is less clear. The processed form of the protein, LL-37, is increased in psoriatic lesions (26) where it may play a role in enhancing inflammation, yet it also has been described to decrease inflammasome activation in response to cytosolic self-DNA in keratinocytes (64). In contrast to psoriasis, atopic dermatitis patients present with lowered expression of LL-37 and suffer increased susceptibility to skin infections (25, 65). Using immunohistochemistry, we previously observed in our *CAMP* transgenic mouse model that wounding increased hCAP18 expression in the skin; while in normal skin, expression was lower and not evident in keratinocytes by immunohistochemistry (42). Our findings are consistent with prior studies showing low expression in normal skin, but elevated levels upon wounding and inflammation (10, 24, 26).

When crossed onto the mouse *Camp* KO background, the *CAMP* transgene restored wound healing in the skin, demonstrating that the human gene can complement the function of the mouse *Camp* in vivo. Comparing the wound healing rate between the transgenic *CAMP* Tg/KO, WT and *Camp* KO mice showed that both the human and mouse forms of cathelicidin promote healing at similar rates at the time points measured while the KO showed a severe lack of healing. Thus, both the mouse and human cathelicidins appear to play similar roles in wound healing. The similar rates of wound healing also suggest that transgenic hCAP18 is processed into a functional peptide in the mouse. Furthermore, topical treatment of Tg/KO mice with $1,25(OH)_2D_3$ improved resistance to *S. aureus* growth in a wound infection model. This suggests that induction of human *CAMP* in the skin of Tg mice by vitamin D has functional significance to protect wound sites from infection. The *CAMP* transgenic mouse may prove useful as a model to further determine the role of $1,25(OH)_2D_3$-induced expression of *CAMP* in the skin against infection and other disease states.

The current study also reinforces an important species-specific difference in regulation of CYP27B1 between mouse and human macrophages. The production of the active hormonal form of vitamin D, $1,25(OH)_2D3$, is controlled by the sequential enzyme activity of CYP2R1 and CYP27B1 (66, 67). In the liver, CYP2R1 activity produces $25(OH)D_3$ which then circulates and can be converted primarily in the kidney by CYP27B1 to $1,25(OH)_2D_3$, the active hormonal form. Extra-renal CYP27B1 activity has been demonstrated in human skin (68), human placenta (69) and human macrophages and dendritic cells (59, 70–72), where it can promote local effects of vitamin D action, likely by autocrine or paracrine signaling. In human macrophages, TLR signaling results in induction of CYP27B1 expression and subsequent synthesis of $1,25(OH)_2D_3$, which then increases production of *CAMP* locally (21). We treated mouse WT and transgenic macrophages with multiple TLR ligands to induce *Cyp27b1* but did not observe any significant increase at either 3 h or 24 h post stimulation. As a comparison, we also replicated prior findings showing that in human monocyte-derived macrophages following TLR stimulation, a robust expression of CYP27B1 lead to a significant induction of *CAMP* in the presence of $25(OH)D_3$. In contrast, transgenic mouse macrophages did not have a significant induction of *CAMP* following TLR stimulation. This correlated with the lack of constitutive or induced *Cyp27b1* expression. This finding is consistent with prior studies showing that LPS stimulation did not induce *Cyp27b1* expression in mouse bone marrow derived macrophages (53, 54); however, we also showed that treatment of murine macrophages with additional TLR agonists did not induce *Cyp27b1* expression.

The lack of *Cyp27b1* expression or induction in murine macrophages raises the question as to how infection may increase the production of $1,25(OH)_2D_3$. Therefore, we examined endogenous *Cyp27b1* expression in several murine tissues using qRT-PCR. As expected, the highest basal expression was found in the kidney, followed by the salivary glands and skin. Expression on the order of 100- to 1000-fold less than the kidney was observed for the lungs, ileum, and colon. Acute inflammatory challenge in mice with a high-dose of LPS did not increase *Cyp27b1* expression in any of the tested tissues, except the lungs (approximately 6-fold induction 24 h post IP-injection). We also tested a lower dose of LPS with a shorter duration of acute challenge. At 3 h post injection we did observe significant

induction of *Cyp27b1* in the kidneys, but not in other tissues. These results suggest acute systemic inflammation may briefly upregulate expression of *Cyp27b1* in the kidney, but it does not appear to be sustained. A recent study modeling endotoxemia in mice demonstrated increased expression of *Cyp27b1* in the kidney that peaked at 4 h post-injection of LPS and resulted in a concomitant increase in circulating 1,25(OH)2D3 (73). Expression of *Cyp27b1* mRNA in the kidney and circulating $1,25(OH)_2D_3$ decreased by about 50% at 16 h post-injection (73). Using *Cyp27b1*-LacZ knock-in mice LPS injection did not increase reporter activity in the kidney when measured at 24 h (54); however, long term chronic inflammatory events caused by DSS induced colitis did increase C*yp27b1*reporter activity in the small intestines and colon of about 30% of the mice, suggesting that chronic inflammation may induce *Cyp27b1* at extrarenal sites. Induction of *Cyp27b1* has been reported in microglia and placenta in mice (74, 75), in both cases, the induction was important for controlling inflammation.

Our findings together with others indicate that certain aspects of immune modulation performed by *CYP27B1* in humans are not conserved, particularly in mouse macrophages. Effects may occur locally in tissues through extra-renal synthesis of $1,25(OH)_2D_3$. Future studies are required to determine if other cell types in the mouse contribute to local production. In mouse skin, wounding does increase the vitamin D responsive gene *CD14* in WT mice, but not in *Cyp27b1* knockout mice, suggesting that inflammatory signals and wound healing may induce *Cyp27b1* (34). In human skin, wounding does increase CD14, CYP27B1, CYP24A1 and CAMP expression, which can be blocked by itraconazole, a CYP27B1 inhibitor. This strongly suggests that $1,25(OH)_2D_3$ exerts local effects in the skin (34). TLR responsiveness in cultured human skin keratinocytes is different than in human macrophages, as keratinocytes need a combination of TGF-β1 and $25(OH)D_3$ to become responsive to TLR2 ligands through induced expression of TLR2 and CYP27B1 (34). Conservation of this pathway in mouse skin needs to be verified. In the *CAMP* transgenic mouse model, we could bypass the uncertainty of local *Cyp27b1* activity by treating topically with $1,25(OH)_2D_3$ to induce *CAMP* expression. In summary, we have developed a mouse model in which we can test the role of vitamin D3 induction of human cathelicidin during infection and non-pathogenic disease states.

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Abbreviations:

CAMP	cathelicidin antimicrobial peptide
TLR	Toll-like receptor
VDRE	vitamin D response element

VDR	vitamin D receptor
SINE	short interspersed nuclear element
BMDM	bone marrow derived macrophage
BM	bone marrow
AMP	antimicrobial peptide
qRT-PCR	quantitative real time polymerase chain reaction
Tg	transgenic
KO	knockout
WT	wildtype
PBMC	peripheral blood mononuclear cells
IP	intraperitoneal
TLA	targeted locus amplification

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Highlights

- We created a transgenic mouse that expresses the human cathelicidin gene
- The human transgene can be regulated by vitamin D3 in the mouse
- Expression of human cathelicidin restores wound healing in a mouse knockout model
- Human cathelicidin transgenic mice show increased resistance to gut infections
- Topical skin treatment with vitamin D3 decreases staph infection in transgenic mice

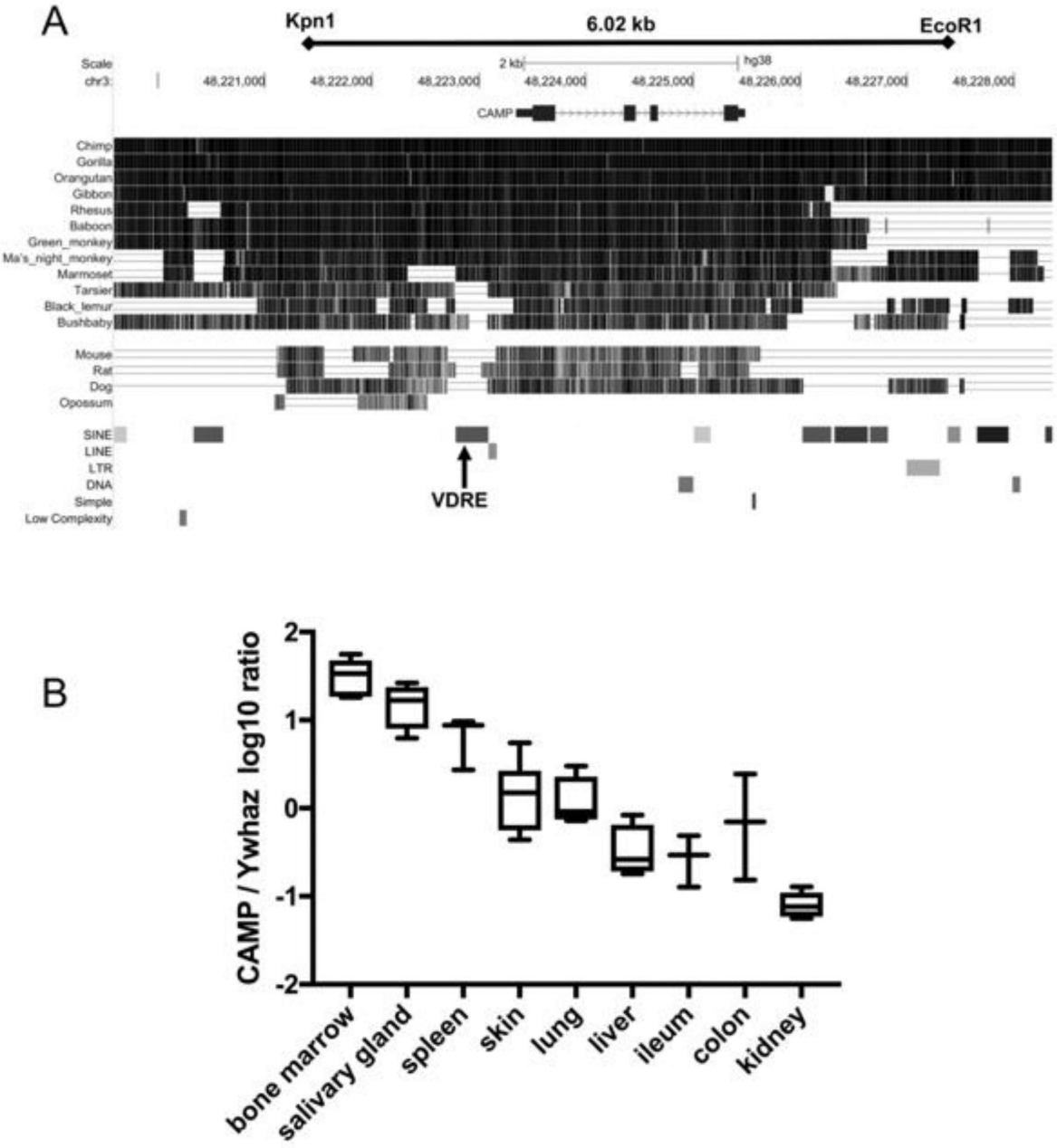

Figure 1. Generation of a *CAMP* transgenic mouse and expression in different tissues.

(A) This schematic generated from the UCSC Genome Browser December 2013 (GRCh38/hg38) Assembly shows that the human 6.02 kb Kpn1-EcoR1 genomic DNA fragment contains the *CAMP* gene located on chromosome 3 (base pairs 48,223,347 −48,225,491). It also shows the conserved 5'- and 3'-regions that possess potentially important transcriptional regulatory elements and the VDRE located in a primate-specific AluSx SINE conserved in only apes (chimp, gorilla, orangutan and gibbon), Old World primates (rhesus macaque, baboon and green monkey) and New World primates (Ma's night monkey and marmoset), but not in prosimians (tarsier, black lemur and bushbaby) or other mammals (mouse, rat, dog and opossum). (B) The human *CAMP* mRNA is expressed in tissues of Tg

mice. Total RNA was isolated from tissues of Tg/WT mice and expression of *CAMP* mRNA was measured by qRT-PCR. The ratio of *CAMP* to the reference gene *Ywhaz* was log10 transformed and graphed as the mean (+S.D.) with the minimum and maximum (Box plots, n=4–6 mice; non-box plots, n=3 mice).

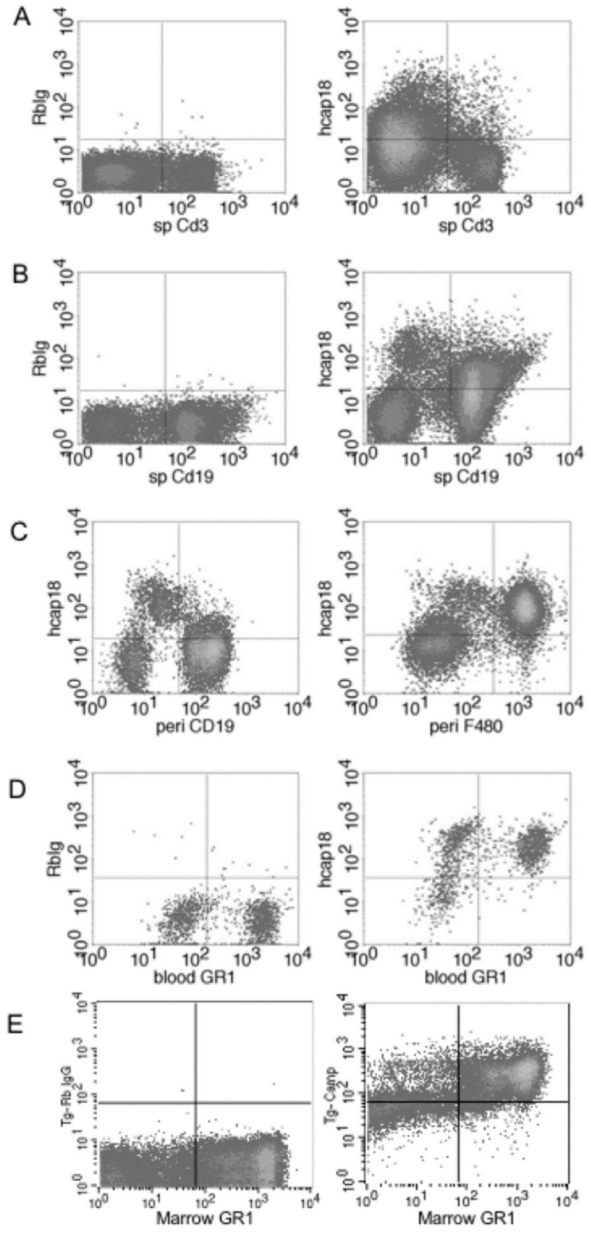

Figure 2. Tg/KO mice express hCAP18 in several lineages of immune cells.

Immune cells from three mice were pooled from spleen, peritoneal cavity, blood, and bone marrow and dual stained with lineage markers and either control rabbit IgG (left panels A, B, D, E) or anti-hCAP18 antibody (left panel C and all right panels) and analyzed by flow cytometry. (A) Splenocytes stained with the T-cell marker CD3. (B) Splenocytes stained with the B-cell marker CD19. (C) Peritoneal cells stained with CD19 (left panel) or F4/80 (right panel). (D) Blood cells stained with the granulocyte marker GR1. (E) Bone marrow cells stained with GR1. Representative plots from three different experiments are shown.

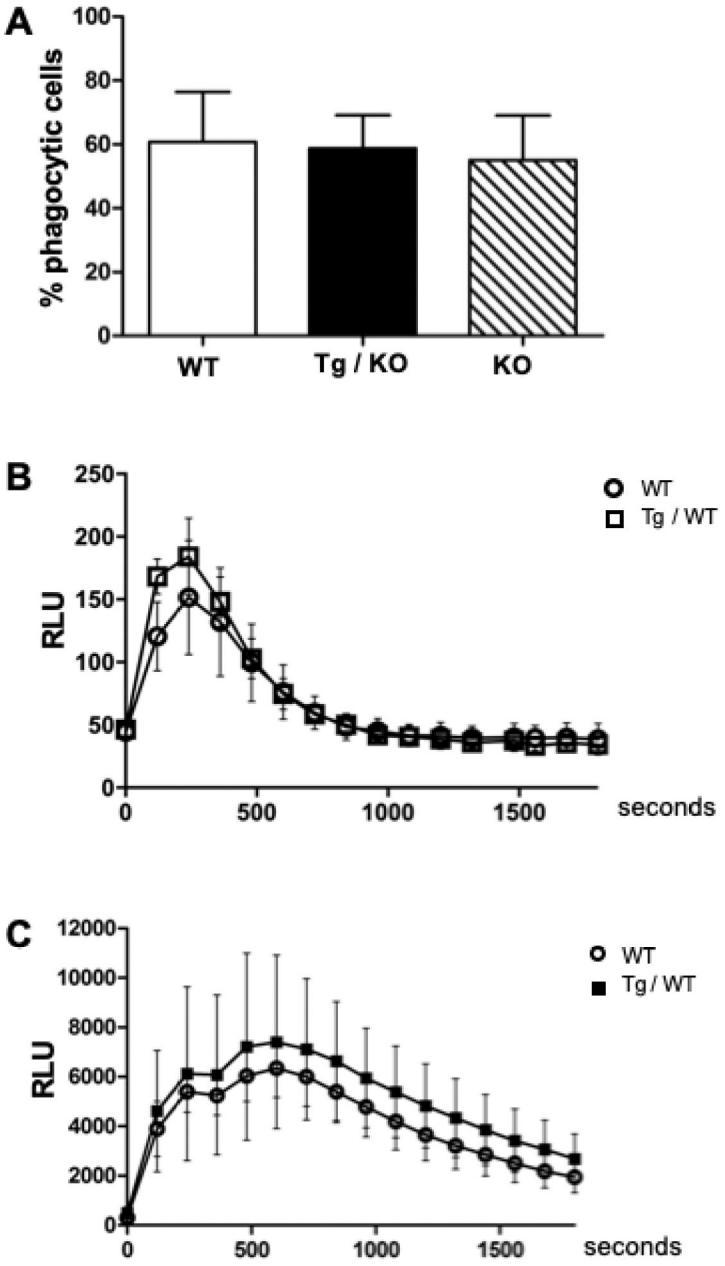

Figure 3. Human *CAMP* gene does not alter phagocytosis or superoxide production in murine phagocytes.

(A) BM-derived macrophages from WT, Tg/KO, and KO genotypes were incubated with fluorescent pHrodo *E. coli* for 60 min and then analyzed by flow cytometry to measure the percentage of phagocytes. Data represent mean (+SD) of four experiments. (B) BM-derived macrophages from WT and Tg/WT mice were incubated with a luminol substrate and stimulated with PMA to induce superoxide as measured by relative light unit (RLU) emission over a time course. Each time point represents the mean (±SD) of three experiments. (C) BM neutrophils from WT and Tg/WT mice were incubated as described in panel B. Each time point represents the mean (±SD) of six biological replicates from two

experiments for each genotype. No significant differences were observed between the genotypes.

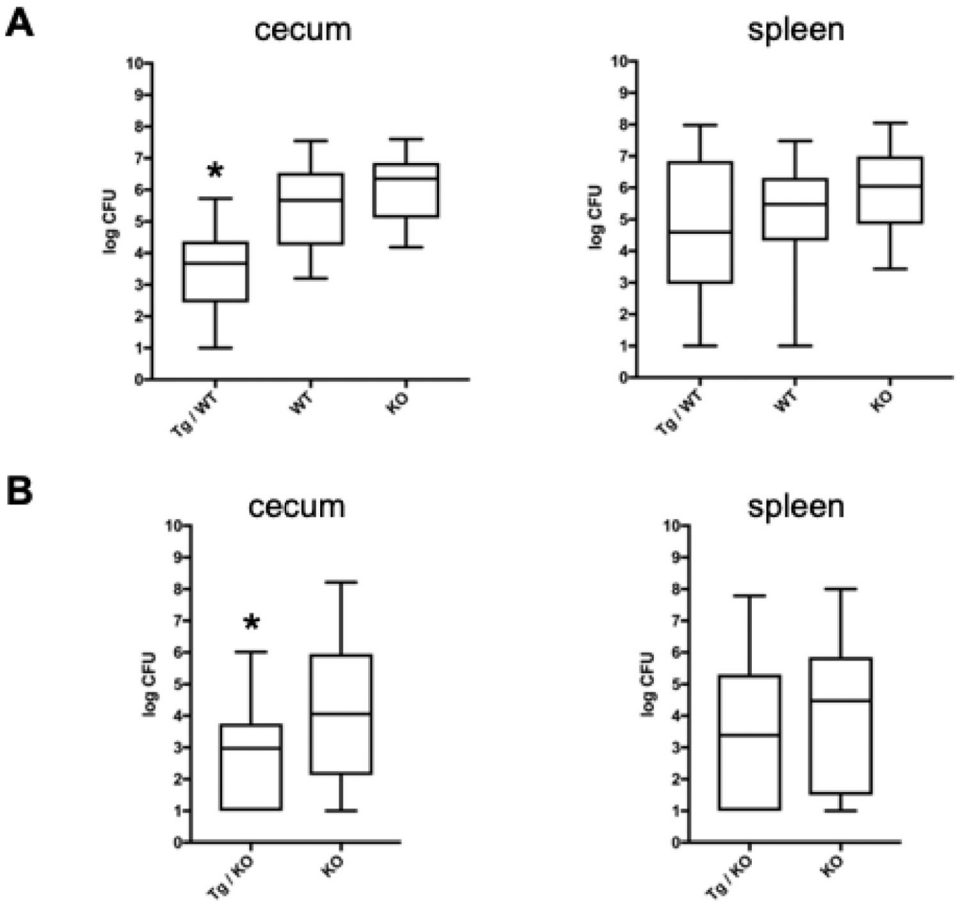

Figure 4. Human *CAMP* gene increases resistance to enteric infection in *Camp* Tg mice.

(A) Mice were infected by oral gavage with 8×10^8 *S. typhimurium*. Five days post infection organs were harvested and plated to measure bacterial CFU shown on a log10 scale. The Tg/WT (n=14) group of mice had a significant decrease in cecum burden compared with both the WT (n=13) and KO (n=14) groups (Asterisk indicates p value <.0001, one-way ANOVA,) (B) Tg/KO and *Camp* KO mice were infected with a lower dose (8×10^7) of *S. typhimurium* for 5 days. The CFU burden in Tg/KO mice (n=30) was significantly lower compared with the KO mice (n=33). The asterisk indicates a p value = 0.007 (two-tailed T-test). Data were plotted as the mean CFU (horizontal line) ±SD (box). The bars show the range of the data.

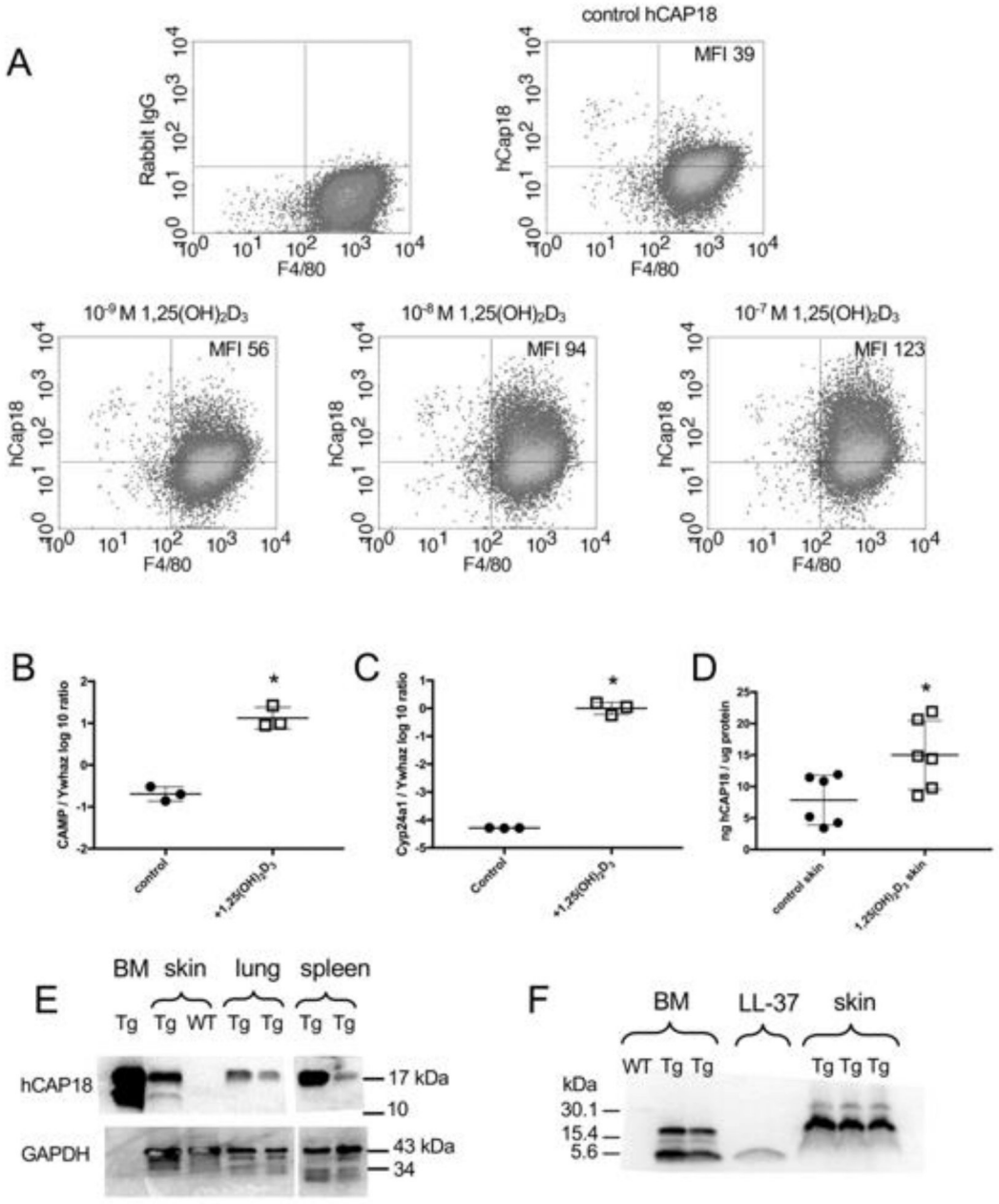

Figure 5. Induction of *CAMP* gene and hCAP18/LL-37 expression with 1,25(OH)$_2$D$_3$ treatment.

(A) BM-derived macrophages from Tg/KO mice were treated 48 h in vitro with increasing concentrations (10^{-9}, 10^{-8} and 10^{-7} M) of 1,25(OH)$_2$D$_3$. hCAP18 and F4/80 staining cells (macrophages) were detected by flow cytometry. Mean fluorescent intensity (MFI) of hCAP18 is noted in the upper right quadrant. Graphs are representative of four experiments. (B-F) Dorsal skin of Tg/KO mice was treated with either vehicle control or 1 nmole 1,25(OH)$_2$D$_3$ for 24 h (mRNA) or 48 h (protein). Expression of *CAMP* (B) and *Cyp24a1* (C) mRNAs (n=3 mice) determined by qRT-PCR was normalized to the housekeeping gene *Ywhaz*. The ratio was log10 transformed and plotted as the mean (+SD). D) hCAP18 protein levels were determined by ELISA (n=6 mice per treatment) and plotted as the mean (ng

hCAP18/mg of total protein, ±SD). (E) hCAP18 expression in tissues from untreated Tg/KO and WT mice was analyzed by Western blot using 50 g protein per lane and an anti-hCAP18 polyclonal antibody. The 18-kDa pro-protein was detected in BM, skin, lung and spleen of the Tg/KO mice. The 14-kDa processed cathelin-domain was detected in the BM and skin. No hCAP18 was detected in the WT skin. GAPDH was used as a loading control, except this particular antibody does not identify GAPDH in bone marrow. (F) BM (untreated) and skin (from treated mice, panel C) samples from WT and Tg/KO mice were analyzed by Western blot with an anti-LL-37 monoclonal antibody. Synthetic LL-37 peptide (4-kDa) was included as a positive control. Both the 18- and 4-kDa forms were detected in the BM. In skin, primarily the 18-kDa was detected with very faint 4-kDa bands. Each Western blot lane represents an individual mouse. Asterisks denote statistical significance ($p<0.05$) determined by a two-tailed T-test.

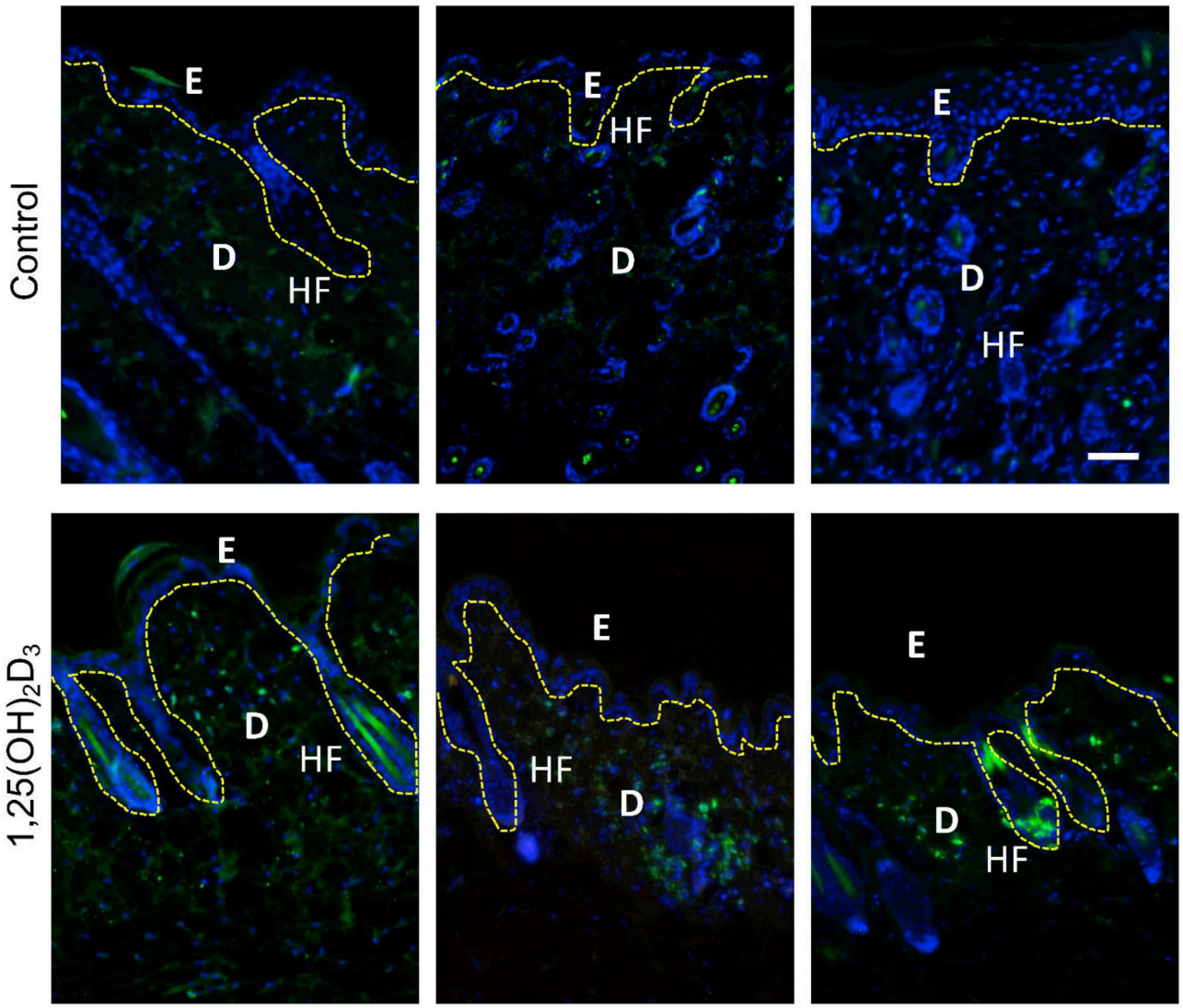

Figure 6. Topical application of $1,25(OH)_2D_3$ induces hCAP18 in Tg/KO mouse skin.

Tg/KO mice were topically treated for 48 h with either 50% ethanol/glycerin vehicle (control) or $1,25(OH)_2D_3$ in vehicle. Skin samples were fixed and paraffin embedded. Sections were stained with anti-hCAP18 polyclonal antibody and then detected with a secondary antibody conjugated to Alexafluor 488 (green) and counterstained with DAPI (blue). The dashed yellow line indicates the border between the epidermis (E), dermis (D) of the skin, and hair follicles (HF), white scale bar = 50 μm. Top panels are multiple sections from vehicle control mice, while the bottom panels are multiple sections from $1,25(OH)_2D_3$-treated mice.

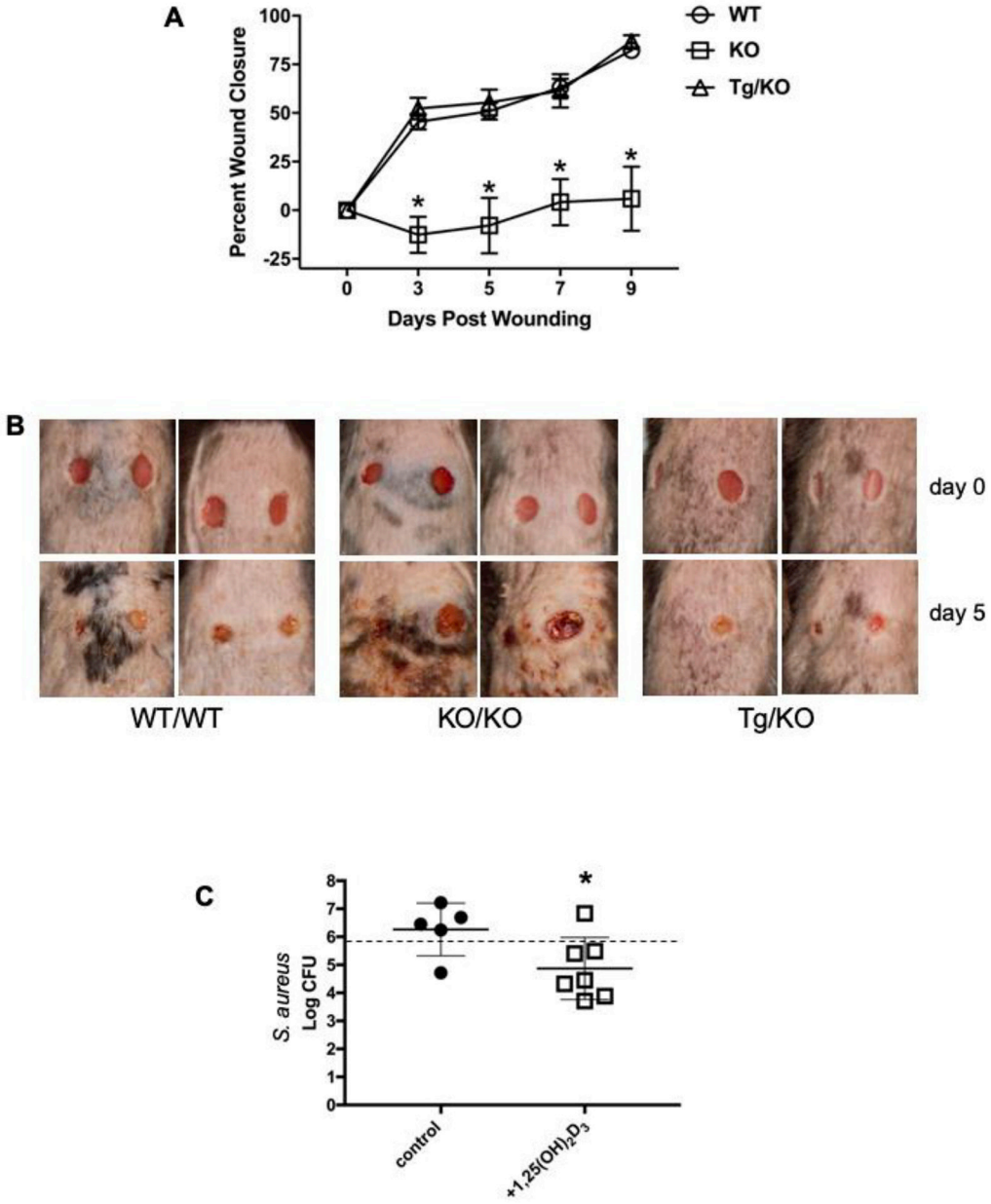

Figure 7. The human *CAMP* gene restores wound healing and topical treatment with $1,25(OH)_2D_3$ lowers *S. aureus* burden in Tg/KO mice.

On Day 0, horizontal dermal wounds were generated on the backs of mice using a 5 mm dermal punch (n=4 per genotype). (A) Percent wound closure was determined by measuring the wound size on days 3, 5, 7, and 9. On Day 5, both WT and Tg/KO mice showed similar wound healing and contraction rates, as measured by total wound area, while a significant lack of wound closure was observed for KO mice (Asterisk indicates $p < 0.05$, Student's t-test). (B) Illustrative examples are shown for each genotype at initial wounding day 0 (top panels) and 5 days post wounding (lower panels). (C) Single 5mm dermal wounds were formed on Tg/KO mice and 1 day later mice were treated with either vehicle control (n=5) or $1,25(OH)_2D_3$ (n=7). The following day 8×10^5 CFU of *S. aureus* were applied to the

wound. At 2 days post infection, the mice were treated again with either vehicle or $1,25(OH)_2D_3$ at the wound site. At 5 days post infection, mice were euthanized and a 10 mm circular biopsy of the wound site was used to enumerate surviving *S. aureus* bacteria expressed as log10 CFU. The dashed line across the graph indicates the starting infectious CFU, while the center line and brackets indicate the mean and S.D. per group of mice. (Asterisk indicates $p < 0.05$, two tailed t-test)

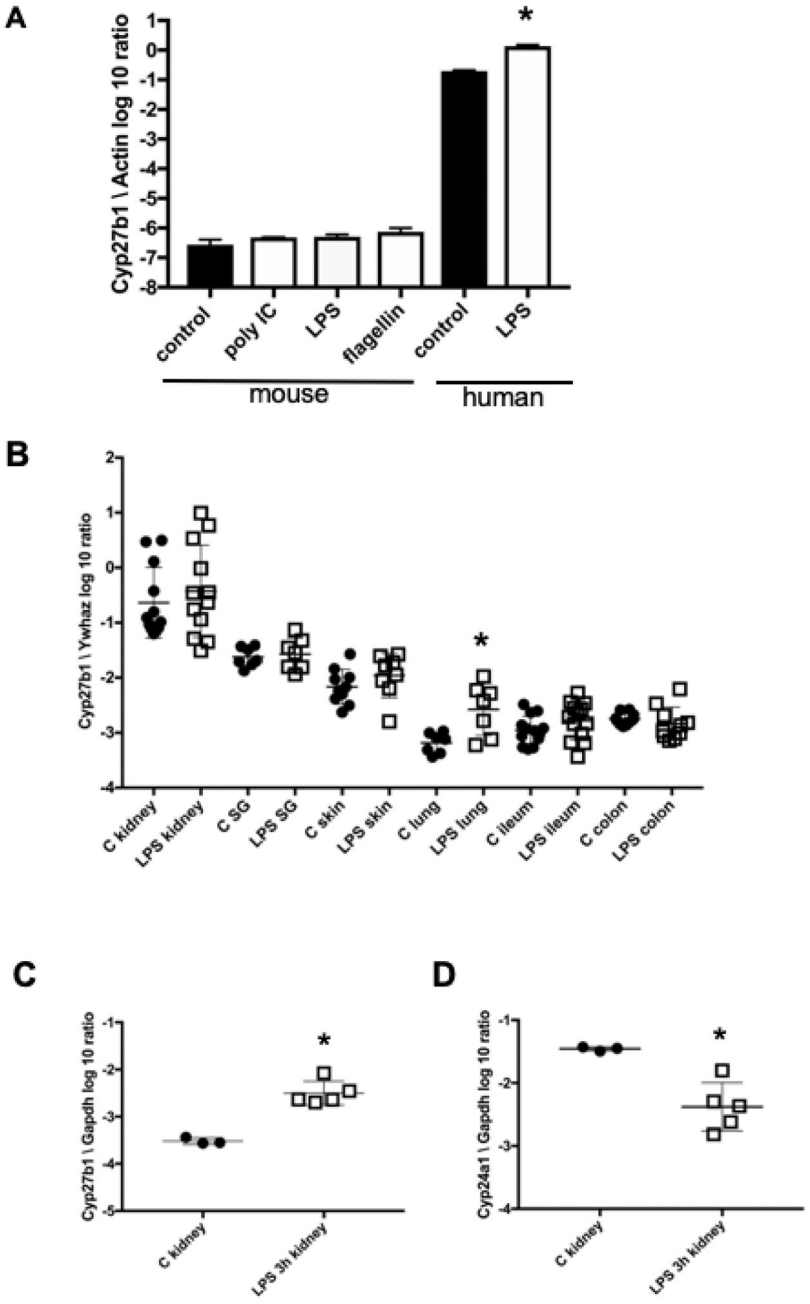

Figure 8. Expression of *Cyp27b1* in mouse macrophages and tissues after TLR ligand stimulation.

TLR ligands do not induce *Cyp27b1* expression in mouse macrophages. (A) BM-derived Tg/WT mouse macrophages and human PBMC-derived macrophages were stimulated with TLR ligands for 24 h and *Cyp27b1* gene expression was measured by qRT-PCR, normalized to β-actin and graphed on a log10 scale. Graphs are a single experiment representative of three independent experiments (Asterisks denote significance from control; $p<0.05$, two-tailed T-test). (B) Tg/WT mice were injected IP with either 10 μg/g body weight LPS or vehicle control for 24 h. Expression of *Cyp27b1* was normalized to *Ywhaz* by qRT-PCR and graphed on a log10 scale. Asterisk denotes a significant increase as compared with the

control ($p<0.05$, T-test with Welch's correction). (C) WT mice were injected IP with either 0.5 g/g body weight LPS or vehicle control. Kidneys were collected 3 h post injection. *Cyp27b1* expression was normalized to *Gapdh* and graphed on a log10 scale. D) *Cyp24a1* expression was normalized to *Gapdh* and graphed on a log10 scale. Data were graphed as the mean (center line) ±SD (brackets). Each symbol represents an individual mouse for B, C and D. Asterisks denote significance from control ($p<0.05$, two-tailed T-test).

Table 1.

Expression of hCAP18 in various tissues from human *CAMP* Tg/KO mice

	hCAP18 (ng/mg total protein) *	**+/− SD**
Bone Marrow	10.25	1.05
*Plasma**	6.87	0.74
Salivary Gland	4.11	2.76
Skin	0.95	0.41
Spleen	0.40	0.11
Lung	0.21	0.07
Ileum	0.05	0.001
Colon	0.04	0.01
Kidney	0.04	0.01
Liver	0.03	0.004

hCAP18 protein was measured by ELISA in tissues from Tg/KO transgenic mice (n=3) and expressed as ng/mg total protein or ng/ml for plasma*.