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1 **ONLINE REPOSITORY TEXT**

2 **Vitamin D_3 represses IgE-dependent mast cell activation via mast cell-CYP27B1 and -**
3 **vitamin D receptor activity**

4

5 **Kwok-Ho Yip, PhD,[*] Natasha Kolesnikoff, PhD,[*] Chunping Yu, BSc(Hons), Nicholas**
6 **Hauschild, BSc(Hons), Houng Taing, BSc(Hons), Lisa Biggs, BSc(Hons), David**
7 **Goltzman, MD, Philip A. Gregory, PhD, Paul H. Anderson, PhD, Michael S. Samuel,**
8 **PhD, Stephen J. Galli, MD, Angel F. Lopez, PhD, and Michele A. Grimbaldeston, PhD[#]**

9

10 Inventory

11 1. Online Repository Methods

12 2. Online Repository Figure Legends

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13

14 **ONLINE REPOSITORY METHODS**

15 **Mice**

16 B6.129S4-*Vdr*^{tm1Mbd}/J mice were backcrossed to C57BL/6 mice for greater than nine
17 generations. As previously reported, adult *Kit*$^{W-sh/Wsh}$ and *Kit*$^{W/W-v}$ mice have a profound
18 deficiency of mast cells, including <1.0% the WT level of mast cells in the dermis[E1-3]. All
19 mice (including *VDR*$^{-/-}$ mice) with the exception of *CYP27B1*$^{-/-}$ mice, were provided
20 commercial mouse chow containing Vitamin D_3 (cholecalciferol) at >2,000 IU/kg *ad libitum*.
21 Derivation of parental strain was undertaken by homologous recombination in embryonic
22 stems where a neomycin resistance gene was inserted in place of exons VI, VII and VIII of
23 the mouse *CYP27B1* gene, replacing both the ligand binding and heme binding domains, as
24 previously described[E4]. These mice were originally maintained on a mixed genetic
25 background with B6 and BALB/c strains and then backcrossed for an additional 3
26 generations with C57BL/6 mice in house in Adelaide. *CYP27B1*$^{-/-}$ mice were maintained on a
27 high calcium diet containing 1.5% calcium in drinking water and chow containing 1%
28 calcium, 0.85% phosphorus, 0% lactose and 2200 IU/kg Vitamin D_3 (Specialty Feeds).
29 Experiments were performed in compliance with the ethical guidelines of the National Health
30 and Medical Research Council of Australia, with approval from the Institute of Medical and
31 Veterinary Science Animal Ethics Committee (Australia).

32

33 **Generation of BMCMCs**

34 As previously described[E2, 5], BMCMCs were obtained by culturing bone marrow cells from
35 femurs and tibias of mice in DMEM (Life Technologies) supplemented with 10% fetal calf
36 serum (FCS; Bovogen) and 20% WEHI-3 conditioned medium (containing 3-4 ng/mL IL-3)

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37 for 4-6 wk, at which time > 95% of the cells were identified as mast cells by May Grünwald-
38 Giemsa staining and by flow cytometric analysis (c-Kit$^+$, FcεRI$^+$).
39
40 **Preparation of vitamin D_3**
41 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and 25-hydroxyvitamin D_3 (25OHD_3) (Sigma-
42 Aldrich) were reconstituted at 1 or 10 mM with 100% absolute ethanol (EtOH; Sigma-
43 Aldrich) and stored, shielded from light, in an airtight tube at -80°C. The chemical integrity
44 of both metabolites was regularly verified using a scanning spectrophotometer.
45
46 **Multiple epicutaneous applications of vitamin D_3 with or without IgE-dependent PCA.**
47 For experiments where 1α,25$(OH)_2D_3$ was epicutaneously applied a total of 9 times every 2 d
48 in the absence of PCA, female C57BL/6J mice received per application a dose of 0.06
49 nmol/ear (3 μM in 20 μL of EPGW vehicle) or 0.25 nmol/ear (12.5 μM in 20 μL of 100%
50 EtOH vehicle) to the right ear or vehicle alone as indicated to the left ear and change in ear
51 thickness from baseline measured prior to each application. For some experiments on the
52 same day as the final application of 1α,25$(OH)_2D_3$, mice were *i.d.* injected with 20 ng IgE
53 anti-DNP in the right ear, or vehicle HMEM-Pipes in the left ear. 16h later, mice were *i.v.*
54 injected with 200 μg of DNP-HSA and changes (*Δ*) in ear thickness 0-6 h were measured and
55 calculated from baseline measured at day 0 prior to first application of 1α,25$(OH)_2D_3$. For all
56 experiments, ear pinnae were collected for histological analysis and gene expression analysis
57 24 h after the final application of 1α,25$(OH)_2D_3$ or 6 h after induction of the PCA reaction.
58
59 **Histology and quantification of mast cell numbers**
60 Mice were killed by CO_2 inhalation and samples of ear pinna were fixed in 10% buffered
61 formalin, embedded in paraffin (with care to ensure a cross-section orientation), and 4-µm

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62 sections were cut. Ear sections were stained with 0.1% Toluidine Blue (pH 1.0) for the
63 detection of mast cells (cytoplasmic granules appear purple). Ear pinna mast cells were
64 counted in 6-9 consecutive fixed fields of 870 µm width using a 20x microscope objective
65 (200× final magnification), and mast cell numbers were expressed per horizontal ear cartilage
66 field length (millimeter), using computer-generated image analysis (NIH Image J software,
67 version 1.46r). The entire length of a strip of skin extending from the base to the tip of the ear
68 pinna (~5.4-8.1 mm) was quantified. After *i.d.* engraftment of BMCMCs, $Kit^{W\text{-}sh/W\text{-}sh}$ or
69 $Kit^{W/W\text{-}v}$ mice exhibited mast cells from the base to the tip of the ear pinnae, in an anatomical
70 distribution similar to that of the native mast cell populations in the corresponding WT mice.

71

72 **Immunofluoresence**

73 For CYP27B1 immunofluorescence in BMCMCs, cells were centrifuged at 500 rpm for 5
74 min onto Polysine™ slides (Menzel-Glaser), fixed with 150 µL IC Fixation buffer (Fixation
75 & Permeabilisation Kit; eBioscience) for 20 min at room temperature before washing in
76 Permeabilization Buffer (eBioscience) for 5 min. Cells were then incubated with 3 µg/mL
77 rabbit anti-CYP27B1 Ab (Santa Cruz Biotechnology) or 3 µg/mL rabbit polyclonal IgG
78 isotype control Ab (Dako) for 16 h at 4°C. Slides were then rinsed three times in
79 Permeabilization Buffer and incubated with Alexa 594-conjugated goat anti-rabbit Ab (1:200
80 dilution; Molecular Probes) for 1 h at room temperature in the dark. Following three
81 additional washes in Permeabilization Buffer, cells were incubated with 1 µg/mL DAPI
82 (Roche) for 2 min at room temperature, rinsed in Permeabilization Buffer, mounted with
83 Fluorescence Mounting Medium (Dako) and imaged using a Nikon Spectral Imaging
84 Confocal Microscope Digital Eclipse C1si and EZ-C1 software (version 3.20).

85

86 **Preparation of human mast cells**

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87 Mature cord blood-derived mast cells (CBMCs) or peripheral blood-derived mast cells
88 (PBMCs) were generated by first isolating $CD34^+$ progenitor cells from human umbilical
89 cord blood or human buffy coat provided by Australian Red Cross, respectively. Briefly,
90 blood was diluted with sterile phosphate buffered saline (PBS) at a ratio of 1:1, layered gently
91 over Histopaque®-1077 (1.77 g/L; Sigma-Aldrich) and after centrifugation (600 *g*, 30 min),
92 the interface containing mononuclear cells was harvested and the remaining red blood cells
93 were disrupted with haemolytic solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA
94 2Na). $CD34^+$ progenitor cells were enriched by positive immunomagnetic selection using
95 CD34 MicroBeads and an autoMACS Separator (Miltenyi Biotec) according to the
96 manufacturer's instructions. The isolated $CD34^+$ cells were then transferred into 12-well
97 plates at a density of 5 x 10^6 cells/mL in IMDM medium (Life Technologies) supplemented
98 with 1% insulin-transferrin-selenium (Life Technologies), 5 x 10^{-5} M 2-mercaptoethanol (Life
99 Technologies), 1% penicillin-streptomycin (Life Technologies), 0.1% bovine serum albumin
100 (BSA; Sigma-Aldrich), 100 ng/ml recombinant human (rh) SCF, 50 ng/ml rhIL-6 and 1
101 ng/ml rhIL-3 (all rh cytokines from Shenandoah Biotechnology INC.) and placed in a CO_2
102 incubator at 37° C. The cytokine-supplemented medium was replaced weekly and rhIL-3 was
103 omitted from the medium after the first 2 wk of culture. From 6 wk, 10% FCS was added to
104 the medium, and CBMCs or PBMCs used at 10 wk of culture. At that time, the populations
105 contained 96% mast cells as determined by May Grünwald-Giemsa staining and by flow
106 cytometric analysis (tryptase$^+$; 10 μg/mL; Millipore).
107

108 **Measurement of histamine and cysteinyl leukotriene**

109 BMCMCs and CBMCs or PBMCs were pre-incubated in 10% charcoal-stripped-FCS
110 complete medium for 72 h, supplemented with the vitamin D_3 metabolites and sensitized with
111 IgE as outlined above for mast cell activation *in vitro*. Following the 16 h IgE-sensitization

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112 BMCMCs or CBMCs or PBMCs (10^6 cells/mL) were re-suspended in Tyrodes buffer and
113 then activated with DNP-HSA (10 ng/ml; BMCMCs) or anti-human IgE Ab (1 μg/mL;
114 CBMCs or PBMCs) for 30 min at 37° C in the presence of $1\alpha,25(OH)_2D_3$ (10^{-8} - 10^{-6} M) or
115 $25OHD_3$ (10^{-8} - 10^{-6} M) or EtOH (0.03%). Histamine or Cys-LT levels in supernatants and
116 corresponding cell lysates (histamine only) were measured using histamine (Beckman
117 Coulter) or Cys-LT EIA (Cayman Chemical) kits according to manufacturers' instructions.
118

119 **Immunoblotting**

120 BMCMCs and CBMCs were pre-incubated in 10% charcoal-stripped-FCS complete medium
121 for 72 h, then treated with $25OHD_3$ (10^{-8} or 10^{-7} M) for 3 and 8 h, and lysed in ice-cold lysis
122 buffer (50 mM Tris-base, 100 mM NaCl, 5 mM EDTA, 67 mM $Na_4P_2O_7$, 0.01% Triton X-
123 100 and complete protease inhibitors cocktail [Roche]). Proteins were separated with SDS-
124 polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes
125 were blocked with 5% non-fat dry milk in Tris-buffered saline that contained 0.1% Tween-
126 20; they were then probed with an rabbit anti-CYP27B1 antibody (600 ng/mL; Santa Cruz
127 Biotechnology) or rabbit anti-β-actin antibody (1:1000 dilution; Cell Signaling) overnight at
128 4°C. Membranes were then probed with horseradish peroxidase–conjugated antibody against
129 rabbit IgG (1:2000 dilution; Cell Signaling) and bands visualized using ECL reagent
130 (Amersham) with a LAS4000 imaging system (Fujifilm).

131 For cells that were sensitized with IgE anti-DNP mAb (SPE-7; 2mg/ml), IgE was
132 added to the cells at the same time as administration of $1\alpha,25(OH)_2D_3$ (10^{-7} M) or vehicle
133 (0.03% EtOH) and incubated for 16 h at 37° C in a CO_2 incubator. Cells were centrifuged 180
134 *x g* for 5 min, resuspended in Tyrode's buffer (129 mM NaCl, 8.4 mM glucose, 10 mM
135 HEPES, 5 mM KCl, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$ and 1% BSA at pH 7.4), centrifuged again
136 and then resuspended with Tyrode's buffer at 4 x 10^6 cells/mL. Cells were activated with 10

137 ng/mL of DNP-HSA–specific antigen for 2 or 15 min at 37 ^oC in the presence of
138 $1\alpha,25(OH)_2D_3$ (10^{-7} M) or EtOH (0.03%). The reaction was quenched by the addition of ice-
139 cold buffer followed immediately by centrifugation at 180 *x g* for 5 min at 4 ^oC. Cells were
140 lysed in ice-cold lysis buffer, electrophoretically transferred and bands visualized using the
141 same protocol as detailed above with the exception that membranes were probed with rabbit
142 anti-phospho-Erk1/2, anti-phospho-p-38, anti-phospho-JNK1/2 and anti-phospho-NF-κB-
143 p65. Stripped membranes were then probed with total form of anti-Erk1/2, anti-p-38, anti-
144 JNK1/2 and anti-NF-κB-p65 (all including phosphor-antibodies were used at 1:1000 dilution;
145 Cell Signaling). Immunoblots presented in figures are representative of 2 or 3 similar
146 independent experiments.
147
148 **Flow cytometric analysis**
149 BMCMCs were incubated with $1\alpha,25(OH)_2D_3$ (10^{-8} - 10^{-7} M) and IgE anti-DNP Ab (2
150 μg/ml) for 16 h before cell surface FcεRI and c-kit expression determination. BMCMCs were
151 washed in FACS buffer (PBS with 2% FCS) and incubated with anti-mouse CD16/CD32
152 mAb (1 μg/mL) on ice for 15 min. After FcR blocking, BMCMCs were incubated with anti-
153 FcεRIα-FITC (2.5 μg/mL; eBioscience) or anti-c-kit-PE (2 μg/mL) antibodies or isotype
154 control American hamster IgG-FITC (2.5 μg/mL) and rat IgG2b-PE (2.5 μg/mL) antibody for
155 30 min on ice and then analysed on a Beckman Coulter Cytomics FC500 and using CXP
156 Cytometry List Mode Data Acquisition and Analysis Software version 2.2 (Beckman
157 Coulter). All antibodies were obtained from eBioscience. For BMCMC-CYP27B1
158 expression, cells were incubated with rabbit anti-CYP27B1 Ab (3 μg/mL; Santa Cruz
159 Biotechnology) or isotype control rabbit IgG Ab (3 μg/mL; Dako) in Permeabilization Buffer
160 (eBioscience) for 30 min on ice, then incubated with Alexa 594-conjugated goat anti-rabbit
161 Ab (1:100 dilution; Molecular Probes) for 30 min on ice, and finally analysed by flow

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162 cytometry. For determination of CBMC-VDR expression, cells were fixed in IC fixation
163 buffer (eBioscience) for 20 min at room temperature, incubated with rat anti-VDR antibody
164 (10 μg/mL; Millipore) or isotype control rat IgG2a antibody (2 μg/mL; eBioscience) in
165 Permeabilization buffer (eBioscience) for 30 min on ice. Cells were washed, incubated with
166 goat anti-rat FITC-conjugated antibody (1:100 dilution; Life Technologies) for 30 min on ice
167 and then analysed by flow cytometry.
168

169 **RNA extraction and real-time PCR**

170 Ear pinnae were finely sliced, sonicated in 500 μl TRIzol reagent (Life Technologies) from
171 which RNA was extracted according to the manufacturer's instructions. For mRNA analysis,
172 0.5 μg of RNA was used for complementary DNA (cDNA) synthesis using the QuantiTect
173 reverse transcription kit (QIAGEN). Quantitative real-time PCR was performed using a 1:4
174 dilution of cDNA with the QuantiTect SYBR Green PCR System (QIAGEN) on a Rotor-
175 Gene 6000 PCR machine (QIAGEN). PCR assays were performed for 45 cycles (95° C for
176 15 s, 55° C for 20 s, and 72 °C for 20 s). Relative expression levels of TSLP mRNA was
177 normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control using the Rotor-
178 Gene Series 6000 Software (QIAGEN). The following oligonucleotide sequences were used:
179 TSLP, forward 5'-AGCTTGTCTCCTGAAAATCGAG-3', reverse 5'-
180 AGGTTTGATTCAGGCAGATGTT-3'; LTC4S, forward 5'-
181 ATGAAGGACGAAGTGGCTCTT-3', reverse 5'-CCTGTAGGGAGAAGTAGGCTTG-3';
182 HDC, forward 5'- AGGAGCAATCCAAGGGAGAT-3', reverse 5'-
183 GGTATCCAGGCTGCACATTT-3',and GAPDH forward 5'-
184 ACATCATCCCTGCATCCACT-3', reverse 5'-ACTTGGCAGGTTTCTCCAG-3'.
185 CYP27B1 cDNA was primed using the Mm_Cyp27b1_1_SG QuantiTect primer assay
186 (QIAGEN) and PCR was conducted according to the manufacturer's instructions.

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187

188

189 **Measurement of 1α,25$(OH)_2D_3$ in BMCMCs and CBMCs**

190 Five wk old BMCMCs (WT [VDR mouse colony] or *VDR*$^{-/-}$ or WT [CYP27B1 mouse
191 colony] or *CYP27B1*$^{-/-}$ or 10 wk old CBMCs (2×10^6 cells/ml) were pre-incubated in 10%
192 charcoal-stripped-FCS complete medium (DMEM or IMDM, respectively) for 72 h in a CO_2
193 incubator at 37° C. Cells were then replenished with the charcoal-stripped-FCS DMEM
194 (supplemented with 3 ng/mL rmIL-3 for BMCMCs) and IMDM (supplemented with 100
195 ng/mL rhSCF, 50 ng/mL rhIL-6 for CBMCs). For CBMCs, 2 x 10^6 cells/mL were incubated
196 with $25OHD_3$ (10^{-7} or 10^{-6} M) or EtOH (0.03%) for 6-7 h, whereas BMCMCs (2 x 10^6
197 cells/mL) were incubated for 24 h and the supernatant replaced with new medium containing
198 $25OHD_3$ (10^{-7} or 10^{-6} M) for a further 6 h incubation. Culture supernatants and cell lysates
199 were collected and snap-frozen in liquid nitrogen. Samples were stored, shielded from light,
200 at -80° C until analysis. Levels of 1α,25$(OH)_2D_3$ supernatants and corresponding cell lysates
201 were measured using a radioimmunoassay kit (Immunodiagnostic Systems) according to the
202 manufacturer's instructions. Mast cell production of 1α,25$(OH)_2D_3$ was determined as the
203 amount measured in the cell lysate + the supernatant and expressed as pM.

204

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222 **ONLINE REPOSITORY FIGURE LEGENDS**

223

224

225 **Figure E1. 1α25(OH)$_2$D$_3$ treatment does not alter key signalling events downstream of**
226 **IgE-FcεRI activation or FcεRI and c-kit expression in BMCMCs.** (**A**) WT or *VDR*$^{-/-}$
227 BMCMCs were preincubated with 1α,25(OH)$_2$D$_3$ (10^{-7} M) or 0.03% EtOH for 16 h and then
228 stimulated with DNP-HSA in the presence of 1α,25(OH)$_2$D$_3$ (10^{-7} M) or 0.03% EtOH for the
229 indicated times. Whole cell lysates were prepared and probed with antibodies to phospho-
230 Erk1/2 (p-Erk1/2) phospho-p-38 (p-p38) phospho-JNK1/2 (p-JNK) and phospho-NF-kB-p65
231 (p-NF-kB). Stripped membranes were re-probed with antibodies to total form of the
232 corresponding phospho-proteins as controls. Results are representative of two similar
233 experiments. (**B**) Flow cytometric analysis of FcεRI and c-kit expression of IgE sensitized
234 WT BMCMCs treated with 10^{-7} M 1α,25(OH)$_2$D$_3$ for 16 h.

235

236 **Figure E2**. **CYP27B1 expression in WT and *VDR*$^{-/-}$ BMCMCs**. Detection of CYP27B1
237 protein in WT and *VDR*$^{-/-}$ BMCMCs by (**A**) immunofluoresence with rabbit anti-CYP27B1
238 Ab or isotype control rabbit IgG Ab (Scale: 50 μm); and (**B**) flow cytometric analysis.
239 Results are representative of 3 similar independent experiments.

240

241 **Figure E3. 1α,25(OH)$_2$D$_3$ does not regulate CYP27B1 expression in BMCMCs**.
242 CYP27B1 mRNA expression analysed by qPCR in 5 wk old WT and *VDR*$^{-/-}$ BMCMCs
243 cultured for 6 h with 1α,25(OH)$_2$D$_3$ (1α25D$_3$) at indicated concentrations or vehicle (0.03%
244 EtOH). Data are expressed as mean values + SD obtained in 2 independent experiments.

245

246 **Figure E4. 1α,25$(OH)_2D_3$ treatment reduced HDC and LTC4S mRNA level in IgE-
247 mediated PCA-affected ears.** (**A**) HDC and (**B**) LTC4S mRNA expression in ear skin of
248 mice were analysed by qPCR at 3 h after induction of IgE-mediated PCA. Data are expressed
249 as mean values + SEM of 4 to 6 mice/group; 3 independent experiments. *, $P < 0.05$; **, $P <$
250 0.01; for the indicated comparisons.

251

252 **Figure E5. Ear swelling responses after injection with vehicle (HMEM-Pipes).** Changes
253 (*Δ*) in ear thickness 0-6 h after *i.v.* injection of 200 μg of DNP-HSA into mice and 16 h after
254 pretreated with topical application of 0.06 nmol/ear 1α,25$(OH)_2D_3$ (1α25D_3; circles) or
255 vehicle (EPGW; squares) that occurred concurrent with *i.d.* injection of vehicle (left ear) in
256 $Kit^{+/+}$ (WT) mice, $Kit^{W\text{-}sh/W\text{-}sh}$, WT BMCMC→$Kit^{W\text{-}sh/W\text{-}sh}$, or $VDR^{-/-}$ BMCMC→$Kit^{W\text{-}sh/W\text{-}sh}$
257 mice. None of the differences between any of the groups achieved statistical significance
258 (defined as $P < 0.05$) by means of two-way ANOVA. These data are from the same mice
259 whose results for IgE anti-DNP Ab injected right ears are shown in Fig 3.

260

261 **Figure E6. Epicutaneous application of 1α,25$(OH)_2D_3$ suppresses IgE-mediated PCA
262 reactions in a mast cell-VDR-dependent manner.** Changes (*Δ*) in ear thickness 0-6 h after
263 *i.v.* injection of 200 μg of DNP-HSA into mice and 16-22 h after topical application of 0.06
264 nmol/ear 1α,25$(OH)_2D_3$ (1α25D_3; circles) or vehicle (EPGW; squares) that occurred
265 concurrent with *i.d.* injection of (**A**) 20 ng IgE anti-DNP antibody into right ears, or (**B**)
266 HMEM-Pipes vehicle in WBB6F_1-$Kit^{+/+}$ (WT) mice (black marker), mast cell-deficient
267 WBB6F_1-$Kit^{W/W\text{-}v}$ (white marker), WT BMCMC→$Kit^{W/W\text{-}v}$ (blue marker) or $VDR^{-/-}$
268 BMCMC→$Kit^{W/W\text{-}v}$ (red marker). Data: n = 6 to 9 mice/group, 2 independent experiments,
269 each of which gave similar results. **, $P < 0.01$***; $P < 0.001$ for comparisons of

270 $1\alpha,25(OH)_2D_3$ *versus* vehicle-treated ears within the same group of mice (as indicated with
271 colour coding) by two-way ANOVA with Bonferroni post-tests.
272
273 **Figure E7. Dermal mast cell numbers in ear pinnae of C57BL/6J-*Kit*$^{+/+}$ mice, mast cell-**
274 **deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice and *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice engrafted with WT (C57BL/6; WT**
275 **BMCMC→*Kit*$^{W\text{-}sh/W\text{-}sh}$ mice), or *VDR*$^{-/-}$ (*VDR*$^{-/-}$ BMCMC→*Kit*$^{W\text{-}sh/W\text{-}sh}$).** BMCMCs were
276 transferred by means of i.d. injection (each ear received 2 injections; 1 x 10^6 cells/20 µL into
277 each of 2 sites) into 4-6 week old *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. Numbers of mast cells per millimetre of
278 ear cartilage were counted in toluidine blue-stained ear pinnae sections at the completion of
279 the PCA experiments outlined in (Fig 3 and Fig E5 Online Repository). Data; n = 9 to 12
280 mice/group are from 3 independent experiments.
281
282 **Figure E8. Chronic epicutaneous application of $1\alpha,25(OH)_2D_3$ suppresses IgE-mediated**
283 **PCA reactions.** (**A**) Changes (*Δ*) in ear thickness of C57BL/6J WT female mice treated with
284 0.06 nmol/ear or 0.25 nmol/ear $1\alpha,25(OH)_2D_3$ ($1\alpha25D_3$; right ears) or relevant vehicle
285 control (EPGW; or EtOH; left ears) every 2 d for 18 d. Data: n = 11 mice/group, 2
286 independent experiments. (**B**) TSLP mRNA expression in ear skin of mice from (**A**) 24 h
287 after the final $1\alpha,25(OH)_2D_3$ topical application. Data: n = 6 mice/group. **, $P < 0.01$ for
288 indicated comparison. (**C**) Changes (*Δ*) in ear thickness of C57BL/6J WT female mice treated
289 with 0.06 nmol/ear, 0.25 nmol/ear $1\alpha25D_3$ or relevant vehicle control (EPGW or EtOH)
290 every 2 d for a total of 9 applications. On day 16 ears were *i.d.* injected with (**C**) 20 ng IgE
291 anti-DNP Ab or (**D**) HMEM-Pipes vehicle. 16 h later, baseline ear thickness was measured
292 and mice were then injected *i.v.* with DNP-HSA and change in ear thickness from measured
293 at intervals over 6 h. Data; n = 3- 4 mice/group from 2 independent experiments. ***; P <

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294 0.001 for comparisons of $1\alpha,25(OH)_2D_3$ *versus* vehicle-treated ears within the same group of
295 mice.
296
297 **Figure E9. A single epicutaneous application of high dose (0.25 nmol) $1\alpha,25(OH)_2D_3$ can**
298 **suppress IgE-mediated PCA reactions.** Changes (*Δ*) in ear thickness 0-6 h after *i.v.*
299 injection of 200 μg of DNP-HSA. C57BL/6J female mice were pretreated with topical
300 application of 0.06 nmol/ear $1\alpha,25(OH)_2D_3$ ($1\alpha25D_3$; grey circles) or 0.25 nmol/ear
301 $1\alpha,25(OH)_2D_3$ ($1\alpha25D_3$; black circles), or corresponding vehicle control (EPGW; grey
302 squares or EtOH; black squares, respectively) and injected *i.d.* with (**A**) 20 ng IgE anti-DNP
303 Ab, or (**B**) HMEM-Pipes vehicle. Data; n = 4 mice/group. ***; $P < 0.001$ for comparisons of
304 $1\alpha,25(OH)_2D_3$ *versus* vehicle-treated ears within the same group of mice.
305
306 **Figure E10. Mast cell-VDR and -CYP27B1 activity are required for epicutaneous**
307 **$25OHD_3$ dampening of IgE-mediated PCA reactions.** Changes (*Δ*) in ear thickness 0-6 h
308 after *i.v.* injection of 200 μg of DNP-HSA into mice and 24-30 h after (**A**) topical application
309 of 0.06 nmol/ear $25OHD_3$ (circles) or vehicle (EPGW; squares) and 16-22 h after *i.d.*
310 injection of (**A**) 20 ng IgE anti-DNP antibody into right ears, or (**B**) HMEM-Pipes vehicle in
311 WBB6F_1-*Kit*$^{+/+}$ (WT) mice (black marker), mast cell-deficient WBB6F_1-*Kit*$^{W/W\text{-}v}$ (white
312 marker), WT BMCMC→*Kit*$^{W/W\text{-}v}$ (blue marker) or *CYP27B1*$^{-/-}$ BMCMC→*Kit*$^{W/W\text{-}v}$ (red
313 marker). Data: n = 3 to 4 mice/group/experiment, 3 independent experiments, each of which
314 gave similar results. *, $P < 0.01$; ***; $P < 0.001$ for comparisons of $25OHD_3$ *versus* vehicle-
315 treated ears within the same group of mice (as indicated with colour coding) by two-way
316 ANOVA with Bonferroni post-tests.
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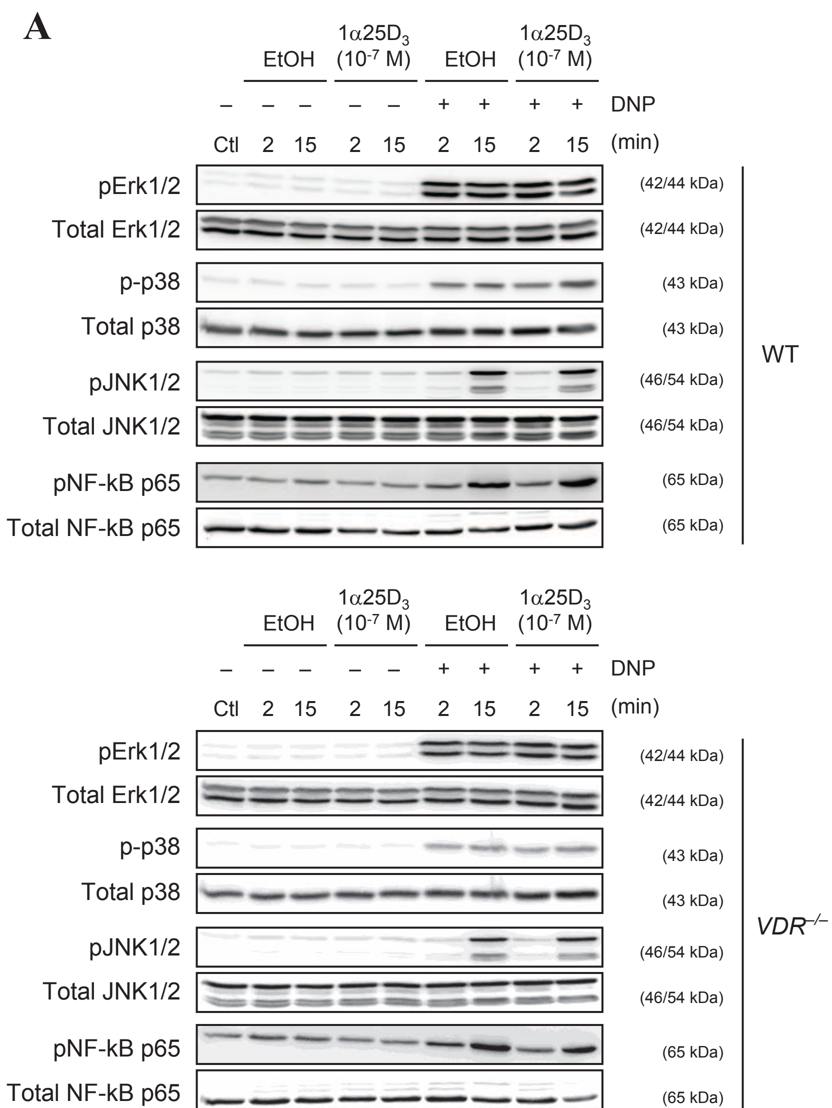
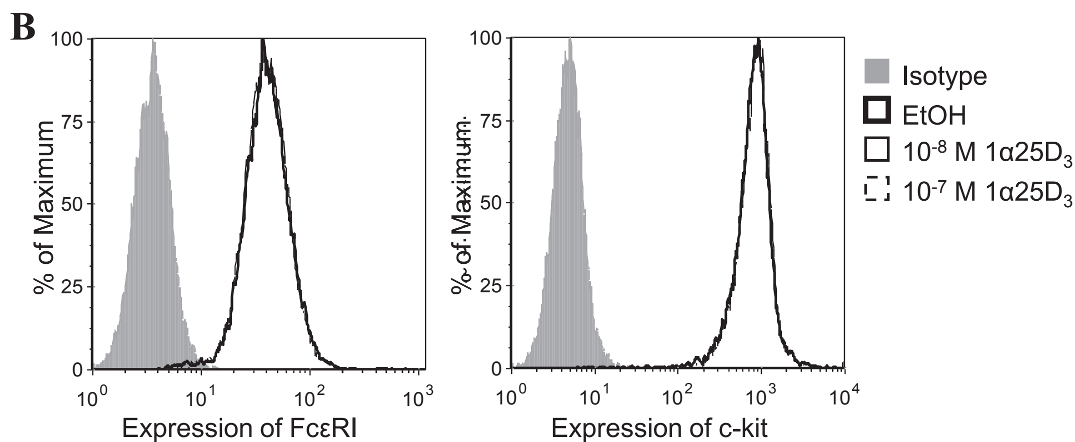
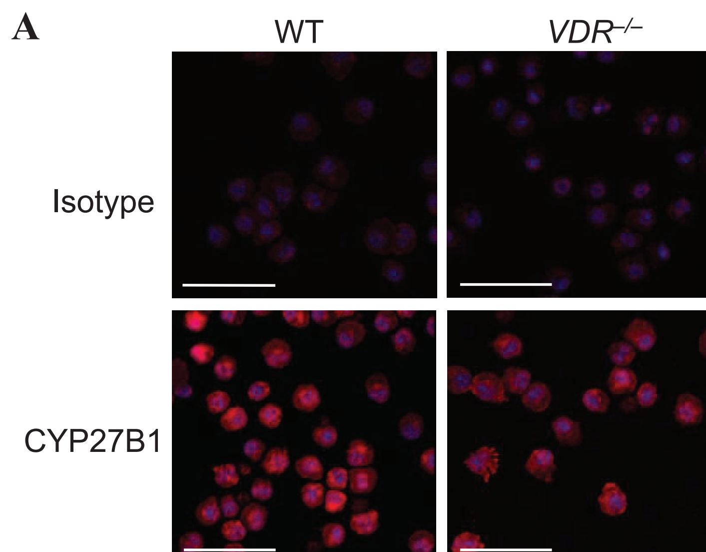
318 **Figure E11. Ear swelling responses after injection with vehicle (HMEM-Pipes) and**
319 **dermal mast cell numbers.** Changes (*Δ*) in ear thickness 0-6 h after *i.v*. injection of 200 μg
320 of DNP-HSA into mice and 24 h after topical application of 0.06 nmol/ear $25OHD_3$ (circles)
321 or vehicle (EPGW; squares) and 16 h after *i.d*. injection of HMEM-Pipes vehicle (left ears) in
322 $Kit^{+/+}$ (WT) mice, $Kit^{W\text{-}sh/W\text{-}sh}$, WT BMCMC→$Kit^{W\text{-}sh/W\text{-}sh}$, (**A**) $VDR^{-/-}$ BMCMC→$Kit^{W\text{-}sh/W\text{-}}$
323 ^{sh}, or (**B**) $CYP27B1^{-/-}$ BMCMC→$Kit^{W\text{-}sh/W\text{-}sh}$ mice. None of the differences between any of
324 the groups achieved statistical significance (defined as $P < 0.05$) by means of two-way
325 ANOVA. (**C** and **D**) Dermal mast cell numbers in ear pinnae of the same groups of mice as
326 indicated in (**A)** and (**B)** and whose results for IgE anti-DNP Ab injected right ears are shown
327 in Fig 4, *A* and *B*. Numbers of mast cells per millimetre of ear cartilage were counted in
328 toluidine blue-stained ear pinnae sections at the completion of the PCA experiments.
329

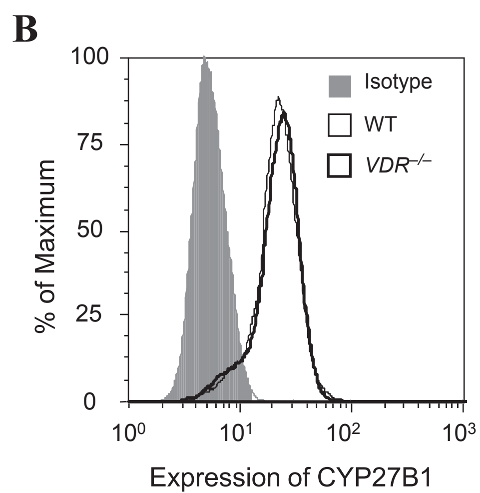
330 **Figure E12. 1α,25(OH)$_2$D$_3$ can impair activation of IgE-mediated peripheral blood-**
331 **derived mast cells.** PBMCs pre-treated with $1\alpha,25(OH)_2D_3$ ($1\alpha25D_3$) or vehicle (0.03%
332 EtOH) at the time of sensitization with human myeloma IgE for 16 h, followed by challenge
333 with anti-human IgE antibody for 30 min and release of (**A**) histamine and (**B**) Cys-LT into
334 the supernatants. Data: 3 to 4 different PBMC batches. *, $P < 0.05$; **, $P < 0.01$; for the
335 indicated comparisons. (**C**) VDR expression in CBMCs. (**D**) CYP27B1 and β-actin protein
336 expression in CBMCs cultured for 8 h with $25OHD_3$ at the indicated concentrations or
337 vehicle (EtOH).

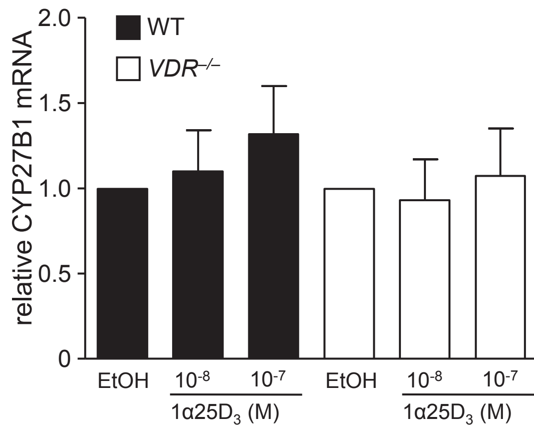
Figure E1 **Yip *et al.***

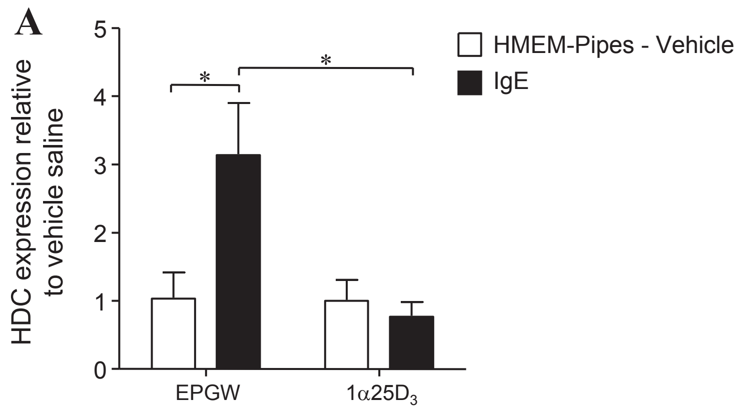





A
WT
VDR−/−
Isotype
CYP27B1


B
100
75
50
25
0
% of Maximum
Isotype
WT
VDR−/−
10⁰
10¹
10²
10³
Expression of CYP27B1


WT
VDR–/–
relative CYP27B1 mRNA
2.0
1.5
1.0
0.5
0
EtOH
10-8
10-7
1α25D3 (M)
EtOH
10-8
10-7
1α25D3 (M)


A
HMEM-Pipes - Vehicle
IgE
HDC expression relative to vehicle saline
0
1
2
3
4
5
*
*
EPGW
1α25D3

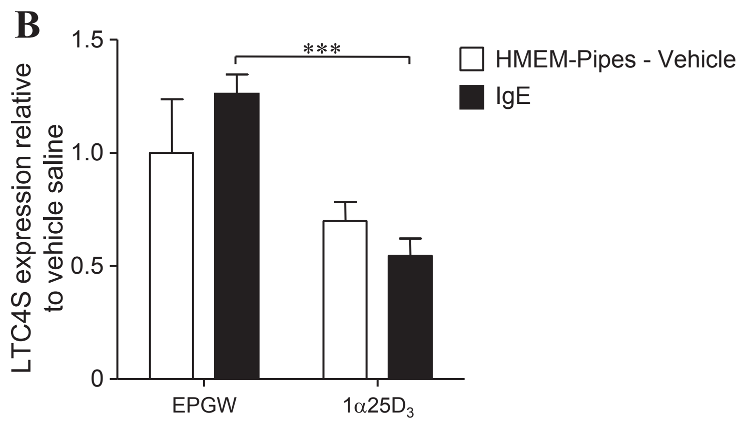
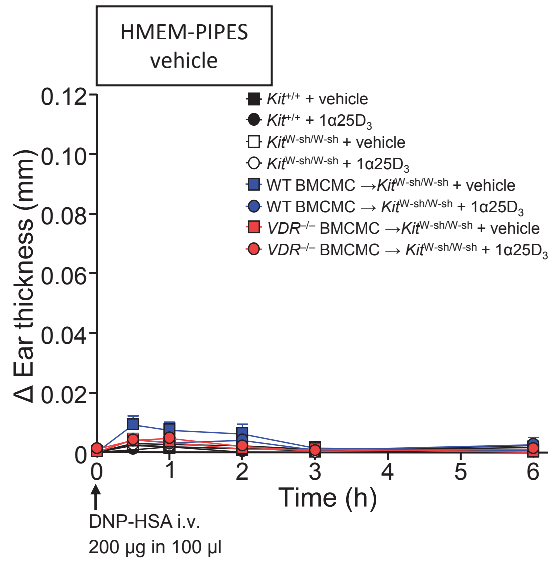

B
HMEM-Pipes - Vehicle
IgE
LTC4S expression relative to vehicle saline
0
0.5
1.0
1.5
***
EPGW
1α25D3

Figure E5



A

IgE αDNP

Δ Ear thickness (mm)

0.18 0.16 0.14 0.12 0.10 0.08 0.06 0.04 0.02 0

0 1 2 3 4 5 6

Time (h)

↑ DNP-HSA i.v. 200 μg in 100 μl

- *Kit*$^{+/+}$ + vehicle
- *Kit*$^{+/+}$ + 1α25D$_3$
- *Kit*$^{W/W-v}$ + vehicle
- *Kit*$^{W/W-v}$ + 1α25D$_3$
- WT BMCMC →*Kit*$^{W/W-v}$ + vehicle
- WT BMCMC → *Kit*$^{W/W-v}$ + 1α25D$_3$
- *VDR*$^{-/-}$ BMCMC →*Kit*$^{W/W-v}$ + vehicle
- *VDR*$^{-/-}$ BMCMC → *Kit*$^{W/W-v}$ + 1α25D$_3$

B

HMEM-PIPES vehicle

Δ Ear thickness (mm)

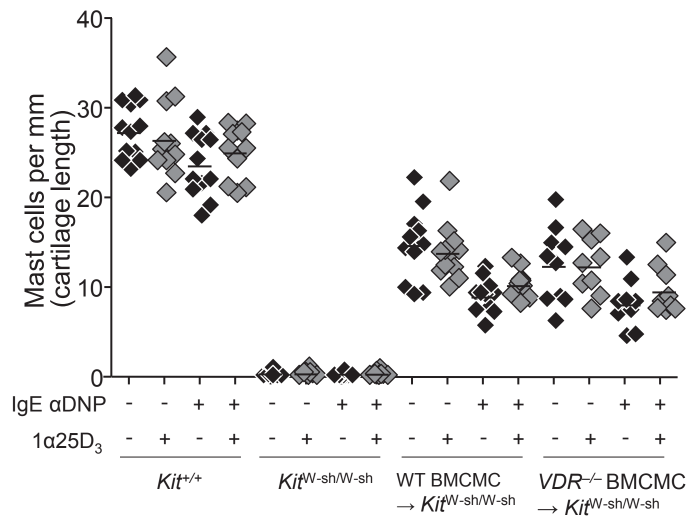
0.18 0.16 0.14 0.12 0.10 0.08 0.06 0.04 0.02 0

0 1 2 3 4 5 6

Time (h)

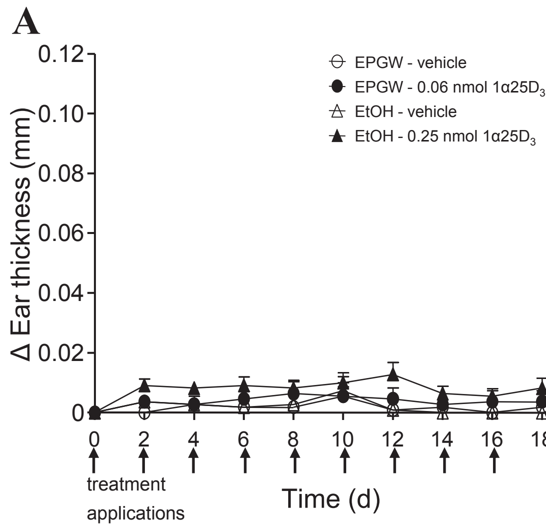
↑ DNP-HSA i.v. 200 μg in 100 μl

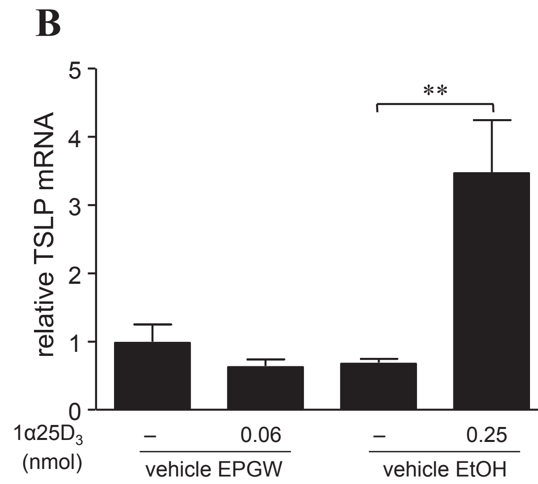
- *Kit*$^{+/+}$ + vehicle
- *Kit*$^{+/+}$ + 1α25D$_3$
- *Kit*$^{W/W-v}$ + vehicle
- *Kit*$^{W/W-v}$ + 1α25D$_3$
- WT BMCMC →*Kit*$^{W/W-v}$ + vehicle
- WT BMCMC → *Kit*$^{W/W-v}$ + 1α25D$_3$
- *VDR*$^{-/-}$ BMCMC →*Kit*$^{W/W-v}$ + vehicle
- *VDR*$^{-/-}$ BMCMC → *Kit*$^{W/W-v}$ + 1α25D$_3$

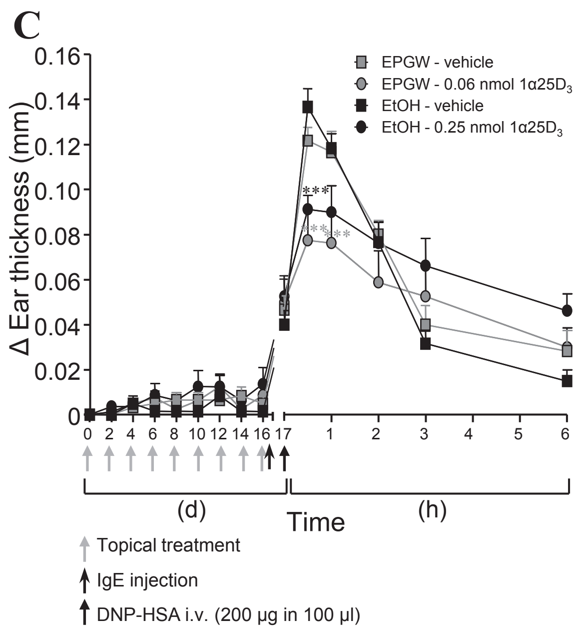


Mast cells per mm (cartilage length)
40
30
20
10
0
IgE αDNP
1α25D3
Kit+/+
KitW-sh/W-sh
WT BMCMC → KitW-sh/W-sh
VDR−/− BMCMC → KitW-sh/W-sh

Figure E6







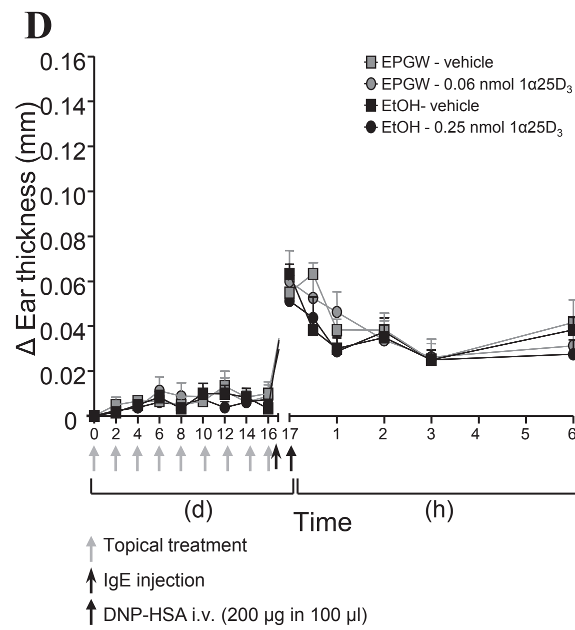
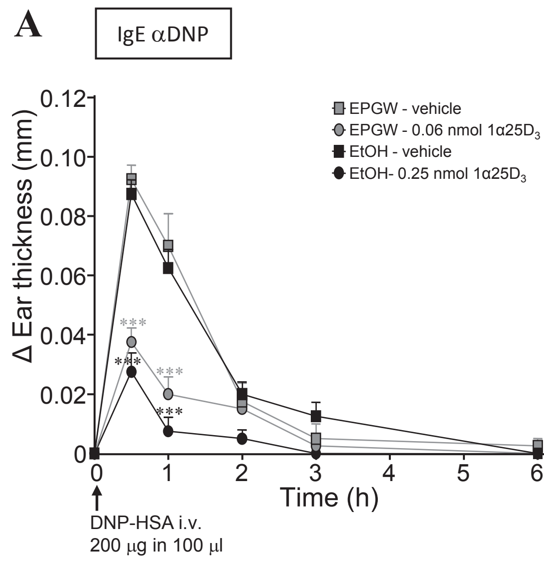
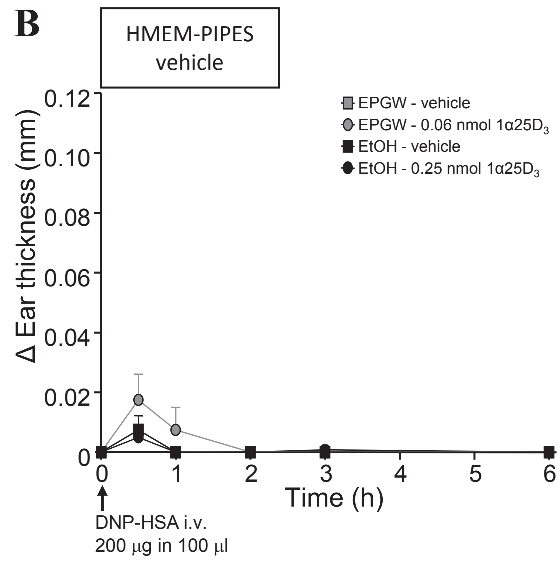


Figure E9





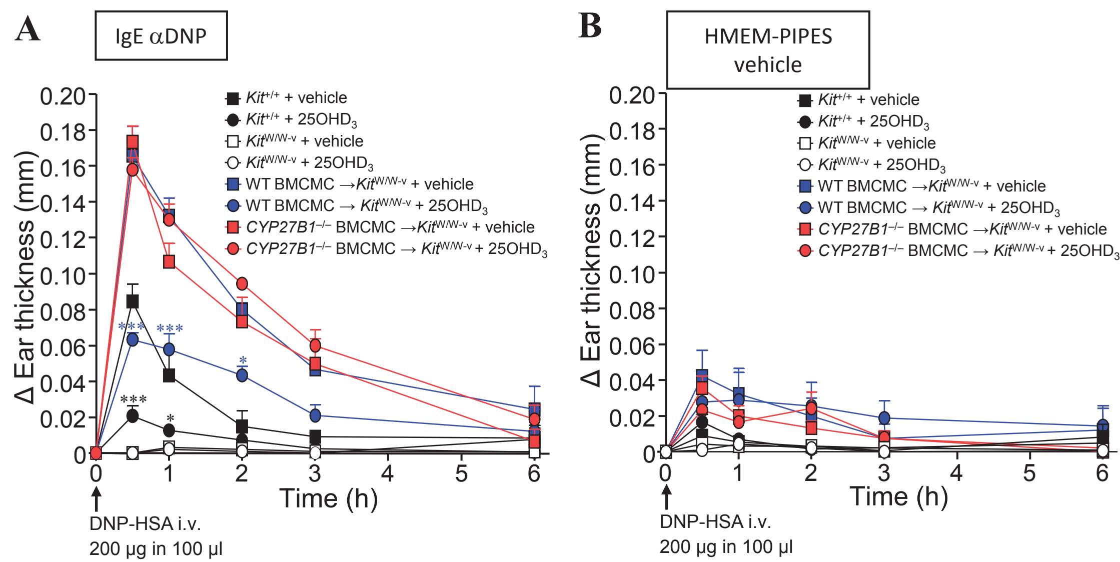
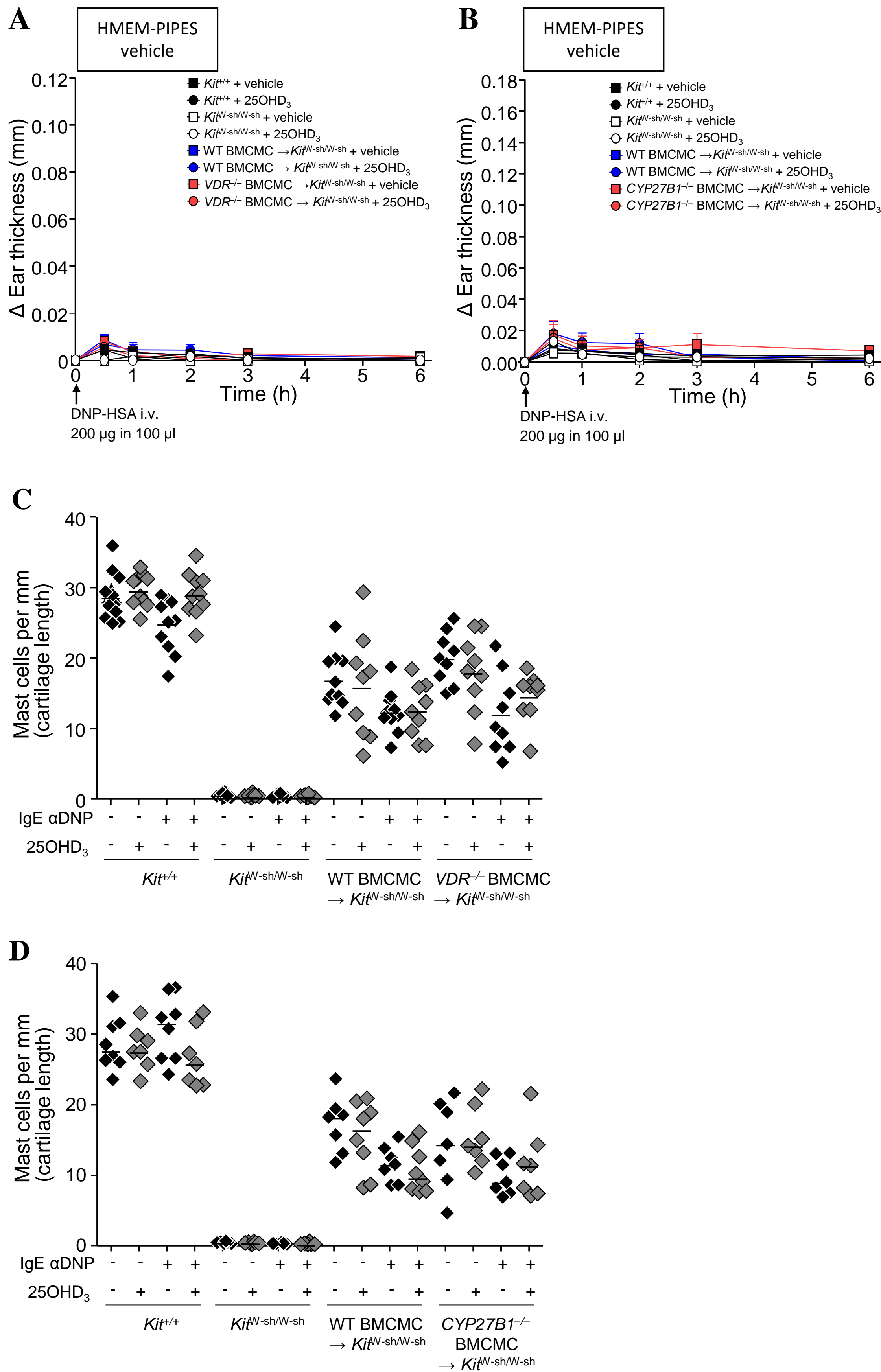
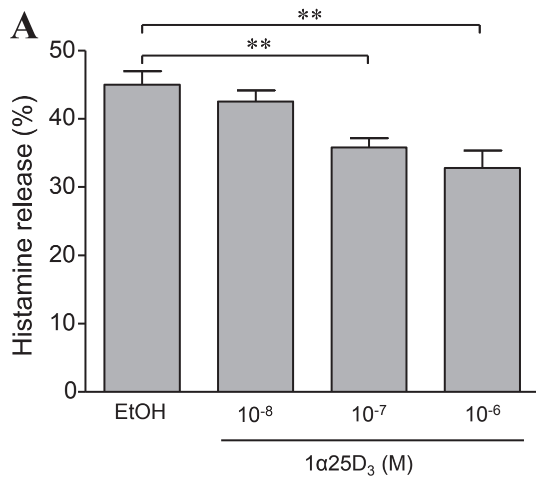
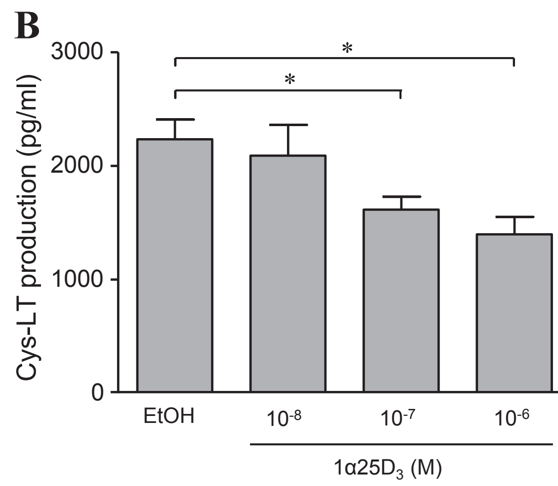
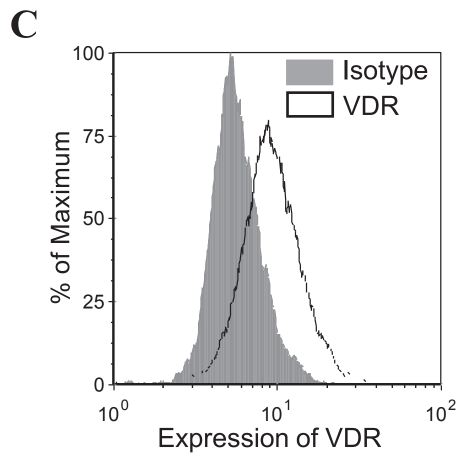

A
IgE αDNP
Δ Ear thickness (mm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Kit+/+ + vehicle
Kit+/+ + 25OHD3
KitW/W-v + vehicle
KitW/W-v + 25OHD3
WT BMCMC → KitW/W-v + vehicle
WT BMCMC → KitW/W-v + 25OHD3
CYP27B1–/– BMCMC → KitW/W-v + vehicle
CYP27B1–/– BMCMC → KitW/W-v + 25OHD3
***
***
***
*
*
Time (h)
0 1 2 3 4 5 6
DNP-HSA i.v.
200 µg in 100 µl
B
HMEM-PIPES
vehicle
Δ Ear thickness (mm)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Kit+/+ + vehicle
Kit+/+ + 25OHD3
KitW/W-v + vehicle
KitW/W-v + 25OHD3
WT BMCMC → KitW/W-v + vehicle
WT BMCMC → KitW/W-v + 25OHD3
CYP27B1–/– BMCMC → KitW/W-v + vehicle
CYP27B1–/– BMCMC → KitW/W-v + 25OHD3
Time (h)
0 1 2 3 4 5 6
DNP-HSA i.v.
200 µg in 100 µl

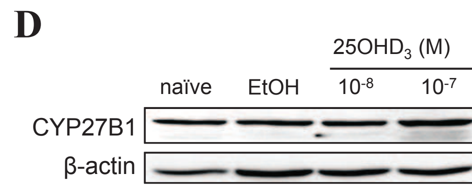
Figure E11




A
Histamine release (%)
50
40
30
20
10
0
**
**
EtOH
10^{-8}
10^{-7}
10^{-6}
$1\alpha25D_3$ (M)


B
Cys-LT production (pg/ml)
3000
2000
1000
0
*
*
EtOH
10^{-8}
10^{-7}
10^{-6}
$1\alpha25D_3$ (M)


C
% of Maximum
100
75
50
25
0
Isotype
VDR
10^0
10^1
10^2
Expression of VDR


D
$25OHD_3$ (M)
naïve
EtOH
10^{-8}
10^{-7}
CYP27B1
β-actin