

Mechanisms of vitamin D₃ metabolite repression of IgE-dependent mast cell activation

Kwok-Ho Yip, PhD,^{a,*} Natasha Kolesnikoff, PhD,^{a,*} Chunping Yu, BSc(Hons),^{a,b} Nicholas Hauschild, BSc(Hons),^a Hounng Taing, BSc(Hons),^{a,b} Lisa Biggs, BSc(Hons),^a David Goltzman, MD,^c Philip A. Gregory, PhD,^a Paul H. Anderson, PhD,^d Michael S. Samuel, PhD,^{a,b,d} Stephen J. Galli, MD,^e Angel F. Lopez, PhD,^{a,b,d} and Michele A. Grimbaldeston, PhD^{a,b,d}
Adelaide, Australia, Montreal, Quebec, Canada, and Stanford, Calif

Background: Mast cells have gained notoriety based on their detrimental contributions to IgE-mediated allergic disorders. Although mast cells express the vitamin D receptor (VDR), it is not clear to what extent 1 α ,25-dihydroxyvitamin D₃ (1 α ,25[OH]₂D₃) or its predominant inactive precursor metabolite in the circulation, 25-hydroxyvitamin D₃ (25OHD₃), can influence IgE-mediated mast cell activation and passive cutaneous anaphylaxis (PCA) *in vivo*.

Objective: We sought to assess whether the vitamin D₃ metabolites 25OHD₃ and 1 α ,25(OH)₂D₃ can repress IgE-dependent mast cell activation through mast cell–25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) and mast cell–VDR activity.

Methods: We measured the extent of vitamin D₃ suppression of IgE-mediated mast cell degranulation and mediator production *in vitro*, as well as the vitamin D₃-induced curtailment of PCA responses in WBB6F₁-*Ki*^{W/W-v} or C57BL/6J-*Ki*^{W-sh/W-sh} mice engrafted with mast cells that did or did not express VDR or CYP27B1.

Results: Here we show that mouse and human mast cells can convert 25OHD₃ to 1 α ,25(OH)₂D₃ through CYP27B1 activity and that both of these vitamin D₃ metabolites suppressed IgE-induced mast cell–derived proinflammatory and vasodilatory mediator production in a VDR-dependent manner *in vitro*. Furthermore, epicutaneously applied vitamin D₃ metabolites significantly reduced the magnitude of skin swelling associated with IgE-mediated PCA reactions *in vivo*; a response that required functional mast cell–VDRs and mast cell–CYP27B1.

Conclusion: Taken together, our findings provide a mechanistic explanation for the anti-inflammatory effects of vitamin D₃ on mast cell function by demonstrating that mast cells can actively metabolize 25OHD₃ to dampen IgE-mediated mast cell activation *in vitro* and *in vivo*. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Mast cells, anaphylaxis, inflammation, IgE, vitamin D₃, vitamin D receptor, CYP27B1

From ^athe Division of Human Immunology, Centre for Cancer Biology, SA Pathology, Adelaide; ^bthe University of Adelaide; ^cthe Departments of Medicine and Physiology, McGill University, Montreal; ^dthe University of South Australia, Adelaide; and ^ethe Departments of Pathology and of Microbiology and Immunology, Stanford University School of Medicine.

*These authors contributed equally to this work.

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Corresponding author: Michele A. Grimbaldeston, PhD, Mast Cell Laboratory, Division of Human Immunology, Centre for Cancer Biology, University of South Australia and SA Pathology, Frome Road, Adelaide, SA 5000, Australia. E-mail: michele.grimbaldeston@health.sa.gov.au.

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Mast cells are key proinflammatory effector cells that can act as potent initiators and amplifiers of IgE-dependent inflammatory and allergic reactions, such as life-threatening anaphylaxis, allergic rhinitis (hay fever), atopic dermatitis (eczema), and allergic asthma.¹ Mast cells express the high-affinity IgE receptor FcεRI, and on activation by polyvalent antigen-induced aggregation of FcεRI-bound IgE, they can release a diverse array of preformed cytoplasmic granule-associated mediators (eg, proteases and vasoactive amines, such as histamine), as well as *de novo* synthesized proinflammatory lipid mediators, cytokines, and chemokines.¹ In certain settings in which invading pathogens require elimination, IgE-mediated mast cell activation is beneficial.² However, the detrimental function of unrestrained IgE-driven activity can contribute to excessive inflammation and the severity of pathology associated with allergic disease.^{1,2} Although factors that promote allergic disorders are being investigated intensively (eg, determinations of genetic predisposition),^{3,4} there is emerging evidence that mast cells can produce anti-inflammatory (ie, IL-10) or regulatory (ie, IL-2) mediators and thereby negatively regulate the magnitude or duration of acute or chronic adverse inflammation in certain settings.⁵⁻⁸ These findings raise the possibility that exogenous agents could be used to engage regulatory pathways in mast cells, which in turn can dampen allergic inflammation through steroid-independent approaches.

In recent years, the secosteroid hormone vitamin D₃ has emerged as a crucial immunoregulatory agent, exerting broad anti-inflammatory actions through the nuclear vitamin D receptor (VDR), which is widely expressed in the immune system.^{9,10} Vitamin D₃ (cholecalciferol) is predominantly generated in the

Abbreviations used

BMCMC:	Bone marrow–derived cultured mast cell
CBMC:	Cord blood–derived mast cell
CYP27B1:	25-hydroxyvitamin D-1 α -hydroxylase
CysLT:	Cysteinyl leukotriene
DMEM:	Dulbecco modified Eagle medium
DNP:	2,4-Dinitrophenol
EPGW:	Ethanol, propylene glycol, and water
Erk:	Extracellular signal-regulated kinase
EtOH:	Ethanol
FITC:	Fluorescein isothiocyanate
IMDM:	Iscove modified Eagle medium
JNK:	c-Jun N-terminal kinase
NF- κ B:	Nuclear factor κ B
1 α ,25(OH) $_2$ D $_3$:	1 α ,25-Dihydroxyvitamin D $_3$
PBMC:	Peripheral blood–derived mast cell
PCA:	Passive cutaneous anaphylaxis
TSLP:	Thymic stromal lymphopoietin
25OHD $_3$:	25-Hydroxyvitamin D $_3$
VDR:	Vitamin D receptor
WT:	Wild-type

epidermis when previtamin D $_3$, the photochemical product derived from 7-dehydrocholesterol in response to ultraviolet B (UVB) irradiation of the skin, undergoes spontaneous thermal isomerization. The conversion of vitamin D $_3$ to its active metabolite, 1 α ,25-dihydroxyvitamin D $_3$ (1 α ,25[OH] $_2$ D $_3$), is carried out in a series of hydroxylation events, first by liver cytochrome P450 proteins (eg, CYP27A1, CYP2D11, CYP3A4, CYP2R1, and CYP2D25) to generate the intermediate metabolite 25-hydroxyvitamin D $_3$ (25OHD $_3$) and then by 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) in the proximal tubule of the kidney to form 1 α ,25(OH) $_2$ D $_3$.¹¹ 1 α ,25(OH) $_2$ D $_3$ exerts its transcriptional activity by binding to the VDR, which leads to the recruitment of its preferred dimerization partner, the retinoid X receptor, to form a heterodimeric complex that targets vitamin D response elements in the promoter regions of genes. Depending on the simultaneous binding of either nuclear coactivators or co-repressors, the DNA-bound complex can function as a ligand-dependent activator or repressor of gene transcription.¹¹⁻¹³

Epidemiologic and experimental data suggest that vitamin D $_3$ insufficiency and suboptimally low levels of circulating 25OHD $_3$ are linked to the pathogenesis of allergic disorders, particularly asthma and eczema in children and infants, respectively.¹⁴⁻¹⁶ At the molecular level, 1 α ,25(OH) $_2$ D $_3$ modifies immune cell functions, including macrophage differentiation, dendritic cell antigen presentation, and enhancement of regulatory T-cell numbers and activity and also dampens T $_H$ 17 differentiation.^{9,17} Surprisingly, it is not known to what extent any potential effect of the vitamin D $_3$ metabolite 1 α ,25(OH) $_2$ D $_3$ or its precursor, 25OHD $_3$, reflects its action on mast cells versus other cell populations during IgE-mediated cutaneous anaphylactic responses *in vivo*. We recently reported that 1 α ,25(OH) $_2$ D $_3$ can induce skin mast cells to produce the anti-inflammatory cytokine IL-10 and thereby curtail IgE-independent inflammation associated with chronic UVB exposure of the skin.⁷ In this study we investigated first whether 1 α ,25(OH) $_2$ D $_3$ can VDR-dependently suppress the extent of IgE-mediated mast cell activation both *in vitro* and during IgE-induced passive cutaneous anaphylaxis (PCA) *in vivo*. Second, we determined whether mast cells express

CYP27B1 and whether its ability to synthesize 1 α ,25(OH) $_2$ D $_3$ is required to mediate 25OHD $_3$ -induced negative regulation of IgE-mediated function *in vitro* and *in vivo*. Finally, we tested whether human mast cells respond like mouse mast cells to the immunoregulatory properties of vitamin D $_3$.

METHODS**Mice**

C57BL/6J (B6), VDR-deficient B6.129S4-*Vdr*^{tm1Mbd/J} (*VDR*^{−/−}), *c-kit* mutant genetically mast cell-deficient (WB/ReJ-*Kit*^{W/+} × C57BL/6-*Kit*^{W/+})F $_1$ -*Kit*^{W/W-v} (WBB6F $_1$ -*Kit*^{W/W-v} [*Kit*^{W/W-v}]), and congenic normal WBB6F $_1$ -*Kit*^{+/+} (*Kit*^{+/+}) mice were obtained from Jackson Laboratories (Bar Harbor, Me). Genetically *c-kit* mutant mast cell-deficient B6-*Kit*^{W-sh/+} mice backcrossed with C57BL/6J (B6J) mice for 12 generations were used as breeding pairs to produce mast cell-deficient B6J-*Kit*^{W-sh/W-sh} mice.^{5,18} *CYP27B1*^{−/−} mice and normal congenic wild-type (WT) littermates were obtained from David Goltzman (McGill University, Montreal, Quebec, Canada). Age-matched female and male mice 8 to 12 weeks of age were used for *in vivo* experiments, and all mice were bred in house at the Institute of Medical and Veterinary Science animal facility (Adelaide, Australia). For additional details, see the **Methods** section in this article's Online Repository at www.jacionline.org.

Adoptive transfer of bone marrow–derived cultured mast cells into mast cell–deficient mice

For mast cell engraftment studies, bone marrow–derived cultured mast cells (BMCMCs) from WT C57BL/6-*VDR*^{+/+} (WT BMCMCs), C57BL/6-*VDR*^{−/−} (*VDR*^{−/−} BMCMCs), WT *CYP27B1*^{+/+} (WT BMCMCs), or *CYP27B1*^{−/−} (*CYP27B1*^{−/−} BMCMCs) mice were transferred by means of intradermal injection (2 injections into each ear with 1 × 10⁶ cells in 25 μ L of Dulbecco modified Eagle medium [DMEM] per injection) into 4- to 6-week-old *Kit*^{W-sh/W-sh} or *Kit*^{W/W-v} mice. PCA experiments were initiated 4 to 6 weeks after intradermal transfer of BMCMCs. For details on the generation of BMCMCs, see the **Methods** section in this article's Online Repository.

Epicutaneous vitamin D $_3$ application and IgE-dependent PCA

For PCA, mice from all groups were injected intradermally with 20 μ L of IgE anti-2,4-dinitrophenol (DNP) mAb (generated from supernatants induced by the hybridoma SPE-7 clone, which produces an IgE mAb to DNP [provided by Zelig Eshar, Weizmann Institute of Science, Rehovot, Israel]) at a concentration of 5 μ g/mL (ie, 100 ng dose) diluted in HMEM-Pipes (Sigma-Aldrich, St Louis, Mo) in the right ear and an equal volume of vehicle in the left ear. Immediately after, both ears of the mice from each group received a single topical application of 1 α ,25(OH) $_2$ D $_3$ (0.06 nmol per ear [3 μ mol/L in 20 μ L diluted in vehicle of 100% ethanol, propylene glycol, and water [EPGW] mixed at 2:1:1 vol/vol/vol] or 0.25 nmol per ear [12.5 μ mol/L in 20 μ L of 100% ethanol [EtOH] vehicle]), and separate control groups were administered vehicle of 20 μ L per of EPGW per ear or 100% EtOH, respectively. For 25OHD $_3$ experiments, mice received the same dose as that used for 1 α ,25(OH) $_2$ D $_3$, but topical application occurred 8 hours before IgE sensitization. Sixteen hours after IgE sensitization, all mice were injected intravenously (retro-orbitally) with 2 mg/mL DNP₃₀₋₄₀-HSA (Sigma-Aldrich) diluted in 100 μ L of sterile 0.9% saline (ie, a dose of 200 μ g). Ear thickness was measured with a dial thickness gauge (model G-1A; Ozaki Manufacturing Company, Tokyo, Japan) before (baseline) and at intervals after intravenous antigen challenge. For further details, see the **Methods** section in this article's Online Repository.

Vitamin D $_3$ treatment and IgE-mediated mast cell activation *in vitro*

Five- to 6-week-old BMCMCs and 10-week-old human cord blood–derived mast cells (CBMCs) or peripheral blood–derived mast cells (PBMCs);

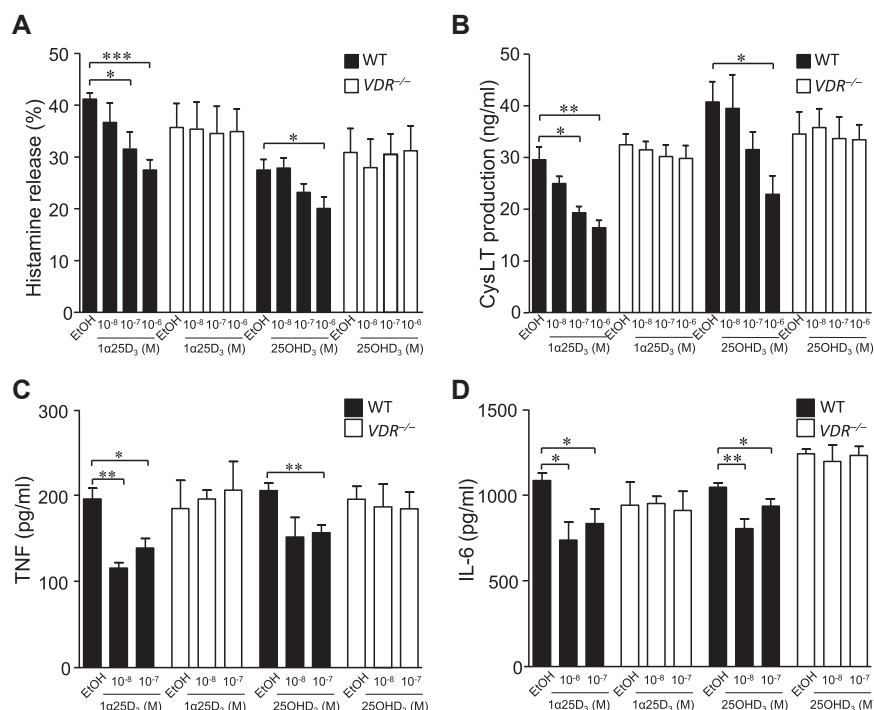


FIG 1. Mast cell-VDRs are required for optimal $1\alpha,25(\text{OH})_2\text{D}_3$ or 25OHD_3 impairment of IgE-mediated mast cell activation *in vitro*. **A-D**, WT (B6J) and $\text{VDR}^{-/-}$ BMCMCs incubated with $1\alpha,25(\text{OH})_2\text{D}_3$ ($1\alpha,25\text{D}_3$) or vehicle (EtOH) 16 or 24 hours before (for 25OHD_3) and during IgE plus DNP-HSA stimulation and release of histamine (30 minutes; Fig 1, A), CysLT (30 minutes; Fig 1, B), TNF (6 hours; Fig 1, C), and IL-6 (6 hours; Fig 1, D). Data are from 3 to 5 independent experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$ for the indicated comparisons.

for details of generation, see the [Methods](#) section in this article's Online Repository) were cultured in DMEM (BMCMCs) or Iscove modified Eagle medium (IMDM; CBMCs or PBMCs) containing 10% charcoal-stripped FCS (and other supplements, as described above) for 72 hours. Cells were replenished with fresh 10% charcoal-stripped FCS complete medium (as indicated for each of BMCMCs, CBMCs, or PBMCs) containing $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} to 10^{-6} mol/L) or vehicle (0.03% EtOH) and simultaneously sensitized with IgE anti-DNP mAb (SPE-7; 2 $\mu\text{g}/\text{mL}$) for BMCMCs or human myeloma IgE (0.5 $\mu\text{g}/\text{mL}$; Calbiochem, San Diego, Calif) for CBMCs or PBMCs and incubated for 16 hours at 37°C . For 25OHD_3 studies, after 72 hours of incubation with 10% charcoal-stripped FCS complete medium, BMCMCs, CBMCs, or PBMCs were replenished with new medium, as noted above, containing 25OHD_3 (10^{-8} to 10^{-6} mol/L) and incubated for 8 hours before the 16-hour IgE sensitization. BMCMCs, CBMCs, or PBMCs were then washed with DMEM or IMDM supplemented with 0.1% BSA, plated at 10^6 cells/mL in the same medium, and stimulated with 20 ng/mL DNP-HSA (BMCMCs) or 1 $\mu\text{g}/\text{mL}$ anti-human IgE antibody (CBMCs or PBMCs; ϵ -chain specific, Sigma-Aldrich) in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$ (10^{-8} to 10^{-6} mol/L) or 25OHD_3 (10^{-8} to 10^{-6} mol/L) or EtOH (0.03%) for 6 or 16 hours, respectively. Supernatants were collected for measurement of mIL-6 and mTNF (BMCMCs) or hTNF and hIL-10 (CBMCs) protein levels by using ELISA (mIL-6 and mTNF OptEIA kits, BD Biosciences, San Jose, Calif; hIL-10 and hTNF, eBioscience, San Diego, Calif). For details pertaining to measurement of histamine and cysteinyl leukotriene (CysLT) release, as well as methods for immunoblotting, flow cytometry, and mast cell production of $1\alpha,25(\text{OH})_2\text{D}_3$, see the [Methods](#) section in this article's Online Repository.

Statistical analysis

Two-way ANOVA with the Bonferroni posttest for repeated measures was used to assess differences in ear swelling between groups of mice over the course of PCA reactions or multiple $1\alpha,25(\text{OH})_2\text{D}_3$ topical applications. For all pairwise comparisons of the data, unless otherwise specified, an unpaired

Student *t* test was performed. For CBMC production of $1\alpha,25(\text{OH})_2\text{D}_3$, we used the Wilcoxon *t* test for comparisons between groups. *P* values of less than .05 were considered statistically significant. Unless otherwise specified, all data are presented as means + SEMs, and for *in vitro* stimulations, data are from at least 3 independent experiments (all measurements for each experiment performed in triplicate).

RESULTS

Vitamin D₃ downregulates IgE-mediated mast cell degranulation and cytokine production in a VDR-dependent manner *in vitro*

Having shown that mast cells can limit inflammation associated with contact hypersensitivity reactions⁵ and that they are responsive to $1\alpha,25(\text{OH})_2\text{D}_3$ in a VDR-dependent manner,^{7,19} we tested whether $1\alpha,25(\text{OH})_2\text{D}_3$, as well as its inactive precursor metabolite 25OHD_3 , can modify IgE plus specific antigen-induced activation of BMCMCs *in vitro*. Exposure to $1\alpha,25(\text{OH})_2\text{D}_3$ for 24 hours reduced IgE-mediated histamine release (by 23% to 34%; Fig 1, A) and CysLT production (by 34% to 44%; Fig 1, B) at the highest concentrations (10^{-7} and 10^{-6} mol/L) tested in WT C57BL/6J (B6) mouse BMCMCs. Reduction of *de novo* TNF (Fig 1, C) and IL-6 (Fig 1, D) production was significant for both 10^{-8} and 10^{-7} mol/L $1\alpha,25(\text{OH})_2\text{D}_3$ in B6-WT (ie, $\text{VDR}^{+/+}$) BMCMCs. The curtailed release of these vasodilatory and proinflammatory mediators was not due to reduced cell viability or repression of the key signaling elements phospho-extracellular signal-regulated kinase (Erk) 1/2, phospho-p38, phospho-c-Jun N-terminal kinase (JNK), or phospho-nuclear factor κB (NF- κB)-p65 downstream of IgE-Fc ϵRI

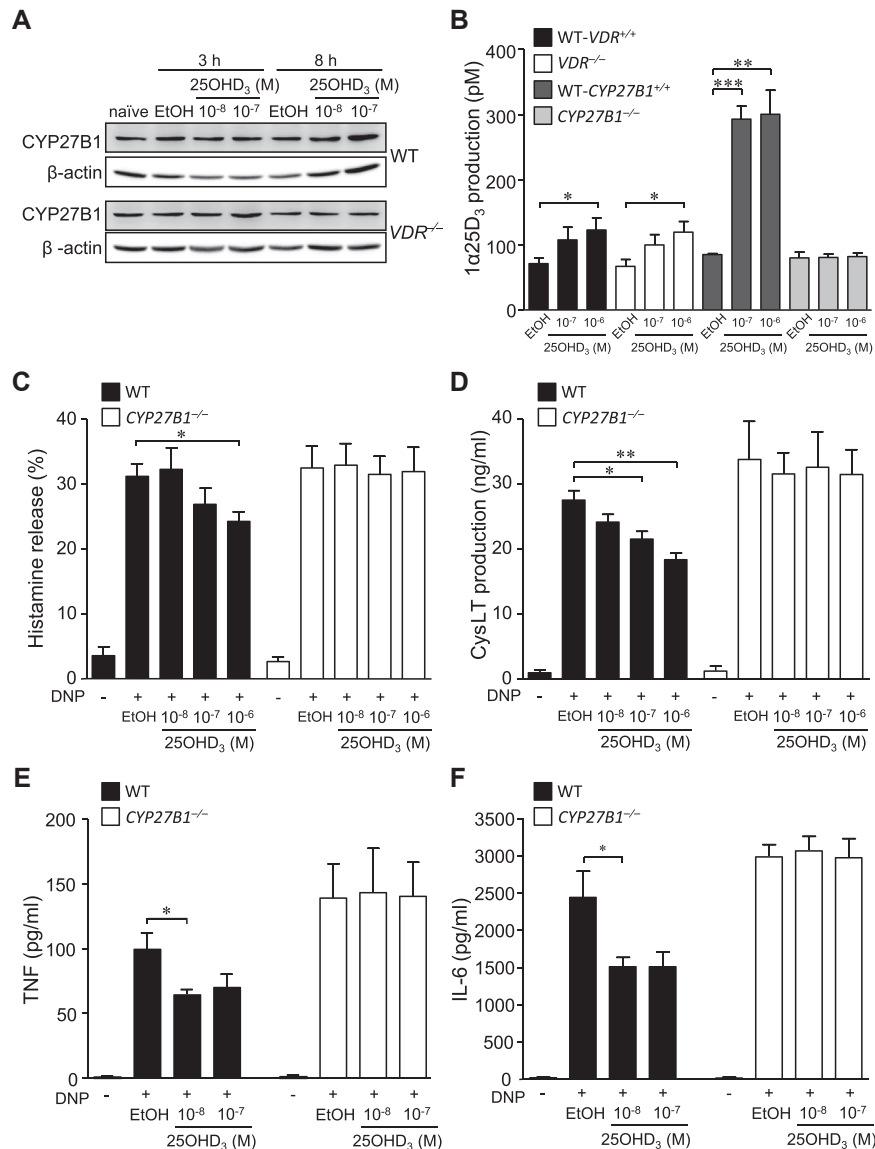


FIG 2. 25OHD₃-driven inhibition of IgE-mediated activation functions through mast cell-CYP27B1 catalytic activity. **A**, CYP27B1 and β-actin protein expression in WT and VDR^{-/-} BMCMCs cultured for 3 or 8 hours with 25OHD₃ at indicated concentrations or vehicle (EtOH). **B**, WT, VDR^{-/-}, or CYP27B1^{-/-} BMCMC production of 1α,25(OH)₂D₃ (1α,25D₃) incubated with 25OHD₃ for 6 hours. **C-F**, WT and CYP27B1^{-/-} BMCMCs pretreated with 25OHD₃ 24 hours before IgE plus DNP-HSA stimulation and release of histamine (30 minutes; Fig 2, C), CysLT (30 minutes; Fig 2, D), TNF (6 hours; Fig 2, E), and IL-6 (6 hours; Fig 2, F) into supernatants. Data are from 3 to 4 independent experiments. **P* < .05, ***P* < .01, and ****P* < .001 for the indicated comparisons.

activation or altered cell-surface expression of FcεRI or reduced c-Kit receptor expression (see Fig E1 in this article's Online Repository at www.jacionline.org); a finding previously reported to occur in BALB/c BMCMCs with long-term 30- to 40-day 1α,25(OH)₂D₃ (10⁻⁷ mol/L) incubation, which causes mast cell apoptosis and inhibits maturation/differentiation of mast cell progenitors.¹⁹ Importantly, we found that functional VDRs were necessary to mediate the negative regulation of mast cells in this setting (Fig 1). Interestingly, the inactive 25OHD₃ metabolite, like its biologically active form, required functional VDR expression to exert similar immunosuppressive activity on IgE-mediated BMCMC function, particularly at the highest concentrations of 10⁻⁷ and 10⁻⁶ mol/L tested (Fig 1).

CYP27B1 hydroxylase activity is required for 25OHD₃-induced suppression of IgE-mediated mast cell activation

It is unclear whether mast cells exhibit CYP27B1 activity and can convert 25OHD₃ to 1α,25(OH)₂D₃. Therefore we first analyzed CYP27B1 expression in BMCMCs using immunoblotting (Fig 2, A), as well as immunofluorescence and flow cytometric analysis (see Fig E2 in this article's Online Repository at www.jacionline.org). Both B6-VDR^{+/+} and VDR^{-/-} BMCMCs constitutively expressed CYP27B1, and although no changes in CYP27B1 protein levels were observed up to 8 hours after incubation with either 10⁻⁸ or 10⁻⁷ mol/L 25OHD₃ (Fig 2, A), VDR^{+/+}, VDR^{-/-}, and, to a greater extent, CYP27B1^{+/+}

BMCs (possibly because of the more diverse genetic background of this mouse colony) all had the ability to convert 10^{-7} and 10^{-6} mol/L 25OHD₃ to $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig 2, B). Importantly, *CYP27B1*^{−/−} BMCs were unable to produce $1\alpha,25(\text{OH})_2\text{D}_3$, showing that *CYP27B1* is essential. Interestingly, unlike *in vivo* findings in the proximal tubule of the kidney, where *CYP27B1* activity can be inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$,²⁰ $1\alpha,25(\text{OH})_2\text{D}_3$ lacked the ability to VDR-dependently transrepress *CYP27B1* mRNA (up to 6 hours; see Fig E3 in this article's Online Repository at www.jacionline.org) or reduce protein expression (up to 8 hours) in WT BMCs (Fig 2, A).

Notably, as determined for *VDR*^{−/−} BMCs (Fig 1), *CYP27B1*^{−/−} BMCs were also unresponsive to the suppressive properties of 10^{-7} or 10^{-6} mol/L 25OHD₃ compared with WT *CYP27B1*-active BMCs, which exhibited significantly reduced histamine release (Fig 2, C) and CysLT (Fig 2, D), TNF (Fig 2, E), and IL-6 (Fig 2, F) production. Thus although these findings do not exclude some suppressive effects mediated by directly bound 25OHD₃-VDR, our *in vitro* data provide evidence that mast cell-*CYP27B1* hydroxylase is required for mast cells to generate $1\alpha,25(\text{OH})_2\text{D}_3$, which, in turn, can repress IgE-mediated BMC activation in a VDR-dependent manner.

Mast cell-VDRs are essential for optimal curtailment of IgE-dependent PCA reactions by epicutaneous $1\alpha,25(\text{OH})_2\text{D}_3$ treatment *in vivo*

Our finding that the vitamin D₃ metabolites $1\alpha,25(\text{OH})_2\text{D}_3$ and 25OHD₃ can restrain IgE-induced mast cell activation *in vitro*, and that this activity requires mast cell-VDRs, raised the possibility that topical application of these secosteroid derivatives could ameliorate mast cell-specific skin reactions associated with PCA *in vivo*. To test this, we used genetic and cell-transfer approaches in 2 types of *c-kit* mutant mice (ie, WBB6F1-*Kit*^{W/W-v} and C57BL/6J-*Kit*^{W-sh/W-sh} mice), which are profoundly mast cell-deficient and can be selectively engrafted with *in vitro*-derived mast cells from WT mice or mice lacking specific mast cell-associated receptors (ie, *VDR*^{−/−}) or enzyme activity (ie, *CYP27B1*^{−/−}). This “mast cell knock-in” approach helps to reveal the extent to which the mast cell-dependent effects in mutant mice can be separated from those caused by other abnormalities^{18,21} associated with their mutation or mutations because only the mast cell deficiency is repaired by engraftment.

A single topical application of $1\alpha,25(\text{OH})_2\text{D}_3$ at a dose of 0.06 nmol per ear (3 $\mu\text{mol/L}$) 16 hours before intravenous challenge with specific antigen (DNP-HSA) significantly reduced the immediate phase (0.5–1 hour) of IgE-induced PCA-associated ear swelling (Fig 3) and this corresponded with attenuated transcription of histidine decarboxylase (*HDC*) and leukotriene C₄ synthase (*LTC4S*) mRNA compared with that seen in untreated PCA ears of WT C57BL/6J (*Kit*^{+/+}) mice (see Fig E4 in this article's Online Repository at www.jacionline.org). In contrast, because of their lack of mast cells, *Kit*^{W-sh/W-sh} mice did not exhibit PCA reactions in IgE-sensitized ears (ear swelling was indistinguishable from that observed in their vehicle-injected ear pinnae, regardless of $1\alpha,25(\text{OH})_2\text{D}_3$ application; Fig 3 and see Fig E5 in this article's Online Repository at www.jacionline.org). However, despite implications that mast cells might be a target of $1\alpha,25(\text{OH})_2\text{D}_3$,²² it remained to be definitively clarified whether, in the course of a PCA reaction, $1\alpha,25(\text{OH})_2\text{D}_3$ could exert direct

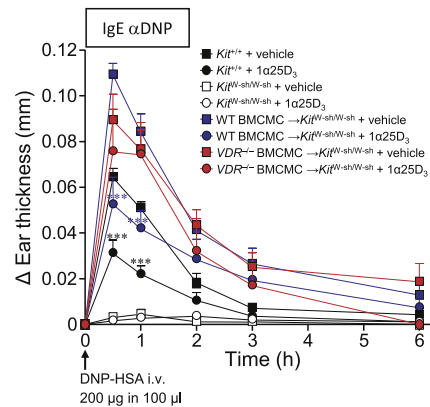


FIG 3. Epicutaneous application of $1\alpha,25(\text{OH})_2\text{D}_3$ suppresses IgE-mediated PCA reactions in a mast cell-VDR-dependent manner. Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μg of DNP-HSA into mice and 16 hours after pretreatment with topical application of 0.06 nmol of $1\alpha,25(\text{OH})_2\text{D}_3$ per ear ($1\alpha,25(\text{OH})_2\text{D}_3$, circles) or vehicle (EPGW; squares) that occurred concurrently with intradermal injection of 100 ng of IgE anti-DNP in *Kit*^{+/+} (WT), *Kit*^{W-sh/W-sh}, WT BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh}, or *VDR*^{−/−} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice. Data are from 9 to 12 mice per group from 3 independent experiments. ****P* < .001 for comparisons of $1\alpha,25(\text{OH})_2\text{D}_3$ versus vehicle-treated ears within the same group of mice.

restraint of mast cell function or acted independently of this, perhaps by altering vascular endothelial permeability.

To address this question, we assessed *Kit*^{W-sh/W-sh} mice engrafted with WT BMCs (WT BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice). Like their WT littermates, they exhibited a marked reduction in the magnitude of the IgE-PCA reaction after $1\alpha,25(\text{OH})_2\text{D}_3$ application (Fig 3). To evaluate whether mast cell-VDRs were required to mediate these suppressive effects of $1\alpha,25(\text{OH})_2\text{D}_3$, we also tested *VDR*^{−/−} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice and found that these animals did not respond to topical $1\alpha,25(\text{OH})_2\text{D}_3$ application, exhibiting comparable levels of ear swelling versus corresponding vehicle-treated IgE-PCA mice (Fig 3). Importantly, we also noted that our findings in *Kit*^{W-sh/W-sh} mice were recapitulated in WBB6F1-*Kit*^{+/+}, mast cell-deficient *Kit*^{W/W-v}, and WT or *VDR*^{−/−} BMCMC $\rightarrow$ *Kit*^{W/W-v} mice (see Fig E6 in this article's Online Repository at www.jacionline.org). This confirmed that notwithstanding the particular range of abnormalities carried by *Kit*^{W/W-v} and *Kit*^{W-sh/W-sh} mice, selective repair of their mast cell deficiency was sufficient to demonstrate the requirement for mast cell-VDRs for the immunoregulatory function of $1\alpha,25(\text{OH})_2\text{D}_3$ we observed in this experimental setting. Furthermore, the differences in ear thickness between the groups of IgE antigen-challenged WT or *VDR*^{−/−} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice were unlikely to be related to disparities in the extent of mast cell engraftment because similar numbers of ear pinna mast cells were present in the 2 groups, irrespective of IgE sensitization or $1\alpha,25(\text{OH})_2\text{D}_3$ treatment (see Fig E7 in this article's Online Repository at www.jacionline.org).

Skin irritation or heightened ear swelling responses were not observed in any of the HMEM-Pipes vehicle-injected ears from the groups of mice tested with a single epicutaneous application of 0.06 nmol of $1\alpha,25(\text{OH})_2\text{D}_3$ per ear (see Fig E5) nor was there evidence of increased ear swelling after 9 applications every 2 days of 0.06 nmol or 0.25 nmol of $1\alpha,25(\text{OH})_2\text{D}_3$ per ear in C57BL/6J WT mice (see Fig E8, A, in this article's Online Repository at www.jacionline.org). In contrast, multiple exposures of

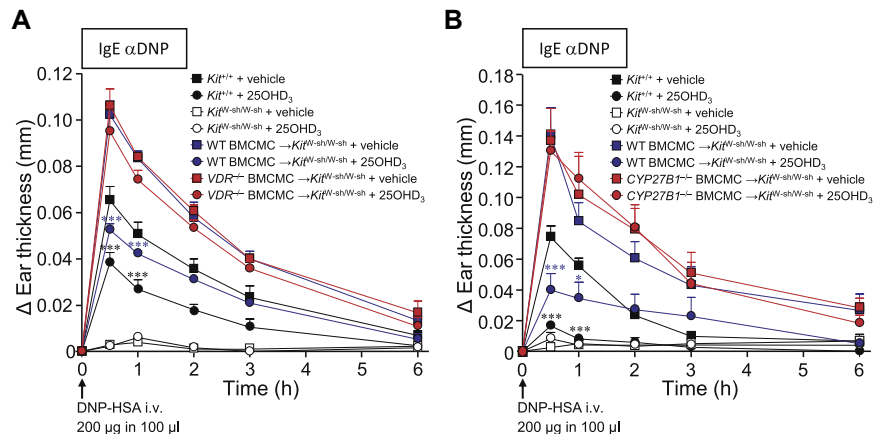


FIG 4. Mast cell–VDR and mast cell–CYP27B1 activity are required for epicutaneous 25OHD₃ dampening of IgE-mediated PCA reactions. Changes (Δ) in ear thickness 0 to 6 hours after intravenous (i.v.) injection of 200 μ g of DNP-HSA into mice and 24 hours after topical application of 0.06 nmol of 25OHD₃ per ear (circles) or vehicle (EPGW; squares) and 16 hours after intradermal injection of 100 ng of IgE anti-DNP antibody (right ears) in *Kit*^{+/+} (WT), *Kit*^{W-sh/W-sh}, WT BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh}, *VDR*^{-/-} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} (A), or *CYP27B1*^{-/-} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} (B) mice. Data are expressed as mean $\pm$ SEM and are from 3 (Fig 4, A; n = 2 to 4 mice per group per experiment) or 4 (Fig 4, B; n = 3 to 4 mice per group per experiment) independent experiments. **P* < .05 and ****P* < .001 for comparisons of 25OHD₃ versus vehicle-treated ears within the same group of mice.

1 α ,25(OH)₂D₃ significantly increased thymic stromal lymphopoietin (TSLP) mRNA levels only in the mice receiving the higher amount tested (0.25 nmol per ear; see Fig E8, B). Notably, although a single (see Fig E9 in this article's Online Repository at www.jacionline.org) or multiple application of 1 α ,25(OH)₂D₃ (dose of 0.25 nmol or 0.06 nmol per ear) markedly curtailed ear swelling responses, each to a similar extent, in the first 30 minutes of the PCA reaction, the extent of the repression was dampened over the 1- to 6-hour time course in C57BL/6J WT mice that received multiple applications of the high-dose 1 α ,25(OH)₂D₃ group compared with the lower-dose group (see Fig E8, C). These findings indicate that the induction of TSLP might reduce the regulatory effect of 1 α ,25(OH)₂D₃ to a limited extent in C57BL/6J WT mice and that the lower dose of 1 α ,25(OH)₂D₃ was required for optimal attenuation of IgE-mediated PCA reactions *in vivo*.

Mast cell–CYP27B1 activity is required for 25OHD₃-induced attenuation of PCA

On the basis of our *in vitro* data (Fig 1), we next assessed whether epicutaneous 25OHD₃ could reduce IgE- and mast cell-dependent PCA reactions *in vivo*. 25OHD₃ pretreatment mirrored the effects of 1 α ,25(OH)₂D₃, causing a significant reduction in IgE-dependent ear swelling in WT mice and WT BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice but not in *VDR*^{-/-} BMCMC $\rightarrow$ *Kit*^{W-sh/W-sh} mice (Fig 4, A). Notably, mast cell–CYP27B1 activity was necessary for reduced tissue swelling in this setting (Fig 4, B). Absence of mast cell–CYP27B1 rendered the BMCMC-engrafted groups of *Kit*^{W-sh/W-sh} (Fig 4, B) and *Kit*^{W/W-v} mice (see Fig E10 in this article's Online Repository at www.jacionline.org) unresponsive to 25OHD₃ compared with WT littermates and WT BMCMC-engrafted groups. Ear swelling in vehicle-injected control ears was minimal, and indistinguishable among the groups of mice tested, and remained unchanged with 25OHD₃ treatment (see Figs E10 and E11 in this article's Online Repository at www.jacionline.org). No significant disparities were evident in the numbers of mast cells in ear pinnae

among the engrafted groups of *Kit*^{W-sh/W-sh} and *Kit*^{W/W-v} mice (see Fig E11, C and D, in this article's Online Repository at www.jacionline.org). Thus our data show that mast cell–VDRs are required to mediate the activity of the vitamin D₃ metabolites 1 α ,25(OH)₂D₃ and 25OHD₃ and strongly suggest that autogenous mast cell 1 α ,25(OH)₂D₃ production through the activity of CYP27B1 contributed to the response to 25OHD₃ (even though other potential extrarenal sources of 1 α ,25(OH)₂D₃ derived from the administered 25OHD₃ might have been available to interact with *CYP27B1*^{-/-} BMCMC–VDRs in the treated ear).

IgE-mediated activation of human mast cells can be negatively regulated by vitamin D₃ metabolites

Because of the substantial immunosuppressive ability of both 1 α ,25(OH)₂D₃ and 25OHD₃ on mouse BMCMCs *in vitro* and *in vivo*, we decided to examine the potential clinical relevance of these findings by testing human CBMCs or PBMCs. Like their mouse counterparts, CBMCs and PBMCs exhibited a dose-dependent reduction in histamine release and CysLT production in response to either of the vitamin D₃ metabolites (Fig 5, A and B, and see Fig E12 in this article's Online Repository at www.jacionline.org). Although TNF production was significantly impaired by 10⁻⁸ and 10⁻⁷ mol/L 1 α ,25(OH)₂D₃ and, to a lesser extent, by 25OHD₃ (Fig 5, C), IgE-dependent CBMC–IL-10 production remained unchanged by 1 α ,25(OH)₂D₃ and was even significantly enhanced in the presence of 10⁻⁸ mol/L 25OHD₃ (Fig 5, D). As in mouse BMCMCs (Fig 2, A),⁷ these vitamin D₃-driven immunomodulatory effects are likely mediated through CBMC–VDRs (see Fig E12) and CBMC–CYP27B1 hydroxylation of 25OHD₃ to biologically active 1 α ,25(OH)₂D₃ (Fig 5, E, and see Fig E12).

DISCUSSION

In this study we have identified that mouse and human mast cells express CYP27B1, which enables them to convert inactive 25OHD₃ to biologically active 1 α ,25(OH)₂D₃. Mast cell–CYP27B1 activity and mast cell–VDRs represent important

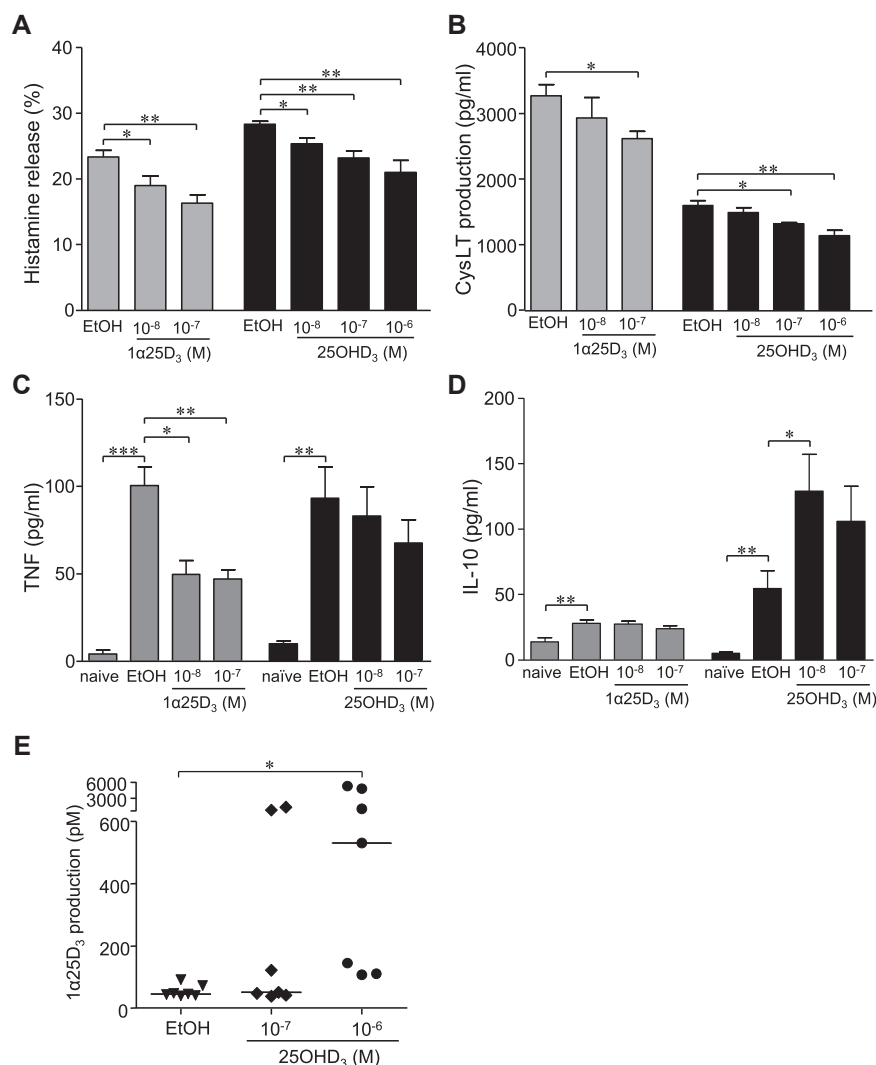


FIG 5. Vitamin D₃ metabolites can impair IgE-mediated human mast cell activation. **A–D**, CBMCs pretreated with 1α,25(OH)₂D₃ (1α25D₃), 25OHD₃, or vehicle (EtOH) at the time of or 8 hours before (for 25OHD₃) sensitization with human myeloma IgE for 16 hours, followed by challenge with anti-human IgE antibody and release of histamine (30 minutes; Fig 5, **A**), CysLT (30 minutes; Fig 5, **B**), TNF (16 hours; Fig 5, **C**), or IL-10 (16 hours; Fig 5, **D**) into supernatants. Data are expressed as mean + SEM and are from 3 to 5 different cord blood donors. **P* < .05, ***P* < .01, and ****P* < .001 for the indicated comparisons. **E**, CBMC production of 1α,25(OH)₂D₃ (1α25D₃) after incubation with 25OHD₃ for 6 to 7 hours. Data are from 7 independent cord blood donors. **P* < .05 for the indicated comparison, as analyzed by using the Wilcoxon *t* test.

mechanisms by which the vitamin D₃ metabolites 25OHD₃ and 1α,25(OH)₂D₃ can repress overt IgE-mediated mast cell activation *in vitro* and *in vivo*. The intrinsic ability to endogenously produce 1α,25(OH)₂D₃ is a previously unrecognized function of mast cells and is likely to have clinical implications in settings of 25OHD₃ insufficiency.

Interestingly, we observed that the inactive 25OHD₃ metabolite, like its biologically active form, required functional VDR expression to exert similar immunosuppressive activity on IgE-mediated BMCMC function, particularly at the highest concentrations of 10⁻⁷ and 10⁻⁶ mol/L tested (Fig 1). 25OHD₃ can bind to VDRs with low affinity and has been described as a weak genomic agonist at this concentration.²³ Therefore it is possible that the 25OHD₃-bound VDR exerted its regulatory effects in a manner similar to that of its more biologically efficacious metabolite 1α,25(OH)₂D₃, albeit at higher

concentrations. However, our data indicate that the most likely mechanistic explanation for 25OHD₃-VDR repression of IgE-mediated mast cell function is through the mast cell's intrinsic ability to convert 25OHD₃ to 1α,25(OH)₂D₃ in a CYP27B1-dependent manner.

Although our *in vitro* investigations provided evidence that treatment of IgE-activated mast cells with 25OHD₃ or 1α,25(OH)₂D₃ appeared to cause a modest reduction in the release of each mediator tested, it is conceivable that the cumulative effect of these individual changes could result in a substantially diminished response in the setting of mast cell-dependent IgE-mediated PCA *in vivo*. However, additional repressive effects on G protein-coupled receptors, such as the anaphylatoxin receptors²⁴ or sphingosine-1-phosphate receptors,^{25,26} which can enhance IgE-dependent skin inflammation *in vivo*, cannot be excluded and are yet to be explored. Importantly, our data show

that mast cell–VDRs at the skin site of topical application are required to mediate the activity of the vitamin D₃ metabolites 1 α ,25(OH)₂D₃ and 25OHD₃ and strongly suggest that endogenous mast cell 1 α ,25(OH)₂D₃ production through the catalytic activity of CYP27B1 contributed to the response to 25OHD₃ (even though other potential extrarenal sources of 1 α ,25(OH)₂D₃ derived from the administered 25OHD₃ might have been available to interact with CYP27B1^{−/−} BMCMC-VDRs in the treated ear).

In other settings application of 1 α ,25(OH)₂D₃ to the skin can induce a regulatory type of environment by enhancing CD4⁺CD25⁺ Treg cell suppressive activity²⁷ and downregulating immune responses in models of contact hypersensitivity.^{22,28} Recently, Li et al²⁹ reported that CD1 WT mice treated every other day for 18 days with 0.25 nmol of 1 α ,25(OH)₂D₃ per ear had atopic dermatitis–like skin inflammation. We found that multiple exposures of 1 α ,25(OH)₂D₃ at only the higher amount tested (0.25 nmol per ear compared with 0.06 nmol per ear) significantly increased TSLP mRNA levels in the treated skin but was not coupled with skin irritation and exacerbated ear swelling responses in the C57BL/6J WT mice tested, a disparity that might be due to the different genetic backgrounds of the mice. Importantly, the lower dose of 0.06 nmol 1 α ,25(OH)₂D₃ per ear used throughout our *in vivo* studies, which elicited marked negative regulatory effects on IgE-dependent PCA reactions, could be applied multiple times without enhancing TSLP expression or skin irritation and was able to attenuate PCA reactions to a similar extent irrespective of whether applied as a single or multiple (9 $\times$) application; a finding which suggests that low-calcemic VDR agonists might have beneficial anti-inflammatory functions when topically applied at much lower amounts than those reported to induce TSLP and trigger changes similar to those of atopic dermatitis in mice.²⁹

The observation that IgE-activated human CBMCs can potentially be altered from a proinflammatory to an IL-10–expressing anti-inflammatory state when exposed to sufficient levels of vitamin D₃ is of interest and might even be clinically relevant. Allergic disorders have dramatically increased in prevalence in industrialized countries. The pathogenesis of such disorders is likely to be multifactorial, but there is growing speculation of a link with vitamin D₃ insufficiency, which is defined by most experts as less than 20 ng/mL (ie, 48 nmol/L) and optimal at greater than 30 ng/mL (72 nmol/L) of 25OHD₃^{10,16}; a concentration range that is similar to those tested in our human CBMCs (10^{−8} to 10^{−6} mol/L; Fig 5, A–D, and see Fig E12). Maternal 25OHD₃ insufficiency during pregnancy has been linked to an increased risk of eczema through alterations in skin barrier function and changes in microbial defense in the first 12 months of infancy,¹⁵ and serum vitamin D₃ levels have been shown to inversely correlate with serum IgE levels in children with asthma.¹⁴ On the basis of our *in vitro* and *in vivo* findings, it is plausible that suboptimal levels of circulating 25OHD₃ could unshackle an intrinsic mechanism of mast cell restraint, resulting in heightened detrimental contributions of mast cells during IgE-mediated disorders.

In conclusion, our study has provided evidence that the vitamin D₃ metabolites 1 α ,25(OH)₂D₃ and 25OHD₃ can attenuate the generation of proinflammatory signals from IgE-activated mouse and human mast cells. Essential to this negative regulatory function of vitamin D₃ in mice is mast cell expression of VDRs and, for 25OHD₃, the hydroxylase activity of CYP27B1. Our results in C57BL/6J-Kit^{W-sh/W-sh} and WBB6F₁-Kit^{W/W-v} mice show

that epicutaneously applied vitamin D₃ metabolites, working through mast cell–associated VDR and CYP27B1 activity, can restrain mast cell–driven PCA *in vivo*. The nature of the mast cell's responses to vitamin D₃ might vary in different species and depend on metabolite concentrations readily available not only in terms of serum levels in circulation but also local levels of biologically active 1 α ,25(OH)₂D₃ in tissues derived from either external or mast cell–intrinsic sources. However, our findings suggest that optimal levels of vitamin D₃ might contribute to the homeostatic regulation of mast cells and that a deficiency in vitamin D₃ might result in dysregulation of this vitamin D/mast cell regulatory axis. These findings also highlight the possibility of using certain low-calcemic agonists of VDRs or perhaps even dietary supplementation with 25OHD₃ itself for the management of mast cell–driven allergic responses and perhaps other inflammatory disorders in which mast cells are implicated.

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Key messages

- Mouse and human mast cells can convert 25OHD₃ to 1 α ,25(OH)₂D₃ through CYP27B1 catalytic activity.
- The vitamin D₃ metabolites 25OHD₃ and 1 α ,25(OH)₂D₃ repress IgE-mediated mast cell–derived proinflammatory and vasodilatory mediator release in a VDR-dependent manner *in vitro*.
- Epicutaneously applied vitamin D₃ metabolites significantly reduce the magnitude of IgE-mediated PCA reactions *in vivo*; a response that requires functional mast cell–VDRs and mast cell–CYP27B1 at the affected skin site.

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METHODS

Mice

B6.129S4-*Vdr*^{tm1Mbd/J} mice were backcrossed to C57BL/6 mice for more than 9 generations. As previously reported, adult *Kit*^{W-sh/W-sh} and *Kit*^{W/W-v} mice have a profound deficiency of mast cells, including less than 1.0% the WT level of mast cells in the dermis.^{E1-E3} All mice (including *VDR*^{-/-} mice), with the exception of *CYP27B1*^{-/-} mice, were provided commercial mouse chow containing vitamin D₃ (cholecalciferol) at greater than 2000 IU/kg *ad libitum*. Derivation of the parental strain was undertaken by means of homologous recombination in embryonic stems, where a neomycin resistance gene was inserted in place of exons VI, VII, and VIII of the mouse *CYP27B1* gene, replacing both the ligand-binding and heme-binding domains, as previously described.^{E4} These mice were originally maintained on a mixed genetic background with B6 and BALB/c strains and then backcrossed for an additional 3 generations with C57BL/6 mice in house in Adelaide. *CYP27B1*^{-/-} mice were maintained on a high-calcium diet containing 1.5% calcium in drinking water and chow containing 1% calcium, 0.85% phosphorus, 0% lactose, and 2200 IU/kg vitamin D₃ (Specialty Feeds). Experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia, with approval from the Institute of Medical and Veterinary Science Animal Ethics Committee (Australia).

Generation of BMCMCs

As previously described,^{E2,E5} BMCMCs were obtained by culturing bone marrow cells from femurs and tibias of mice in DMEM (Life Technologies, Carlsbad, Calif) supplemented with 10% FCS (Bovogen, East Keilor, Australia) and 20% WEHI-3 conditioned medium (containing 3–4 ng/mL IL-3) for 4 to 6 weeks, at which time greater than 95% of the cells were identified as mast cells by using May Grünwald-Giemsa staining and flow cytometric analysis (c-Kit⁺FcεRI⁺).

Preparation of vitamin D₃

1α,25(OH)₂D₃ and 25OHD₃ (Sigma-Aldrich) were reconstituted at 1 or 10 mmol/L with 100% absolute EtOH (Sigma-Aldrich) and stored, shielded from light, in an airtight tube at -80°C. The chemical integrity of both metabolites was regularly verified with a scanning spectrophotometer.

Multiple epicutaneous applications of vitamin D₃ with or without IgE-dependent PCA

For experiments in which 1α,25(OH)₂D₃ was epicutaneously applied a total of 9 times every 2 days in the absence of PCA, female C57BL/6J mice received per application a dose of 0.06 nmol per ear (3 μmol/L in 20 μL of EPGW vehicle) or 0.25 nmol per ear (12.5 μmol/L in 20 μL of 100% EtOH vehicle) to the right ear or vehicle alone as indicated to the left ear, and change in ear thickness from baseline was measured before each application. For some experiments on the same day as the final application of 1α,25(OH)₂D₃, mice were intradermally injected with 100 ng of IgE anti-DNP in the right ear or vehicle HMEM-Pipes in the left ear. Sixteen hours later, mice were intravenously injected with 200 μg of DNP-HSA, and changes (Δ) in ear thickness at 0 to 6 hours were measured and calculated from the baseline measured at day 0 before the first application of 1α,25(OH)₂D₃. For all experiments, ear pinnae were collected for histologic analysis and gene expression analysis 48 hours after the final application of 1α,25(OH)₂D₃ or 6 hours after induction of the PCA reaction.

Histology and quantification of mast cell numbers

Mice were killed by means of CO₂ inhalation, samples of ear pinna were fixed in 10% buffered formalin and embedded in paraffin (with care to ensure a cross-section orientation), and 4-μm sections were cut. Ear sections were stained with 0.1% toluidine blue (pH 1.0) for the detection of mast cells (cytoplasmic granules appear purple). Ear pinna mast cells were counted in 6 to 9 consecutive fixed fields of 870 μm in width with a 20× microscope objective (×200 final magnification), and mast cell numbers were expressed per

horizontal ear cartilage field length (millimeters) by using computer-generated image analysis (National Institutes of Health Image J software, version 1.46⁵). The entire length of a strip of skin extending from the base to the tip of the ear pinna (approximately 5.4–8.1 mm) was quantified. After intradermal engraftment of BMCMCs, *Kit*^{W-sh/W-sh} or *Kit*^{W/W-v} mice exhibited mast cells from the base to the tip of the ear pinnae in an anatomic distribution similar to that of the native mast cell populations in the corresponding WT mice.

Immunofluorescence

For CYP27B1 immunofluorescence in BMCMCs, cells were centrifuged at 500 rpm for 5 minutes onto Polysine slides (Menzel-Gläser, Thermo Fisher Scientific, Waltham, Mass) and fixed with 150 μL of IC Fixation Buffer (Fixation & Permeabilization Kit, eBioscience) for 20 minutes at room temperature before washing in Permeabilization Buffer (eBioscience) for 5 minutes. Cells were then incubated with 3 μg/mL rabbit anti-CYP27B1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) or 3 μg/mL rabbit polyclonal IgG isotype control antibody (Dako, Hørsholm, Denmark) for 16 hours at 4°C. Slides were then rinsed 3 times in Permeabilization Buffer and incubated with Alexa Fluor 594-conjugated goat anti-rabbit antibody (1:200 dilution; Molecular Probes, Eugene, Ore) for 1 hour at room temperature in the dark. After 3 additional washes in Permeabilization Buffer, cells were incubated with 1 μg/mL DAPI (Roche, Mannheim, Germany) for 2 minutes at room temperature, rinsed in Permeabilization Buffer, mounted with Fluorescence Mounting Medium (Dako), and imaged with a Nikon Spectral Imaging Confocal Microscope Digital Eclipse C1si and EZ-C1 software (version 3.20).

Preparation of human mast cells

Mature CBMCs or PBMCs were generated by first isolating CD34⁺ progenitor cells from human umbilical cord blood or human buffy coats provided by the Australian Red Cross, respectively. Briefly, blood was diluted with sterile PBS at a ratio of 1:1 and layered gently over Histopaque-1077 (1.77 g/L, Sigma-Aldrich), and after centrifugation (600g, 30 minutes), the interface containing mononuclear cells was harvested and the remaining red blood cells were disrupted with hemolytic solution (155 mmol/L NH₄Cl, 10 mmol/L KHCO₃, 0.1 mmol/L EDTA 2Na). CD34⁺ progenitor cells were enriched by means of positive immunomagnetic selection with CD34 MicroBeads and an autoMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The isolated CD34⁺ cells were then transferred into 12-well plates at a density of 5 × 10⁶ cells/mL in IMDM medium (Life Technologies) supplemented with 1% insulin-transferrin-selenium (Life Technologies), 5 × 10⁻⁵ mol/L 2-mercaptoethanol (Life Technologies), 1% penicillin-streptomycin (Life Technologies), 0.1% BSA (Sigma-Aldrich), 100 ng/mL recombinant human stem cell factor, 50 ng/mL rhIL-6, and 1 ng/mL rhIL-3 (all recombinant human cytokines were from Shendoh Biotechnology, Warwick, Pa) and placed in a CO₂ incubator at 37°C. The cytokine-supplemented medium was replaced weekly, and rhIL-3 was omitted from the medium after the first 2 weeks of culture. From 6 weeks, 10% FCS was added to the medium, and CBMCs or PBMCs were used at 10 weeks of culture. At that time, the populations contained 96% mast cells, as determined by using May Grünwald-Giemsa staining and flow cytometric analysis (tryptase positive, 10 μg/mL; Millipore, Billerica, Mass).

Measurement of histamine and CysLT

BMCMCs and CBMCs or PBMCs were preincubated in 10% charcoal-stripped FCS complete medium for 72 hours, supplemented with the vitamin D₃ metabolites, and sensitized with IgE, as outlined above, for mast cell activation *in vitro*. After the 16 hour IgE sensitization, BMCMCs, CBMCs, or PBMCs (10⁶ cells/mL) were resuspended in Tyrode's buffer and then activated with DNP-HSA (10 ng/mL, BMCMCs) or anti-human IgE antibody (1 μg/mL, CBMCs or PBMCs) for 30 minutes at 37°C in the presence of 1α,25(OH)₂D₃ (10⁻⁸ to 10⁻⁶ mol/L) or 25OHD₃ (10⁻⁸ to 10⁻⁶ mol/L) or EtOH (0.03%). Histamine or CysLT levels in supernatants and corresponding cell lysates (histamine only) were measured with histamine (Beckman

Coulter, Fullerton, Calif) or CysLT EIA (Cayman Chemical, Ann Arbor, Mich) kits, according to the manufacturer's instructions.

Immunoblotting

BMCMCs and CBMCs were preincubated in 10% charcoal-stripped FCS complete medium for 72 hours, treated with 25OHD₃ (10^{-8} or 10^{-7} mol/L) for 3 and 8 hours, and lysed in ice-cold lysis buffer (50 mmol/L Tris-base, 100 mmol/L NaCl, 5 mmol/L EDTA, 67 mmol/L Na₂P₂O₇, 0.01% Triton X-100, and complete protease inhibitors cocktail [Roche]). Proteins were separated with SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline that contained 0.1% Tween-20; they were then probed with a rabbit anti-CYP27B1 antibody (600 ng/mL, Santa Cruz Biotechnology) or rabbit anti- β -actin antibody (1:1000 dilution; Cell Signaling Technology, Beverly, Mass) overnight at 4°C. Membranes were then probed with horseradish peroxidase-conjugated antibody against rabbit IgG (1:2000 dilution, Cell Signaling), and bands were visualized with ECL reagent (Amersham, GE Healthcare Life Sciences, Pittsburgh, Pa) with a LAS4000 imaging system (Fujifilm, Tokyo, Japan).

For cells that were sensitized with IgE anti-DNP mAb (SPE-7, 2 μ g/mL), IgE was added to the cells at the same time as administration of 1 α ,25(OH)₂D₃ (10^{-7} mol/L) or vehicle (0.03% EtOH) and incubated for 16 hours at 37°C in a CO₂ incubator. Cells were centrifuged at 180g for 5 minutes, resuspended in Tyrode's buffer (129 mmol/L NaCl, 8.4 mmol/L glucose, 10 mmol/L HEPES, 5 mmol/L KCl, 1 mmol/L MgCl₂, 1.4 mmol/L CaCl₂, and 1% BSA at pH 7.4), centrifuged again, and then resuspended with Tyrode buffer at 4×10^6 cells/mL. Cells were activated with 10 ng/mL DNP-HSA-specific antigen for 2 or 15 minutes at 37°C in the presence of 1 α ,25(OH)₂D₃ (10^{-7} mol/L) or EtOH (0.03%). The reaction was quenched by the addition of ice-cold buffer, followed immediately by centrifugation at 180g for 5 minutes at 4°C. Cells were lysed in ice-cold lysis buffer and electrophoretically transferred, and bands were visualized with the same protocol as detailed above, with the exception that membranes were probed with rabbit anti-phospho-Erk1/2, anti-phospho-p38, anti-phospho-JNK1/2, and anti-phospho-NF- κ B-p65. Stripped membranes were then probed with the total form of anti-Erk1/2, anti-p38, anti-JNK1/2, and anti-NF- κ B-p65 (all including phosphorylated antibodies were used at 1:1000 dilution, Cell Signaling). Immunoblots presented in figures are representative of 2 or 3 similar independent experiments.

Flow cytometric analysis

BMCMCs were incubated with 1 α ,25(OH)₂D₃ (10^{-8} to 10^{-7} mol/L) and IgE anti-DNP antibody (2 μ g/mL) for 16 hours before cell-surface Fc ϵ RI and c-kit expression determination. BMCMCs were washed in FACS buffer (PBS with 2% FCS) and incubated with anti-mouse CD16/CD32 mAb (1 μ g/mL) on ice for 15 minutes. After FcR blocking, BMCMCs were incubated with anti-Fc ϵ RI α -fluorescein isothiocyanate (FITC; 2.5 μ g/mL, eBioscience) or anti-c-kit-phycoerythrin (2 μ g/mL) antibodies or isotype control American hamster IgG-FITC (2.5 μ g/mL) and rat IgG_{2b}-phycoerythrin (2.5 μ g/mL) antibody for 30 minutes on ice and then analyzed on a Beckman Coulter Cytomics FC500 and using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2 (Beckman Coulter). All antibodies were obtained from eBioscience. For BMCMC-CYP27B1 expression, cells were incubated with rabbit anti-CYP27B1 antibody (3 μ g/mL, Santa Cruz Biotechnology) or isotype control rabbit IgG antibody (3 μ g/mL, Dako) in Permeabilization Buffer (eBioscience) for 30 minutes on ice, then incubated with Alexa Fluor 594-conjugated goat anti-rabbit antibody (1:100 dilution, Molecular Probes) for 30 minutes on ice, and finally analyzed by using flow cytometry. For determination of CBMC-VDR expression, cells were fixed in IC Fixation buffer (eBioscience) for 20 minutes at room temperature and incubated with rat anti-VDR antibody (10 μ g/mL, Millipore) or isotype control rat IgG_{2a} antibody (2 μ g/mL, eBioscience) in Permeabilization Buffer (eBioscience) for 30 minutes on ice. Cells were washed, incubated

with goat anti-rat FITC-conjugated antibody (1:100 dilution, Life Technologies) for 30 minutes on ice, and then analyzed by using flow cytometry.

RNA extraction and real-time PCR

Ear pinnae were finely sliced and sonicated in 500 μ L of TRIzol reagent (Life Technologies) from which RNA was extracted, according to the manufacturer's instructions. For mRNA analysis, 0.5 μ g of RNA was used for cDNA synthesis by using the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed by using a 1:4 dilution of cDNA with the QuantiTect SYBR Green PCR System (Qiagen) on a Rotor-Gene 6000 PCR machine (Qiagen). PCR assays were performed for 45 cycles (95°C for 15 seconds, 55°C for 20 seconds, and 72°C for 20 seconds). Relative expression levels of *TSLP* mRNA were normalized to the glyceraldehyde-3-phosphate dehydrogenase control by using Rotor-Gene Series 6000 Software (Qiagen). The following oligonucleotide sequences were used: *TSLP*—forward, 5'-AGCTTGCTCTCCTGAAATCGAG-3'; reverse, 5'-AGGTTTGATTTCAGGCAGATGTT-3'; leukotriene C₄ synthase (*LTC4S*)—forward, 5'-ATGAAGGACGAAGTGGCTCTT-3'; reverse, 5'-CCTGTAGGGAGAAGTAGGCTTG-3'; histidine decarboxylase (*HDC*)—forward, 5'-AGGAGCAATCCAAGGGAGAT-3'; reverse, 5'-GGTATCCAGGCTGCACATTT-3'; and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)—forward, 5'-ACATCATCCCTGCATCCACT-3'; reverse, 5'-ACTTGGCAGGTTTCTCCAG-3'. CYP27B1 cDNA was primed with the Mm_Cyp27b1_1_SG QuantiTect primer assay (Qiagen), and PCR was conducted according to the manufacturer's instructions.

Measurement of 1 α ,25(OH)₂D₃ in BMCMCs and CBMCs

Five-week-old BMCMCs (WT [VDR mouse colony] or *VDR*^{-/-} or WT [CYP27B1 mouse colony] or *CYP27B1*^{-/-}) or 10-week-old CBMCs (2×10^6 cells/mL) were preincubated in 10% charcoal-stripped FCS complete medium (DMEM or IMDM, respectively) for 72 hours in a CO₂ incubator at 37°C. Cells were then replenished with the charcoal-stripped FCS DMEM (supplemented with 3 ng/mL rmlL-3 for BMCMCs) and IMDM (supplemented with 100 ng/mL recombinant human stem cell factor and 50 ng/mL rhIL-6 for CBMCs). For CBMCs, 2×10^6 cells/mL were incubated with 25OHD₃ (10^{-7} or 10^{-6} mol/L) or EtOH (0.03%) for 6 to 7 hours, whereas BMCMCs (2×10^6 cells/mL) were incubated for 24 hours, and the supernatant was replaced with new medium containing 25OHD₃ (10^{-7} or 10^{-6} mol/L) for a further 6 hour incubation. Culture supernatants and cell lysates were collected and snap-frozen in liquid nitrogen. Samples were stored, shielded from light, at -80°C until analysis. Levels of 1 α ,25(OH)₂D₃ supernatants and corresponding cell lysates were measured with a radioimmunoassay kit (Immunodiagnostic Systems, Frankfurt, Germany), according to the manufacturer's instructions. Mast cell production of 1 α ,25(OH)₂D₃ was determined as the amount measured in the cell lysate plus the supernatant and expressed as picomoles per liter.

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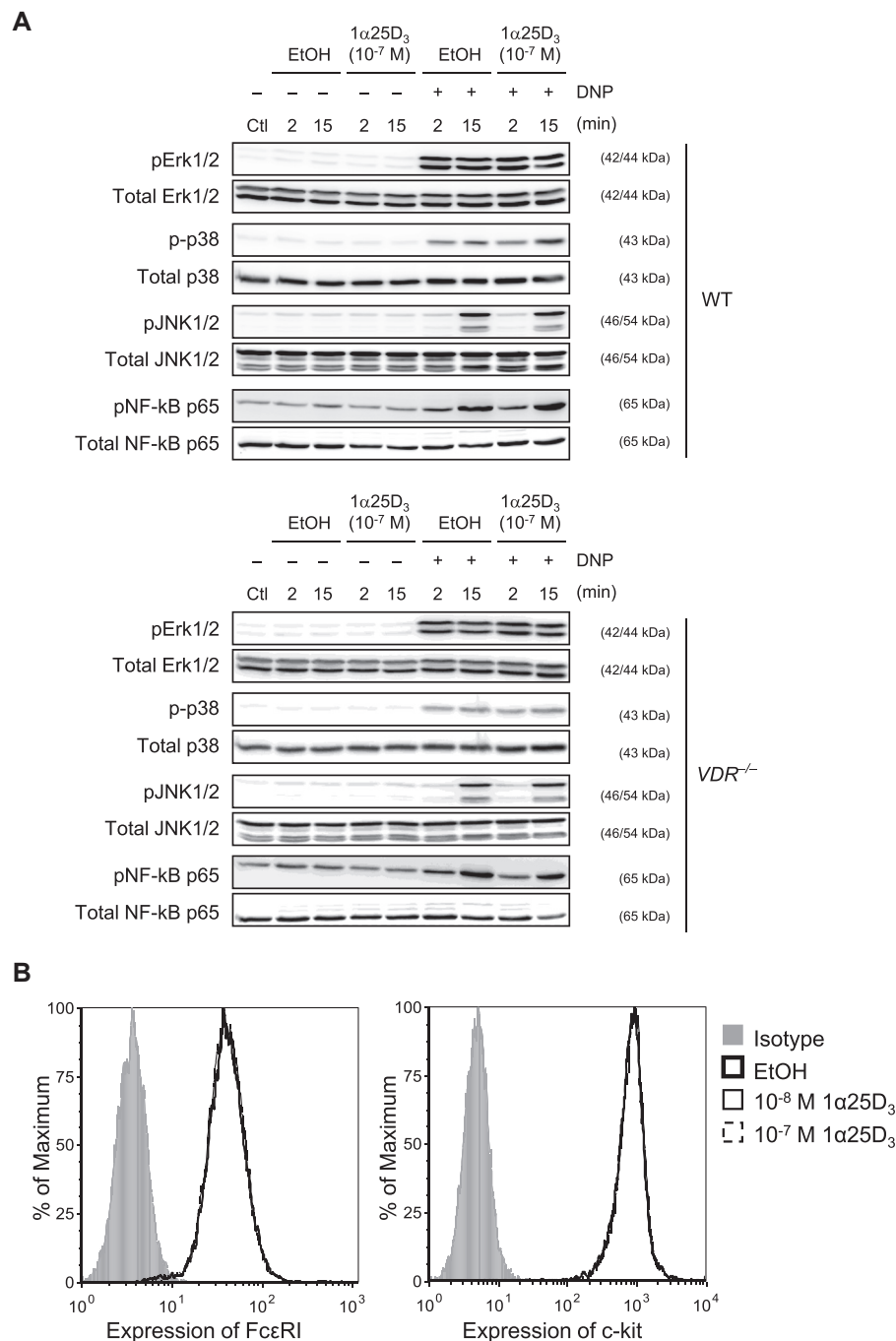


FIG E1. 1 α 25(OH)₂D₃ treatment does not alter key signaling events downstream of IgE-Fc ϵ RI activation or Fc ϵ RI and c-kit expression in BMCMCs. **A**, WT or VDR^{-/-} BMCMCs were preincubated with 1 α ,25(OH)₂D₃ (10⁻⁷ mol/L) or 0.03% EtOH for 16 hours and then stimulated with DNP-HSA in the presence of 1 α ,25(OH)₂D₃ (10⁻⁷ mol/L) or 0.03% EtOH for the indicated times. Whole cell lysates were prepared and probed with antibodies to phospho-Erk1/2 (*p-Erk1/2*), phospho-p38 (*p-p38*), phospho-JNK1/2 (*p-JNK*), and phospho-NF- κ B-p65 (*p-NF- κ B*). Stripped membranes were reprobbed with antibodies against the total form of the corresponding phosphoproteins as controls. Results are representative of 2 similar experiments. **B**, Flow cytometric analysis of Fc ϵ RI and c-kit expression of IgE-sensitized WT BMCMCs treated with 10⁻⁸ or 10⁻⁷ mol/L 1 α ,25(OH)₂D₃ for 16 hours.

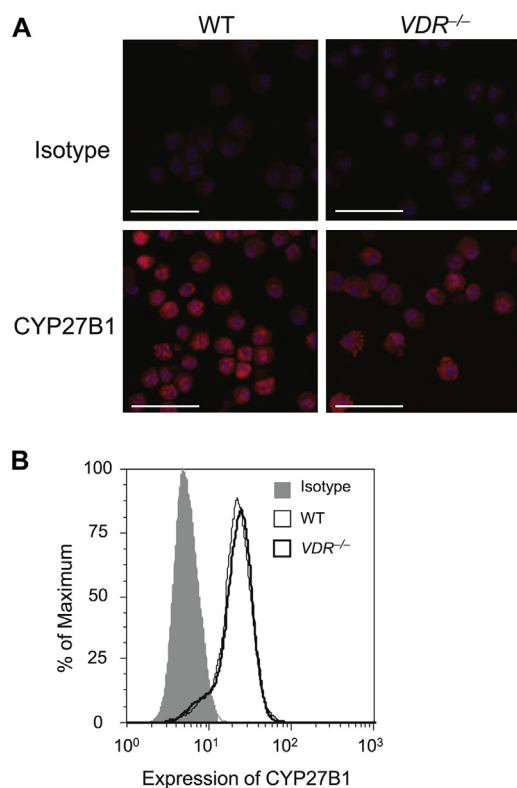


FIG E2. CYP27B1 expression in WT and *VDR*^{-/-} BMCMCs. Detection of CYP27B1 protein in WT and *VDR*^{-/-} BMCMCs by means of immunofluorescence with rabbit anti-CYP27B1 antibody or isotype control rabbit IgG antibody (scale = 50 μ m; **A**) and flow cytometric analysis (**B**) is shown. Results are representative of 3 similar independent experiments.

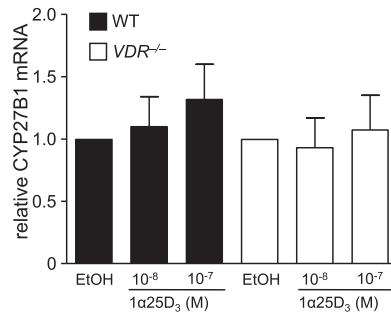


FIG E3. $1\alpha,25(\text{OH})_2\text{D}_3$ does not regulate *CYP27B1* expression in BMCMCs. *CYP27B1* mRNA expression analyzed by using quantitative PCR in 5-week-old WT and *VDR*^{-/-} BMCMCs cultured for 6 hours with $1\alpha,25(\text{OH})_2\text{D}_3$ (*1α25D₃*) at indicated concentrations or vehicle (0.03% EtOH) is shown. Data are expressed as mean + SD obtained in 2 independent experiments.

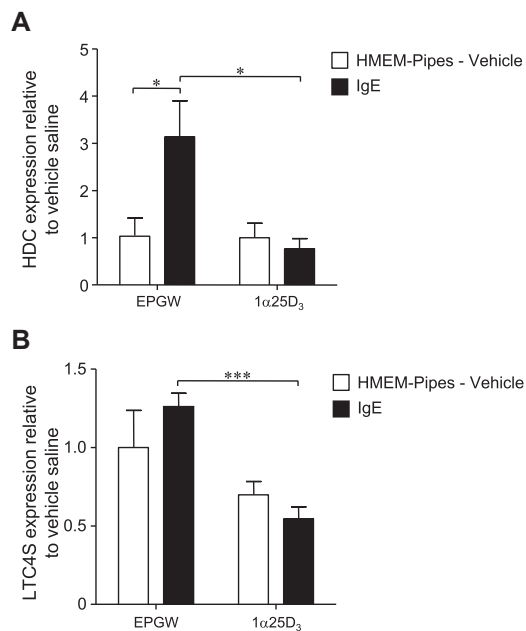


FIG E4. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment reduced histidine decarboxylase (*HDC*) and leukotriene C_4 synthase (*LTC4S*) mRNA levels in IgE-mediated PCA-affected ears. *HDC* (**A**) and *LTC4S* (**B**) mRNA expression in ear skin of mice was analyzed by using quantitative PCR at 3 hours after induction of IgE-mediated PCA. Data are expressed as mean + SEM of 4 to 6 mice per group from 3 independent experiments. * $P < .05$ and *** $P < .001$ for the indicated comparisons.

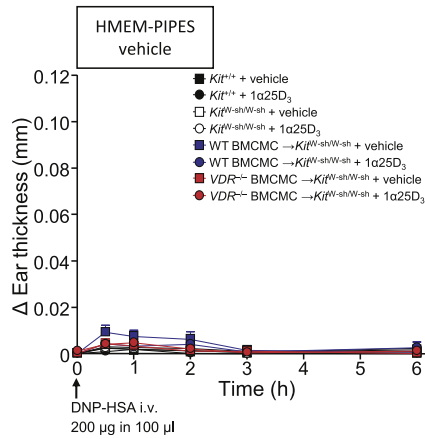


FIG E5. Ear-swelling responses after injection with vehicle (HMEM-Pipes). Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μ g of DNP-HSA into mice and 16 hours after pretreatment with topical application of 0.06 nmol $1\alpha,25(OH)_2D_3$ ($1\alpha 25D_3$; circles) per ear or vehicle (EPGW; squares) that occurred concurrently with intradermal injection of vehicle (left ear) in $Kit^{+/+}$ (WT), $Kit^{W-sh/W-sh}$, WT BMCMC $\rightarrow Kit^{W-sh/W-sh}$, or $VDR^{-/-}$ BMCMC $\rightarrow Kit^{W-sh/W-sh}$ mice are shown. Data expressed as mean $\pm$ SEM. None of the differences between any of the groups achieved statistical significance (defined as $P < .05$), as determined by using 2-way ANOVA. These data are from the same mice whose results for IgE anti-DNP antibody-injected right ears are shown in Fig 3.

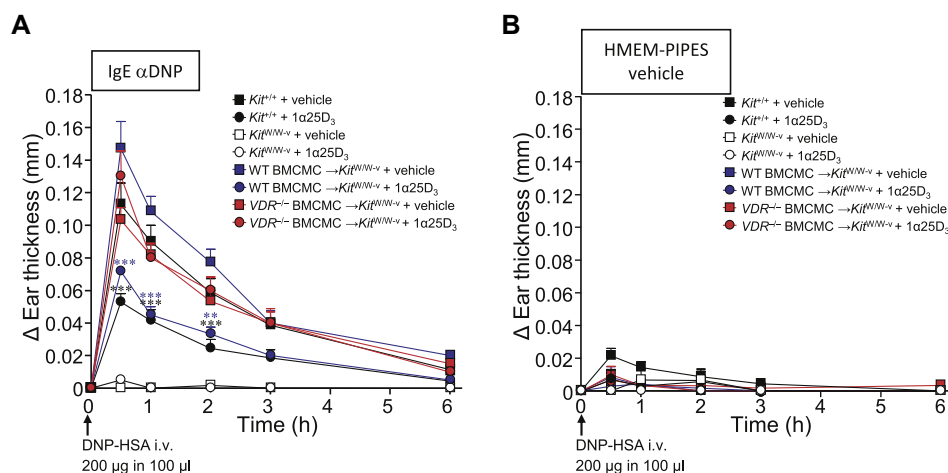


FIG E6. Epicutaneous application of $1\alpha,25(\text{OH})_2\text{D}_3$ suppresses IgE-mediated PCA reactions in a mast cell-VDR-dependent manner. Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μg of DNP-HSA into mice and 16 hours after topical application of 0.06 nmol of $1\alpha,25(\text{OH})_2\text{D}_3$ ($1\alpha25\text{D}_3$; circles) per ear or vehicle (EPGW; squares) that occurred concurrently with intradermal injection of 100 ng of IgE anti-DNP antibody into right ears (A) or HMEM-Pipes vehicle (B) in WBB6F₁-*Kit*^{+/+} (WT; black marker), mast cell-deficient WBB6F₁-*Kit*^{W/W-v} (white marker), WT BMCMC $\rightarrow$ *Kit*^{W/W-v} (blue marker), or *VDR*^{-/-} BMCMC $\rightarrow$ *Kit*^{W/W-v} (red marker) mice are shown. Data are expressed as mean + SEM and are from 6 to 9 mice per group and 2 independent experiments, each of which produced similar results. ***P* < .01 and ****P* < .001 for comparisons of $1\alpha,25(\text{OH})_2\text{D}_3$ versus vehicle-treated ears within the same group of mice, as indicated with color coding, by means of 2-way ANOVA with Bonferroni posttests.

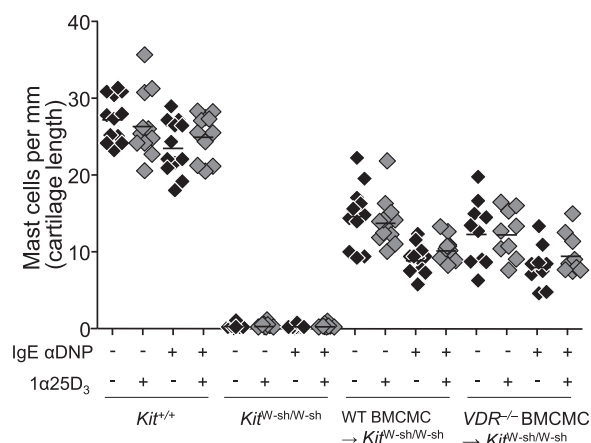


FIG E7. Dermal mast cell numbers in ear pinnae of C57BL/6J-*Kit*^{+/+} mice, mast cell-deficient *Kit*^{W-sh/W-sh} mice, and *Kit*^{W-sh/W-sh} mice engrafted with WT (C57BL/6; WT BMCMC → *Kit*^{W-sh/W-sh} mice) or *VDR*^{-/-} (*VDR*^{-/-} BMCMC → *Kit*^{W-sh/W-sh}) cells. BMCMCs were transferred by means of intra-dermal injection (each ear received 2 injections of 1×10^6 cells/20 μ L into each of 2 sites) into 4- to 6-week-old *Kit*^{W-sh/W-sh} mice. Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue-stained ear pinnae sections at the completion of the PCA experiments outlined in Fig 3 and Fig E5. Data are from 9 to 12 mice per group and 3 independent experiments.

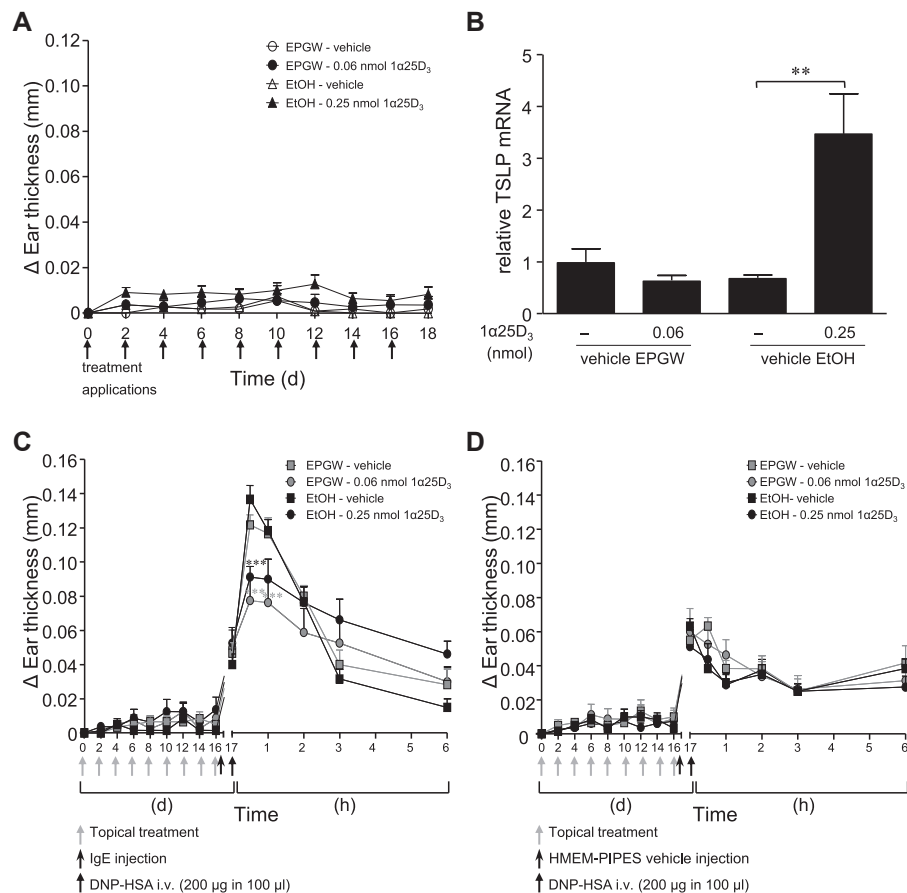


FIG E8. Chronic epicutaneous application of $1\alpha,25(\text{OH})_2\text{D}_3$ suppresses IgE-mediated PCA reactions. **A**, Changes (Δ) in ear thickness of C57BL/6J WT female mice treated with 0.06 nmol or 0.25 nmol $1\alpha,25(\text{OH})_2\text{D}_3$ ($1\alpha25\text{D}_3$; right ears) per ear (or relevant vehicle control [EPGW or EtOH; left ears]) every 2 days for 18 days. Data are from 11 mice per group and 2 independent experiments. **B**, *TSLP* mRNA expression in ear skin of mice from 48 hours after the final $1\alpha,25(\text{OH})_2\text{D}_3$ topical application. Data are from 6 mice per group. $**P < .01$ for the indicated comparison. **C** and **D**, Changes (Δ) in ear thickness of C57BL/6J WT female mice treated with 0.06 nmol or 0.25 nmol $1\alpha25\text{D}_3$ per ear or relevant vehicle control (EPGW or EtOH) every 2 days for a total of 9 applications. On day 16, ears were intradermally injected with 100 ng of IgE anti-DNP antibody (Fig E8, C) or HMEM-Pipes vehicle (Fig E8, D). Sixteen hours later, baseline ear thickness was measured. Mice were then injected intravenously (*i.v.*) with DNP-HSA, and change in ear thickness was measured at intervals over 6 hours. Data are from 3 to 4 mice per group and 2 independent experiments. $***P < .001$ for comparisons of $1\alpha,25(\text{OH})_2\text{D}_3$ versus vehicle-treated ears within the same group of mice. All data expressed as mean + SEM.

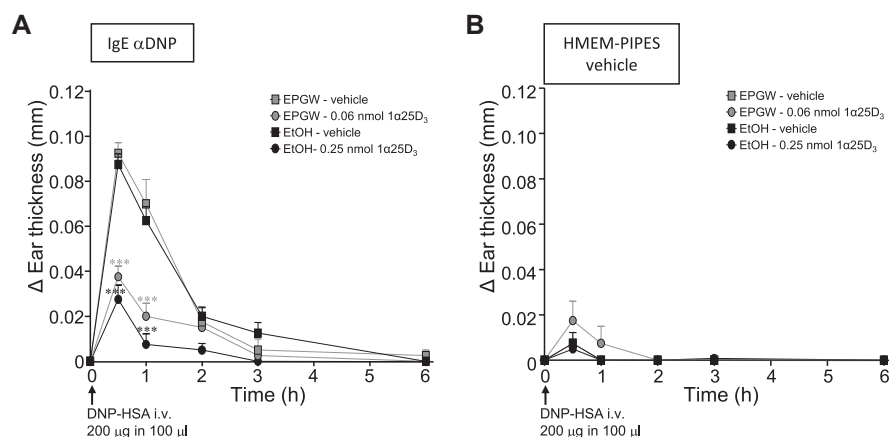


FIG E9. A single epicutaneous application of high-dose (0.25 nmol) $1\alpha,25(\text{OH})_2\text{D}_3$ can suppress IgE-mediated PCA reactions. Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μg of DNP-HSA are shown. C57BL/6J female mice were pretreated with topical application of 0.06 nmol of $1\alpha,25(\text{OH})_2\text{D}_3$ ($1\alpha25\text{D}_3$; gray circles) or 0.25 nmol $1\alpha,25(\text{OH})_2\text{D}_3$ ($1\alpha25\text{D}_3$; black circles) per ear or corresponding vehicle control (EPGW: gray squares or EtOH: black squares, respectively) and injected intradermally with 100 ng of IgE anti-DNP antibody (**A**) or HMEM-Pipes vehicle (**B**). Data expressed as mean $\pm$ SEM and are from 4 mice per group. *** $P < .001$ for comparisons of $1\alpha,25(\text{OH})_2\text{D}_3$ versus vehicle-treated ears within the same group of mice.

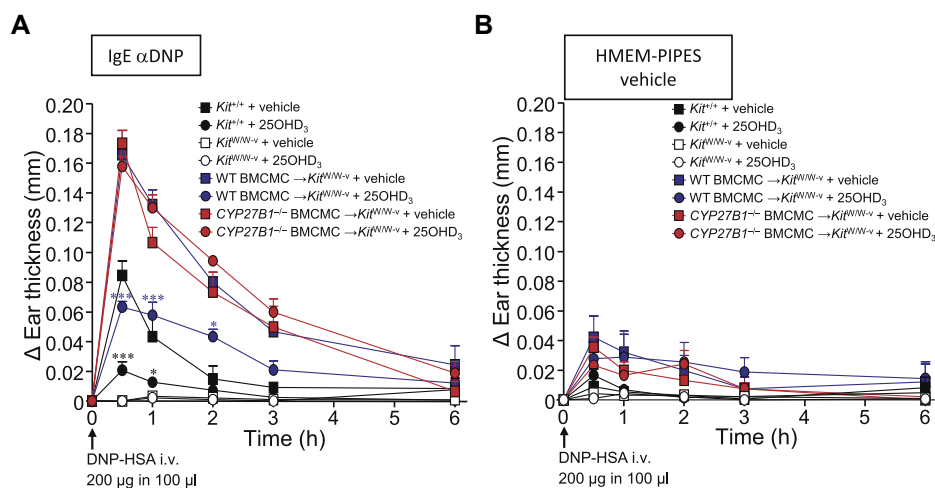


FIG E10. Mast cell–VDR and mast cell–CYP27B1 activities are required for epicutaneous 25OHD₃ dampening of IgE-mediated PCA reactions. Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μ g of DNP-HSA into mice and 30 hours after topical application of 0.06 nmol 25OHD₃ per ear (*circles*) or vehicle (EPGW; *squares*) and 16 hours after intradermal injection of (A) 100 ng of IgE anti-DNP antibody into right ears (A) or HMEM-Pipes vehicle (B) in WBB6F₁- $Kit^{+/+}$ (WT) mice (*black marker*), mast cell-deficient WBB6F₁- $Kit^{W/W-v}$ (*white marker*), WT BMCMC $\rightarrow Kit^{W/W-v}$ (*blue marker*), or CYP27B1^{-/-} BMCMC $\rightarrow Kit^{W/W-v}$ (*red marker*) are shown. Data are expressed as mean + SEM and are from 3 to 4 mice per group per experiment and 3 independent experiments, each of which provided similar results. * $P < .01$ and *** $P < .001$ for comparisons of 25OHD₃ versus vehicle-treated ears within the same group of mice (as indicated with color coding) by means of 2-way ANOVA with Bonferroni posttests.

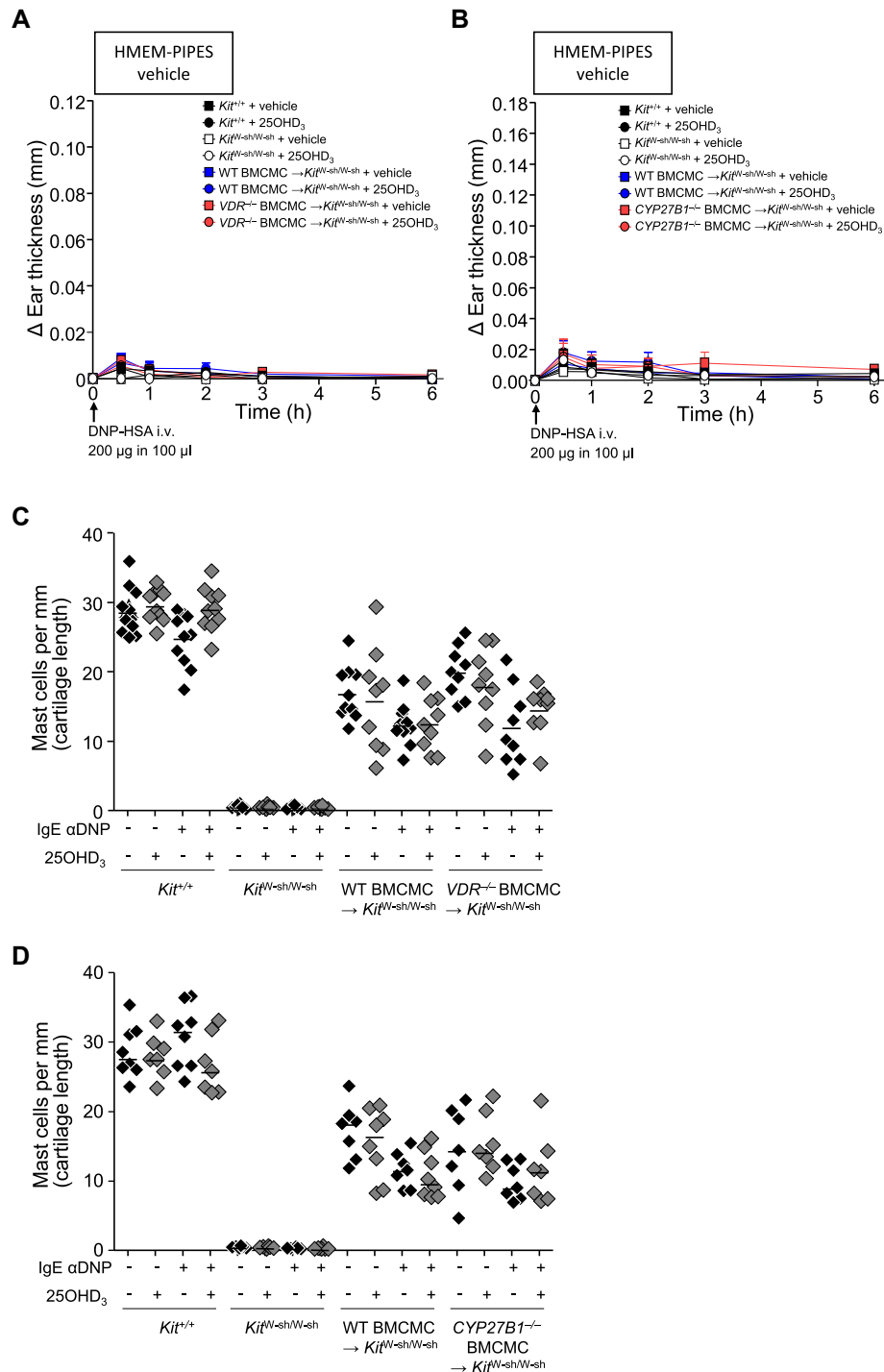


FIG E11. Ear-swelling responses after injection with vehicle (HMEM-Pipes) and dermal mast cell numbers. **A** and **B**, Changes (Δ) in ear thickness 0 to 6 hours after intravenous (*i.v.*) injection of 200 μ g of DNP-HSA into mice and 30 hours after topical application of 0.06 nmol of 25OHD₃ (circles) or vehicle (EPGW; squares) per ear and 16 hours after intradermal injection of HMEM-Pipes vehicle (left ears) in $Kit^{+/+}$ (WT), $Kit^{W-sh/W-sh}$, WT BMCMC $\rightarrow Kit^{W-sh/W-sh}$, $VDR^{-/-}$ BMCMC $\rightarrow Kit^{W-sh/W-sh}$ (Fig E11, **A**) or $CYP27B1^{-/-}$ BMCMC $\rightarrow Kit^{W-sh/W-sh}$ (Fig E11, **B**) mice. Data expressed as mean $\pm$ SEM. None of the differences between any of the groups achieved statistical significance (defined as $P < .05$) by means of 2-way ANOVA. **C** and **D**, Dermal mast cell numbers in ear pinnae of the same groups of mice, as indicated in Fig E11, **A** and **B**, and whose results for IgE anti-DNP antibody-injected right ears are shown in Fig 4, **A** and **B**. Numbers of mast cells per millimeter of ear cartilage were counted in toluidine blue-stained ear pinnae sections at the completion of the PCA experiments.

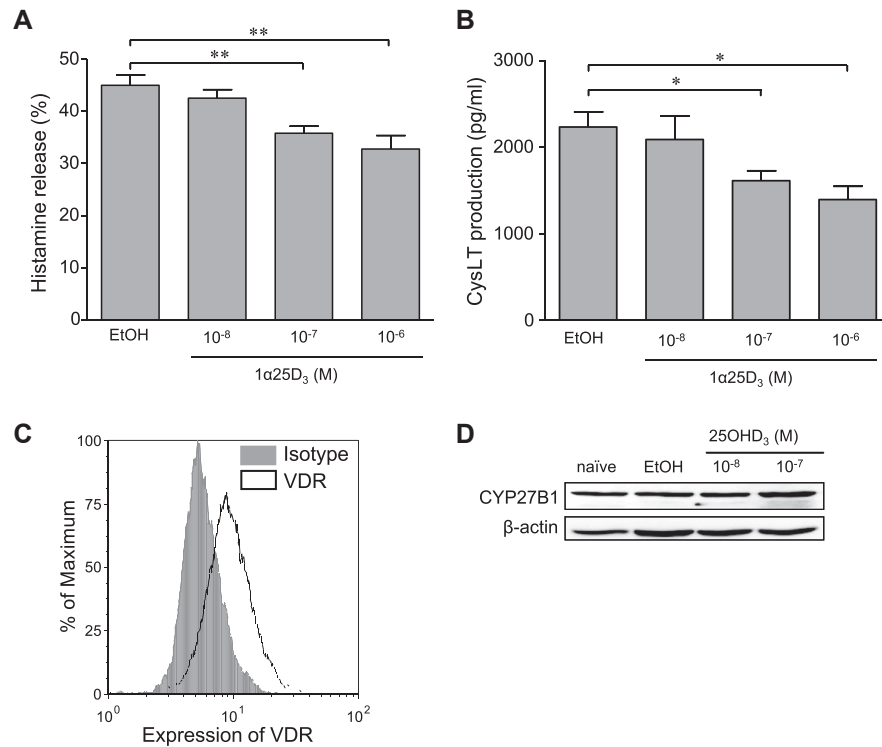


FIG E12. $1\alpha,25(OH)_2D_3$ can impair activation of IgE-mediated PBMCs. **A** and **B**, PBMCs pretreated with $1\alpha,25(OH)_2D_3$ ($1\alpha 25D_3$) or vehicle (0.03% EtOH) at the time of sensitization with human myeloma IgE for 16 hours, followed by challenge with anti-human IgE antibody for 30 minutes and release of histamine (Fig E12, **A**) and CysLT (Fig E12, **B**) into supernatants. Data are expressed as mean + SEM and are from 3 to 4 different PBMC batches. * $P < .05$ and ** $P < .01$ for the indicated comparisons. **C**, VDR expression in CBMCs. **D**, CYP27B1 and β -actin protein expression in CBMCs cultured for 8 hours with 25OHD₃ at the indicated concentrations or vehicle (EtOH).