

CANCER

Vitamin D–dependent induction of cathelicidin in human macrophages results in cytotoxicity against high-grade B cell lymphoma

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Infiltration by macrophages represents a characteristic morphological hallmark in high-grade lymphatic malignancies such as Burkitt's lymphoma (BL). Although macrophages can, in principle, target neoplastic cells and mediate antibody-dependent cellular cytotoxicity (ADCC), tumor-associated macrophages (TAMs) regularly fail to exert direct cytotoxic functions. The underlying mechanisms responsible for this observation remain unclear. We demonstrate that inflammatory M1 macrophages kill proliferating high-grade B cell lymphoma cells by releasing the antimicrobial peptide cathelicidin in a vitamin D–dependent fashion. We show that cathelicidin directly induces cell death by targeting mitochondria of BL cells. In contrast, anti-inflammatory M2 macrophages and M2-like TAMs in BL exhibit an altered vitamin D metabolism, resulting in a reduced production of cathelicidin and consequently in inability to lyse BL cells. However, treatment of M2 macrophages with the bioactive form of vitamin D, 1,25D3, or a vitamin D receptor agonist effectively induces cathelicidin production and triggers tumoricidal activity against BL cells. Furthermore, rituximab-mediated cytotoxicity of vitamin D–treated M2 macrophages is cathelicidin-dependent. Finally, vitamin D treatment of 25-hydroxyvitamin D (25D)–deficient volunteers in vivo or primary TAMs in vitro improves rituximab-mediated ADCC against B cell lymphoma cells. These data indicate that activation of the vitamin D signaling pathway activates antitumor activity of TAMs and improves the efficacy of ADCC.

INTRODUCTION

Macrophages are generally believed to play a central role in host defense against pathogens. However, their function is rather different in the case of neoplastic growth, where macrophages are thought to be protumorigenic because they promote angiogenesis and metastasis (1). These observations are puzzling in light of recent evidence that macrophages can directly kill tumor cells (2) and participate in antitumor immune responses as effector cells (3). Moreover, macrophages are key immune effector cells for the therapeutic effect of the anti-CD20 antibody rituximab (4), which is a standard therapy for B cell non-Hodgkin's lymphomas (NHL). In particular, macrophages can mediate rituximab-induced cytotoxicity through phagocytosis (5) or antibody-dependent cellular cytotoxicity (ADCC) (6). Despite both their intrinsic tumoricidal potential and the fact that they constitute the majority of tumor-infiltrating leukocytes in most malignancies, tumor-associated macrophages (TAMs) consistently fail to eliminate tumor cells (7). This phenomenon has been linked to the preponderance of an anti-inflammatory M2-like phenotype of TAMs (8) that promotes proliferation and survival of tumor cells. In murine models, the tumoricidal effector mechanisms of macrophages and the functional heterogeneity of the proinflammatory M1

versus M2 macrophages are well understood (5, 9). However, how human macrophages contribute to antitumor responses, as well as the functional phenotype of TAMs in human cancers, remains unclear (10).

Vitamin D plays a key role in regulating effector functions of human macrophages (11, 12). This is closely linked to the expression of the vitamin D-1–hydroxylase CYP27B1, which catalyzes the conversion of 25-hydroxyvitamin D (25D) to the bioactive 1,25-dihydroxyvitamin D (1,25D3). Active 1,25D3 binds to the vitamin D receptor (VDR) and induces the expression of a variety of target genes containing vitamin D response elements in their promoters. In human macrophages, vitamin D regulates phagocytosis (13), activates superoxide synthesis (14), and induces expression of cathelicidin (15). Cathelicidin, known as LL-37 in its active form, is a host defense peptide with diverse immunomodulatory effects (16). Its membrane-destabilizing and apoptosis-inducing effects in various cancer cell lines have raised hopes that it may have antineoplastic properties (17).

Vitamin D deficiency has been linked to clinical progression of different B cell lymphoma subtypes (18). In a recent study, vitamin D deficiency was associated with reduced event-free survival and overall survival in NHL patients receiving rituximab, suggesting that vitamin D-mediated mechanisms may play a role in rituximab-induced ADCC (19). Moreover, the expression of VDR, CYP27B1, and the vitamin D-24–hydroxylase CYP24A1 is frequently dysregulated in various human tumors (20), highlighting the importance of vitamin D metabolism in cancer.

Studies of bacterial infections in humans document that the functional phenotype of macrophages determines the activity of the intracellular vitamin D metabolism, the subsequent induction of cathelicidin, and the resulting antimicrobial activity (21). This finding prompted us to investigate if M1 or M2 polarization is responsible for the tumoricidal

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activities of human macrophages, and whether this activity is linked to the vitamin D-mediated induction of cathelicidin. We used Burkitt's lymphoma (BL) as a model disease because it is characterized by dense macrophage infiltration histologically resulting in the "starry sky" appearance of the lymphoma. We show that M1 macrophages are able to kill BL cells via vitamin D-mediated induction of the LL-37/cathelicidin peptide; LL-37 selectively affects proliferating lymphoma cells by targeting the mitochondria. By contrast, M2 macrophages fail to kill lymphoma cells in vitro. BL specimens show an absence of M1 and a preponderance of M2-like macrophages. The deficiency in cytotoxicity of M2 macrophages could be overcome by the bioactive 1,25D3 or by a synthetic VDR agonist (BXL-628). Moreover, we show that rituximab-mediated ADCC of macrophages depends on the expression of cathelicidin and that vitamin D supplementation of 25D-deficient individuals improves rituximab-mediated cytotoxicity by M2 macrophages and TAMs. Together, these data indicate that the therapeutic activation of the vitamin D pathway may restore tumoricidal effector mechanisms of TAMs.

RESULTS

TAMs in BL display reduced capacity to metabolize vitamin D

It remains unexplained why the tumoricidal effector mechanisms are compromised in human TAMs, but are effective in M1 macrophages. Because vitamin D signaling is a key factor in the regulation of effector functions of macrophages (11, 14), we reasoned that reduced activity of the vitamin D pathway may be responsible for the low tumoricidal activity of human TAMs. Given the important roles of VDR, CYP27B1, and CYP24A1 in vitamin D metabolism, we studied their mRNA expression in BL sections. Lymph nodes of patients with benign reactive lymph nodes were analyzed as a control group. RNA analysis of formalin-fixed paraffin-embedded tissues from BL ($n = 9$) and reactive lymph nodes ($n = 9$) revealed significantly lower mRNA expression of VDR and CYP27B1 in lymph node samples from BL patients compared to reactive lymph nodes [VDR: 47.05 ± 29.70 arbitrary units (AU) versus 1.57 ± 1.91 AU, $P = 0.026$; CYP27B1: 1.28 ± 0.67 AU versus 0.007 ± 0.003 AU, $P = 0.0001$], but no significant difference in the mRNA expression of CYP24A1 (control: $11,768 \pm 7800$ AU versus 5382 ± 4354 AU, $P = 0.177$; Fig. 1A). Next, we performed double immunostaining for the macrophage marker CD68 and for VDR, CYP27B1, and CYP24A1 in lymph nodes from BL patients ($n = 5$) or reactive lymph nodes ($n = 5$). Immunohistochemistry revealed lower VDR (Fig. 1B) and CYP27B1 expression in BL macrophages (Fig. 1C) as compared to controls, but no difference in the expression of CYP24A1 (Fig. 1D). These findings suggest that the lower expression of CYP27B1 in TAMs results in decreased conversion of 25D into bioactive 1,25D3. To further characterize the phenotype of these macrophages, we studied the expression of CD68 and CD163 in lymph node specimens from BL patients ($n = 9$). Although the number of infiltrating CD68⁺ macrophages in most BL tissues was comparable to the number of macrophages in the control group (BL: 270 ± 41 macrophages/mm² versus control: 283 ± 57 macrophages/mm²; $P = 0.77$; Fig. 1E and fig. S1A), the frequency of CD163⁺ macrophages was significantly increased (BL: 253 ± 46 macrophages/mm² versus control: 83 ± 21 macrophages/mm², $P = 0.005$) in BL patients (Fig. 1F and fig. S1B). In addition, TAMs in BL expressed the M2 marker CD206 (mannose receptor), whereas the M1 activation marker iNOS (inducible nitric oxide synthase) was ab-

sent or only occasionally found in BL specimens (fig. S1C). On the basis of their high interleukin-10 (IL-10) production, as well as their ability to promote B cell proliferation (22) and the M2-promoting cytokine milieu in lymph nodes of BL patients (fig. S2), we concluded that TAMs in BL display an M2-like phenotype.

In line with our in vivo observations, in vitro-generated M2 macrophages displayed a significantly reduced mRNA expression of VDR (M1: 292 ± 12.7 AU versus M2: 132 ± 28.6 AU, $P = 0.008$) and CYP27B1 (M1: 275 ± 26.4 AU versus M2: 119 ± 18.3 AU, $P = 0.01$), but no difference in the mRNA expression of CYP24A1 (M1: 436 ± 28.7 AU versus M2: 508 ± 22.6 AU, $P = 0.078$) (Fig. 1G). These results were confirmed on the protein level by Western blotting (Fig. 1H). To determine whether the high expression of CYP27B1 in M1 macrophages results in enhanced conversion of vitamin 25D to 1,25D3 in vitro, granulocyte-macrophage colony-stimulating factor (GM-CSF)-generated M1 and M-CSF-generated M2 macrophages were incubated with vitamin D-sufficient serum [25D ≈ 100 nM (23)] for 24 hours, and 1,25D3 secretion was measured in the supernatant by enzyme-linked immunosorbent assay (ELISA). In Fig. 1I, M1 macrophages produced significantly higher amounts of 1,25D3 than did M2 macrophages (240 ± 107 nmol/ml versus 30 ± 14 nmol/ml, $P = 0.03$). In summary, these findings indicate that TAMs in BL and in vitro-generated M2 macrophages have a reduced capacity to metabolize 25D as compared to M1 macrophages.

M1 macrophages kill lymphoma cells in vitro

Although in murine models the tumoricidal effector mechanisms of macrophages are well understood, it remains unexplained which effector mechanisms are operative in human tumoricidal macrophages. We used a well-characterized human macrophage model representing two extremes in the spectrum of macrophage polarization (24) and measured cytotoxic activity of in vitro-generated M1 or M2 macrophages against several BL cell lines. The in vitro polarization model was chosen because macrophages in BL share important features with in vitro-generated M2 macrophages, such as the high expression of CD163 and CD206 (fig. S3A) and the lack of iNOS (fig. S1C), and, upon stimulation with bacterial lipopolysaccharide, the low expression of tumor necrosis factor (TNF) and IL-12, and high expression of IL-10 (fig. S3B). Furthermore, in vitro-generated M2 macrophages, like macrophages in BL (22), secrete BAFF (B cell-activating factor of the TNF family) and APRIL (a proliferation-inducing ligand) as B cell-promoting growth factors (fig. S3C). To test their cytotoxicity, M1 or M2 macrophages were cultured in the presence of BL cells or freshly isolated B cells as targets in different effector-target (E/T) ratios. Viability of target cells was monitored by annexin-V and 7-AAD (7-aminoactinomycin D) staining using flow cytometry (Fig. 2, A and B). When BL cells were cultured with M1 macrophages, significant cytotoxicity was detected after 48 hours at an E/T ratio of 5:1 for all lymphoma cell lines tested (Raji: $20 \pm 4\%$, $P = 0.005$; BL30: $8 \pm 7\%$, $P = 0.04$; BL41: $17 \pm 5\%$, $P = 0.02$; BL70: $20 \pm 5\%$, $P = 0.003$; normal B cells: $2 \pm 1\%$, $P = 0.7$). In contrast, M2 macrophages failed to kill BL cells over a broad range of E/T ratios. Furthermore, in vitro-generated M1 macrophages were also cytotoxic for primary lymphoma cells freshly obtained from bone marrow of patients suffering from diffuse large B cell lymphoma (DLBCL). Similarly to the results shown with BL cell lines, M2 and freshly isolated autologous TAMs from patients were unable to kill primary lymphoma B cells (Fig. 2C; E/T 5:1: $68 \pm 5\%$, $P = 0.002$). The cytotoxic activity of M1 macrophages was tumor-specific, because

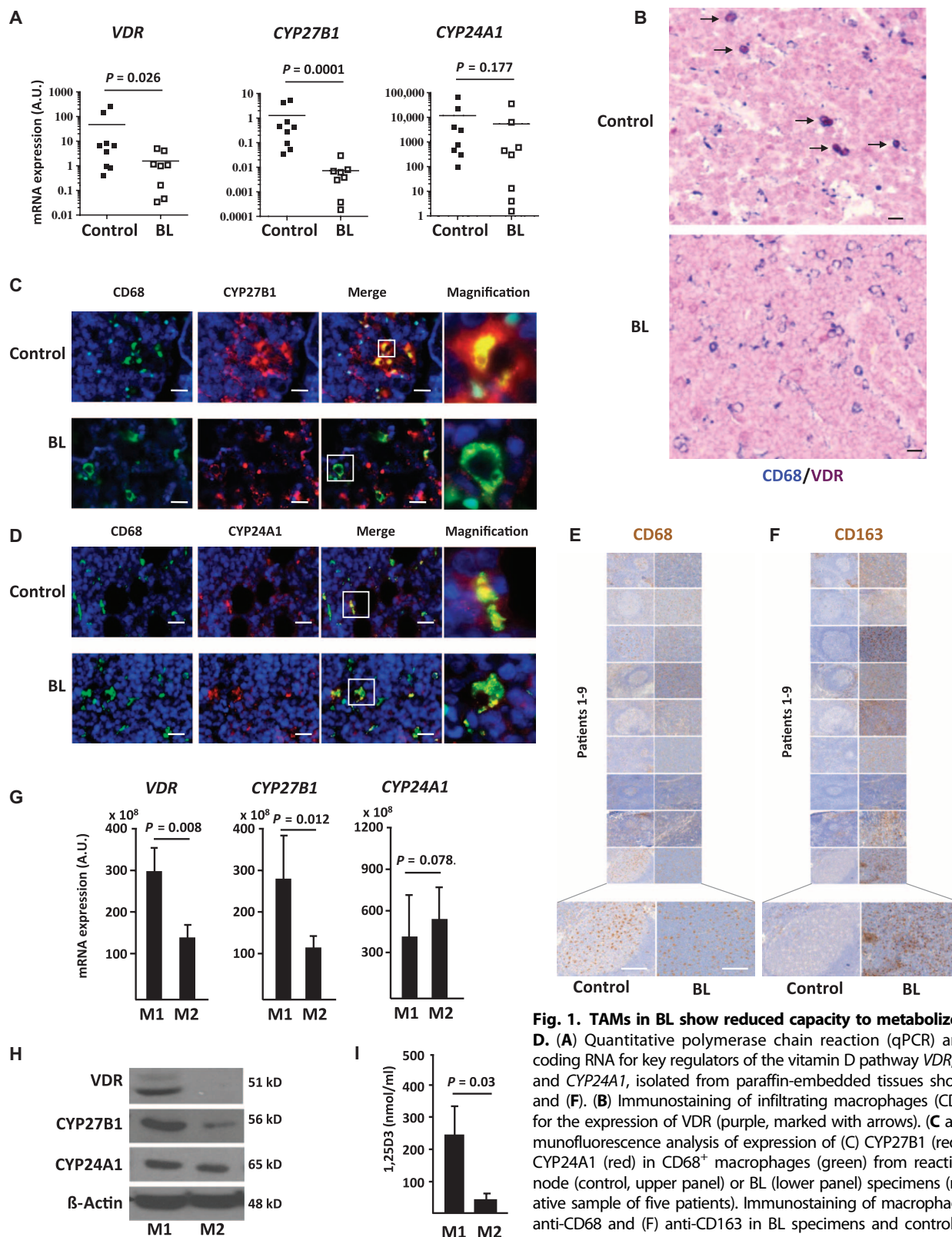


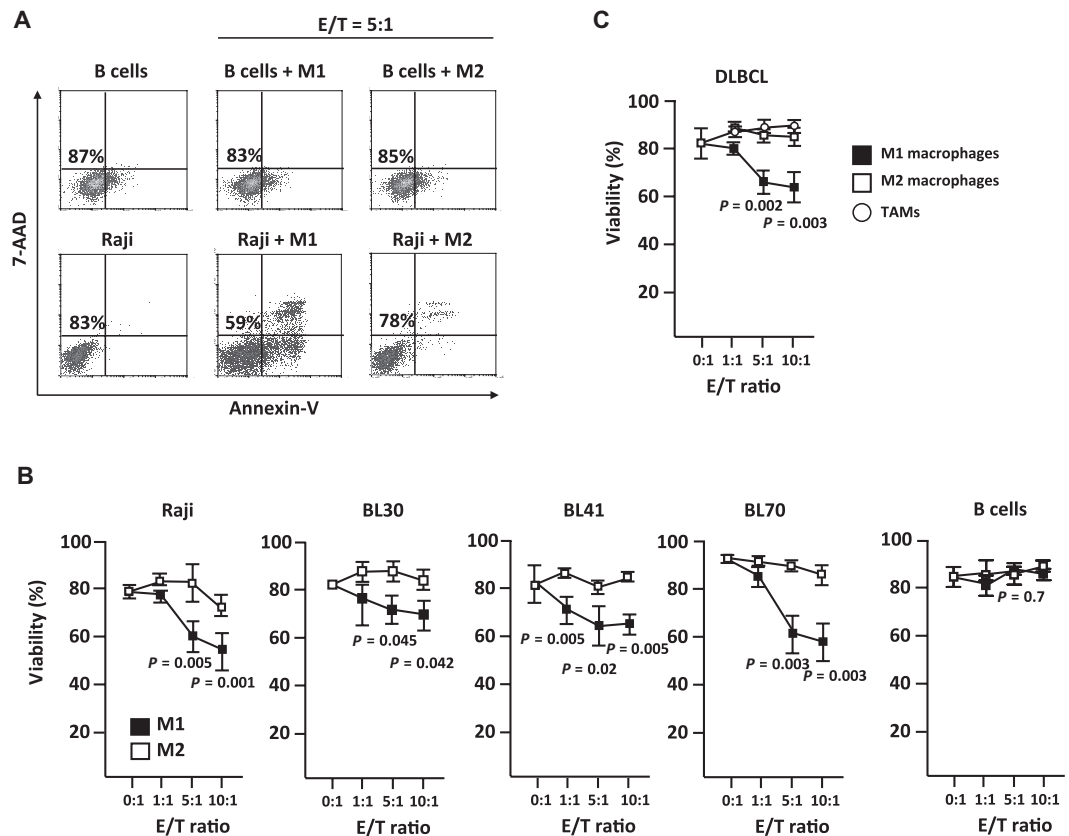
Fig. 1. TAMs in BL show reduced capacity to metabolize vitamin

D. (A) Quantitative polymerase chain reaction (qPCR) analysis of coding RNA for key regulators of the vitamin D pathway *VDR*, *CYP27B1*, and *CYP24A1*, isolated from paraffin-embedded tissues shown in (E) and (F). (B) Immunostaining of infiltrating macrophages (CD68, blue) for the expression of VDR (purple, marked with arrows). (C and D) Immunofluorescence analysis of expression of (C) *CYP27B1* (red) and (D) *CYP24A1* (red) in CD68⁺ macrophages (green) from reactive lymph node (control, upper panel) or BL (lower panel) specimens (representative sample of five patients). Immunostaining of macrophages by (E) anti-CD68 and (F) anti-CD163 in BL specimens and control tissue of reactive lymph nodes. (G) qPCR analysis of in vitro-generated M1 and M2 macrophages. (H) Western blot analysis of in vitro-generated M1 and M2 macrophages confirmed low expression of VDR and CYP27B1 in M2 macrophages at the protein level ($n = 4$). (I) Supernatants from in vitro-generated M1 and M2 macrophages were analyzed for the active form of vitamin D (1,25D3) by ELISA ($n = 5$). Scale bars, 10 μ m (B to D) and 100 μ m (E and F). Concentration of 25D in the medium used in (G) to (I) was ≈ 100 nM.

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Fig. 2. M1 macrophages kill BL cells in vitro.

(A) Representative flow cytometric analysis of Raji or B cells from healthy donors incubated alone or in the presence of M1 or M2 macrophages at a 5:1 E/T ratio for 48 hours. Viability of Raji or B cells (gated on CD11b⁺) was determined by annexin-V/7-AAD staining by flow cytometric analysis. Scatter plots show representative results from 1 of 10 independent experiments. (B) The graphs show the viability of various BL cell lines and B cells (annexin-V⁻ and 7-AAD⁺) compiled from all experiments ($n = 10$). Error bars show SEM. (C) Freshly isolated TAMs of DLBCL patients, or generated M1 or M2 macrophages from healthy donors were incubated with primary lymphoma B cells from DLBCL patients ($n = 4$). The graph shows the viability of lymphoma B cells compiled from four patients as compared to the untreated controls (E/T = 0:1). Concentration of 25D in the medium used in (A) to (C) was ≈ 70 nM.



killing of allogeneic normal B cells from healthy donors remained less than 3% for all E/T ratios tested (Fig. 2B). In comparison to the cytotoxicity of natural killer (NK) cells, which are believed to be key antitumor effector cells, macrophage-mediated antitumor cytotoxicity is a slower, but equally effective process (25). In addition, our E/T cell ratio (5:1) is comparable with earlier macrophage studies from others (2) and similar to NK cell studies (26). These data indicate that human M1 macrophages, in contrast to M2 macrophages and TAMs, display a cytotoxic activity toward lymphoma cells. Furthermore, these data suggest that human M1, but not M2 macrophages or TAMs, have tumoricidal effector molecules or mechanisms.

M1 macrophages kill lymphoma cells by secretion of cathelicidin

Given that vitamin D plays a key role in regulating effector functions of human macrophages, and given that M2 macrophages have a reduced capacity to metabolize vitamin D and failed to kill BL cells, we reasoned that the decisive effector molecule in M1 and M2 macrophages is regulated by vitamin D. We therefore analyzed vitamin D-dependent effector molecules differently expressed in M1 and M2 macrophages. The expression of cathelicidin, an antimicrobial peptide with known cytotoxic function (17), was evaluated by real-time qPCR and immunofluorescence microscopy when M1 and M2 macrophages were cultured in the presence of serum containing low (≤ 40 nM, “deficient”) and high (≈ 100 nM, “sufficient”) concentrations of 25D (23). M2 macrophages showed significantly lower mRNA and intracellular protein expression of cathelicidin compared to M1 macrophages, even in the presence of sufficient 25D levels (M2 mRNA: 144 ± 6.7 AU ver-

sus M1 mRNA: 425 ± 90.5 AU, $P = 0.001$) (Fig. 3, A to C). Because cathelicidin is secreted upon stimulation (27), we hypothesized that macrophages released cathelicidin upon contact with BL Raji target cells. To test this, we analyzed coculture supernatant of BL Raji cells and macrophages by immunoblot for the presence of cathelicidin. We detected increasing amounts of cathelicidin in the supernatant of M1 macrophages after coculture with BL Raji target cells. In contrast, we found only a minimal expression of cathelicidin in the supernatant of M2 macrophages cocultured with BL Raji cells (Fig. 3, D and E). To assess whether cathelicidin is secreted by macrophages toward target cells in a polarized fashion, we examined the localization of cathelicidin in M1 and M2 macrophages during coculture with CFSE (carboxyfluorescein diacetate succinimidyl ester)-stained Raji targets. M1 and M2 macrophages were found in close contact to Raji cells and displayed activation upon cell contact as shown by tyrosine phosphorylation (fig. S4A) and expression of CD107A (fig. S4B). However, only M1 macrophages exhibited a strong polarized accumulation of cathelicidin toward BL Raji targets (Fig. 3, F and G), suggesting that M1 macrophages release cathelicidin at the immunological synapse. To confirm the requirement of cathelicidin for macrophage-mediated tumor killing, we investigated the cytotoxicity of M1 macrophages in the presence of a neutralizing anti-cathelicidin monoclonal antibody or an isotype control monoclonal antibody (mAb). Cytotoxicity of M1 macrophages was markedly inhibited in the presence of the anti-cathelicidin antibody (medium: $18 \pm 6\%$ killing versus anti-cathelicidin: $4 \pm 3\%$ killing, $P = 0.004$), whereas isotype control treatment had no effect [immunoglobulin G1 (IgG1): $20 \pm 8\%$ killing] (Fig. 3, H and I). The cytotoxicity of M1 macrophages was independent of FasL (Fas ligand), TRAIL (TNF-related apoptosis-inducing

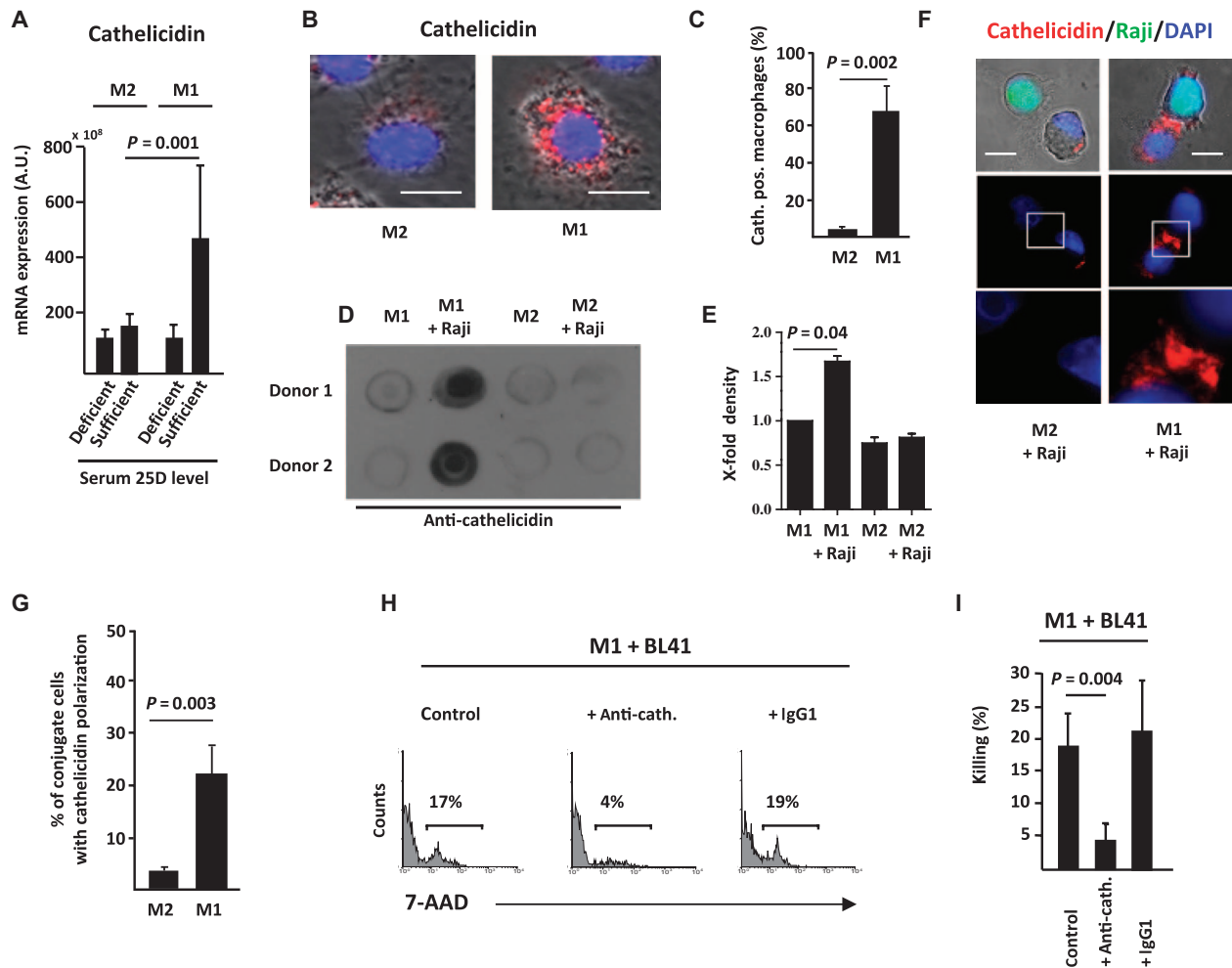


Fig. 3. M1 macrophages kill lymphoma cells by cathelicidin. (A) M1 or M2 macrophages were generated in the presence of deficient (25D ≤ 40 nM) or sufficient (25D ≈ 100 nM) sera for 6 days, and cathelicidin expression was determined by real-time qPCR ($n = 4$). (B) M1 or M2 macrophages were stained for cathelicidin in the presence of vitamin D-sufficient sera (25D ≈ 100 nM) (Hoechst 33258, nuclei, blue). Slides were analyzed by fluorescence microscopy. The panel shows representative areas from independent experiments performed with four donors. (C) The percentage of cathelicidin⁺ cells was calculated from microscopically counted macrophages (minimum 100 per donor, $n = 4$) in unblinded fashion. (D) M1 or M2 macrophages were generated from four different donors and incubated with BL Raji cells (E/T = 5:1) for 24 hours. Supernatants were harvested and dot blots were stained with anti-cathelicidin antibody. The panel shows a typical result from four different donors. (E) Dots were analyzed with a densitometer, and the results were presented as x-fold change in density. (F)

M1 and M2 macrophages were incubated with CFSE-labeled BL Raji cells (green) (E/T = 5:1) for 24 hours in an eight-chamber slide. Macrophages were stained with an anti-cathelicidin antibody (red) and analyzed by fluorescence microscopy. Magnifications ($\times 630$) present representative areas from one of five independent experiments performed with different donors. (G) The percentage of conjugated cells with cathelicidin polarization calculated from microscopically counted cells (minimum 100 per donor, $n = 5$) in unblinded fashion. (H) M1 macrophages were incubated with BL41 (E/T = 5:1) for 48 hours in the presence or absence of a monoclonal anti-cathelicidin antibody (10 μ g/ml) or an isotype control (IgG1, 10 μ g/ml). Viability of the lymphoma cell line was determined with 7-AAD staining by flow cytometric analysis. (I) The graph shows the average result of experiments performed as in (H), using cells from different donors ($n = 5$). Error bars show SEM. Scale bars, 10 μ m (B and F). Concentration of 25D in the medium used in (B) to (I) was ≈ 70 nM.

ligand), TNF, and ROS (reactive oxygen species), and we found only a low level of phagocytosis of tumor targets in our experimental settings (figs. S5 to S7). In summary, these data indicate that cathelicidin is an important effector molecule in M1 macrophages for combating lymphoma cells.

LL-37 is cytotoxic for BL cells through targeting mitochondria

To assess whether LL-37 can directly induce cell death, we treated various BL cell lines with different concentrations of synthetic LL-37 (Fig. 4A).

The lowest significantly cytotoxic LL-37 concentration in these experiments was 2.5 μ M (fig. S8; $P = 0.03$). Although the viability of nonmalignant B cells was not affected, LL-37 (2.5 μ M) induced cell death in all BL cell lines tested (Fig. 4A). However, BL cell lines showed variable sensitivity toward LL-37-mediated cytotoxicity (Raji: $40 \pm 25\%$, BL30: $12 \pm 6\%$, BL41: $27 \pm 8\%$, and BL70: $23 \pm 7\%$). The difference in sensitivity toward LL-37 was not due to variable expression of potential LL-37 receptors on lymphoma cells (fig. S9, A to C), because all cell lines tested expressed comparable amounts of fMLP,

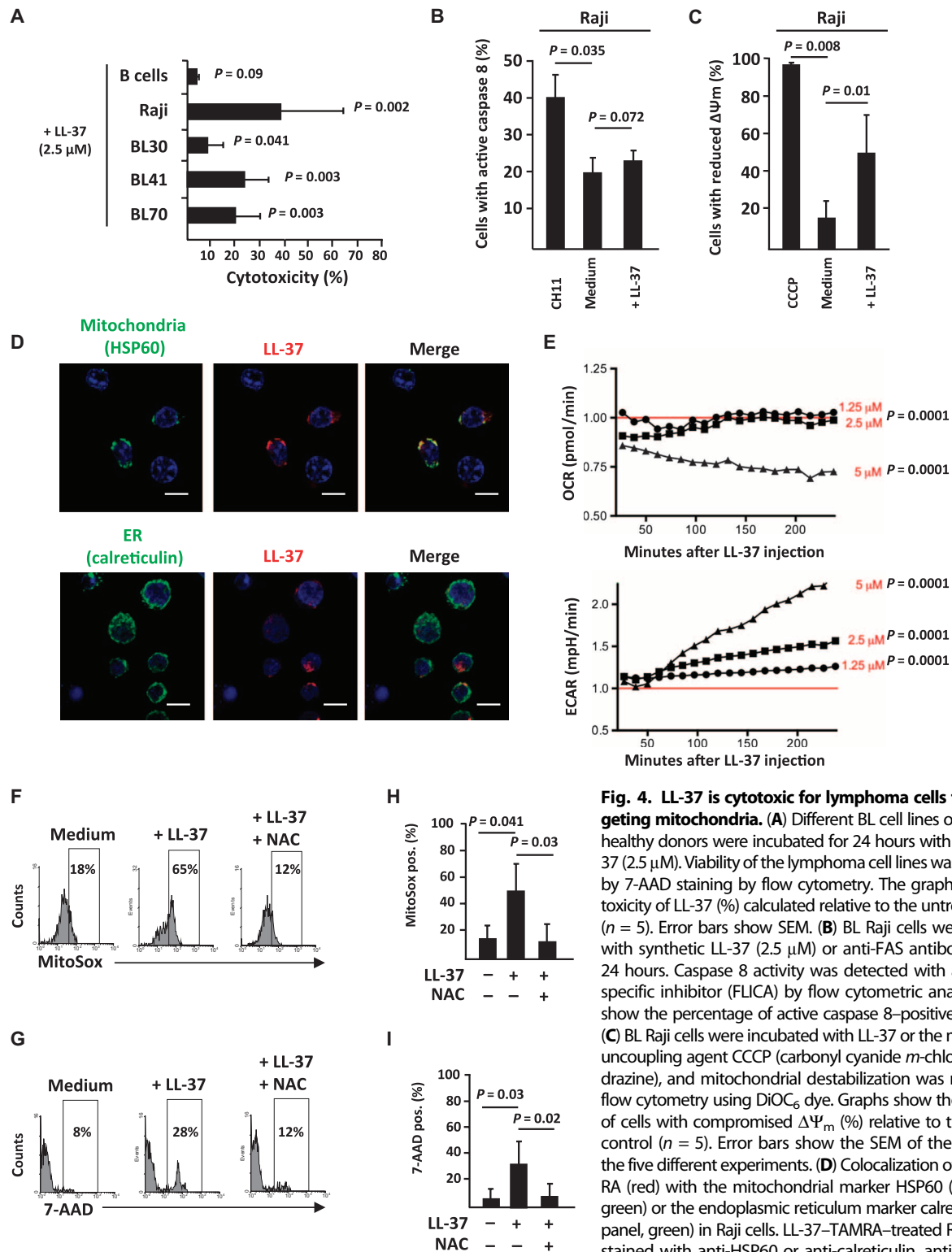


Fig. 4. LL-37 is cytotoxic for lymphoma cells through targeting mitochondria. (A) Different BL cell lines or B cells from healthy donors were incubated for 24 hours with synthetic LL-37 (2.5 μ M). Viability of the lymphoma cell lines was determined by 7-AAD staining by flow cytometry. The graph shows cytotoxicity of LL-37 (%) calculated relative to the untreated control ($n = 5$). Error bars show SEM. (B) BL Raji cells were incubated with synthetic LL-37 (2.5 μ M) or anti-FAS antibody CH11 for 24 hours. Caspase 8 activity was detected with a fluorescent specific inhibitor (FLICA) by flow cytometric analysis. Graphs show the percentage of active caspase 8-positive cells ($n = 5$). (C) BL Raji cells were incubated with LL-37 or the mitochondrial uncoupling agent CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine), and mitochondrial destabilization was measured by flow cytometry using DiOC₆ dye. Graphs show the percentage of cells with compromised $\Delta\Psi_m$ (%) relative to the untreated control ($n = 5$). Error bars show the SEM of the results from the five different experiments. (D) Colocalization of LL-37-TAMRA (red) with the mitochondrial marker HSP60 (upper panel, green) or the endoplasmic reticulum marker calreticulin (lower panel, green) in Raji cells. LL-37-TAMRA-treated Raji cells were stained with anti-HSP60 or anti-calreticulin, anti-rabbit Alexa

647 antibodies, and Hoechst nuclear stain. Slides were analyzed by confocal laser microscopy and evaluated for colocalization of LL-37-TAMRA and HSP60 or calreticulin. Photographs show representative slides from three independent experiments. Scale bar, 10 μ m. (E) Extracellular flux analysis. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were simultaneously measured in Raji cells treated with synthetic LL-37 for 4 hours (1 = untreated cells, shown by the red line). Shown are the average data of three independent experiments ($n = 14$ to 18). (F) Raji cells were incubated with synthetic LL-37 in the presence of NAC. (G to I) Mitochondrial superoxides were detected by MitoSox (F and H), and viability was determined by 7-AAD exclusion (G and I). The histograms (F and G) show a representative result from four independent experiments. The graphs (H and I) show the average result of all experiments. Error bars show SEM.

P2X7R, and IGF-1 (insulin-like growth factor 1) receptor. To identify the mechanism by which LL-37 induced cell death in BL cells, we investigated whether the extrinsic or intrinsic apoptosis pathway was affected. Given the key role of caspase 8 in the extrinsic pathway, we analyzed caspase 8 activity in BL Raji cells using flow cytometric analysis. An anti-Fas antibody (CH11) induced caspase 8 activity, but we found no significant difference in the enzymatic activation of caspase 8 after treatment with LL-37 compared to the medium control (Fig. 4B; $P = 0.072$). To further examine the activation of the mitochondria-associated pathway of apoptosis, we analyzed changes in mitochondrial membrane potential $\Delta\Psi_m$ after LL-37 exposure. The DiOC₆ dye enters the mitochondria in viable cells but leaks into the cytosol of the cell on $\Delta\Psi_m$ dissipation, resulting in decreased fluorescence intensity. LL-37 led to significant ($P = 0.01$) dissipation in $\Delta\Psi_m$ in BL cells ($52 \pm 18\%$), as determined by flow cytometry (Fig. 4C). This is in line with our observations that a BCL-2 overexpressing murine BL cell line overcomes the LL-37-mediated cytotoxicity (fig. S10, A and B). The finding that LL-37 permeabilizes microbial membranes (28) and induces mitochondrial depolarization in BL cells prompted us to investigate whether LL-37 targets mitochondria. To test this, colocalization of fluorescent LL-37 (LL-37-TAMRA) with the mitochondrial markers MitoTracker or HSP60 was studied in BL cells. Analysis by confocal microscopy demonstrated that LL-37 colocalizes with mitochondria in BL cells, but not in B cells from healthy donors (Fig. 4D and fig. S11). Mitochondria of the relatively LL-37-insensitive BL30 cells showed only a weak colocalization with LL-37 (fig. S11). Binding of synthetic LL-37 to mitochondria was specific, because we detected neither colocalization of LL-37 with the endoplasmic reticulum marker calreticulin nor colocalization of a scrambled control peptide with mitochondria (Fig. 4D and figs. S12 and S13). In line with the proposed mitochondrial targeting, we observed a significant ($P < 0.0001$) dose-dependent decline of cellular respiration upon LL-37 application, as assessed by oxygen consumption rate (Fig. 4E, upper panel). Furthermore, treatment-related inhibition of oxidative phosphorylation led to a compensatory increase of anaerobic glycolysis, highlighted by the significantly ($P < 0.0001$) increased production of lactic acid (Fig. 4E, lower panel). To further validate mitochondrial targeting by LL-37, we investigated the ability of LL-37 to stimulate the production of ROS. During apoptosis, mitochondrial damage and the accompanying loss of $\Delta\Psi_m$ exacerbate the uncoupling of the electron transport chain, which results in the excessive production of ROS. We found a direct correlation between LL-37-induced cell death and ROS generation in BL cells monitored by flow cytometry using MitoSox (Fig. 4F) and 7-AAD (Fig. 4G) staining. *N*-acetyl-L-cysteine (NAC), a well-characterized scavenger of oxygen-free radicals, efficiently depleted ROS after LL-37 treatment in BL cells (Fig. 4, F and H, and fig. S14A). Pretreatment of BL cells with NAC significantly ($P = 0.02$) inhibited LL-37-induced cell death (Fig. 4, G and I, and fig. S14B). Collectively, our data indicate that LL-37 targets the mitochondria of lymphoma cells, resulting in the release of ROS and subsequent cell death.

LL-37 is cytotoxic for proliferating B cells

As shown above, LL-37 is cytotoxic for BL cells but not for non-malignant B cells. Because LL-37 inserts into the membrane of BL cells and into the wall of septating bacteria (29), we hypothesized that LL-37 acts on proliferating, but not on resting, cells. Therefore, we analyzed the cell cycle distribution and proliferation of BL cells in comparison with B cells from healthy donors by flow cytometry. As expected, a

substantial portion of BL cells were in S phase (BL30: $25 \pm 2\%$, Raji: $42 \pm 6\%$, BL41: $50 \pm 11\%$, BL70: $52 \pm 8\%$), whereas unstimulated B cells remained in the G₀/G₁ phase (S phase: $5 \pm 3\%$; Fig. 5A). These results are in line with our finding that BL30 cells are less sensitive to LL-37 than, for example, Raji and BL70 cells (Fig. 4A and fig. S14, A and B), because a smaller percentage of BL30 cells was found in S phase. Notably, we did not observe any difference in the expression of BCL-2, BCL-xL, or MCL-1 in BL30 cells (fig. S15), suggesting that the lesser sensitivity of BL30 cells to LL-37 is not a consequence of an increased expression of antiapoptotic proteins. To determine whether the effect of LL-37 on tumor cells is dependent on their proliferation, we used the Epstein-Barr virus-transformed B cell line P493-6. These cells are derived from normal human B cells and were transfected with a vector containing a human *c-MYC* gene negatively regulated by tetracycline. Proliferation of P493-6 cells can be inhibited by switching off *c-MYC* expression through tetracycline in the medium (Fig. 5B) (30). We analyzed the ability of LL-37 to enter mitochondria and induce cell death in proliferating and resting P493-6 cells. Mitochondria of proliferating P493-6 cells showed a strong colocalization with LL-37-TAMRA (Fig. 5C, upper panel), and cell death was significantly increased after treatment with synthetic LL-37 ($2.5 \mu\text{M}$) ($18 \pm 7\%$ with and $36 \pm 7\%$ without tetracycline, $P = 0.021$; Fig. 5D). However, reduction of proliferation by tetracycline resulted in reduced binding of LL-37-TAMRA (Fig. 5C, lower panel) and reduced cell death as shown in Fig. 5D. Similarly, the induction of proliferation in non-malignant B cells by pokeweed mitogen (PWM; Fig. 5E) led to increased binding of LL-37 (Fig. 5F) and cell death ($17 \pm 2\%$ without versus $29 \pm 3\%$ after PWM treatment, $P = 0.004$; Fig. 5G). These findings indicate that LL-37 preferentially targets proliferating B cells.

Vitamin D triggers killing of BL cells by M2 macrophages

Because tumoricidal macrophages required cathelicidin to eliminate lymphoma cells, and because TAMs in BL have a reduced capacity to metabolize vitamin D, we reasoned that TAMs in BL may express only low levels of the cytotoxic peptide cathelicidin. Initially, we analyzed the expression of cathelicidin mRNA in formalin-fixed paraffin-embedded lymph nodes from BL patients ($n = 9$) and reactive lymph nodes as nonlymphoma controls ($n = 8$). TaqMan real-time PCR revealed a significantly higher expression of cathelicidin in reactive lymph nodes than in BL samples (cathelicidin: 1463 ± 943 AU versus 14 ± 6 AU, $P = 0.02$; Fig. 6A). To directly examine whether TAMs express cathelicidin, we stained the lymph nodes of BL patients or reactive lymph nodes simultaneously for cathelicidin and CD68. Whereas a considerable number of macrophages in reactive lymph nodes expressed cathelicidin (CD68⁺cathelicidin⁺: $20 \pm 3\%$), we found no cathelicidin-expressing TAMs in BL tissue ($P = 0.001$; Fig. 6, B and C). Because cathelicidin is up-regulated by the bioactive form of vitamin D, 1,25D₃, we hypothesized that addition of 1,25D₃ would increase the cytotoxicity of M2 macrophages against lymphoma cells. Activation of M2 macrophages with 1,25D₃ (10^{-9} to 10^{-7} M) enhanced gene expression of cathelicidin (10^{-8} M: 2.4-fold; 10^{-7} M: 3.2-fold) compared to unstimulated controls. BXL-628, a low-calcemic clinically tested analog of 1,25D₃, was even more effective in the induction of cathelicidin gene expression (10^{-9} M: 1.4-fold, 10^{-8} M: 3.7-fold, and 10^{-7} M: 4.3-fold, $P = 0.0028$) compared to medium control (Fig. 6D). The up-regulation of the cathelicidin mRNA correlated with increased expression of the cathelicidin protein as detected by fluorescence microscopy (fig. S16A). Finally, activation of macrophages by 1,25D₃ or

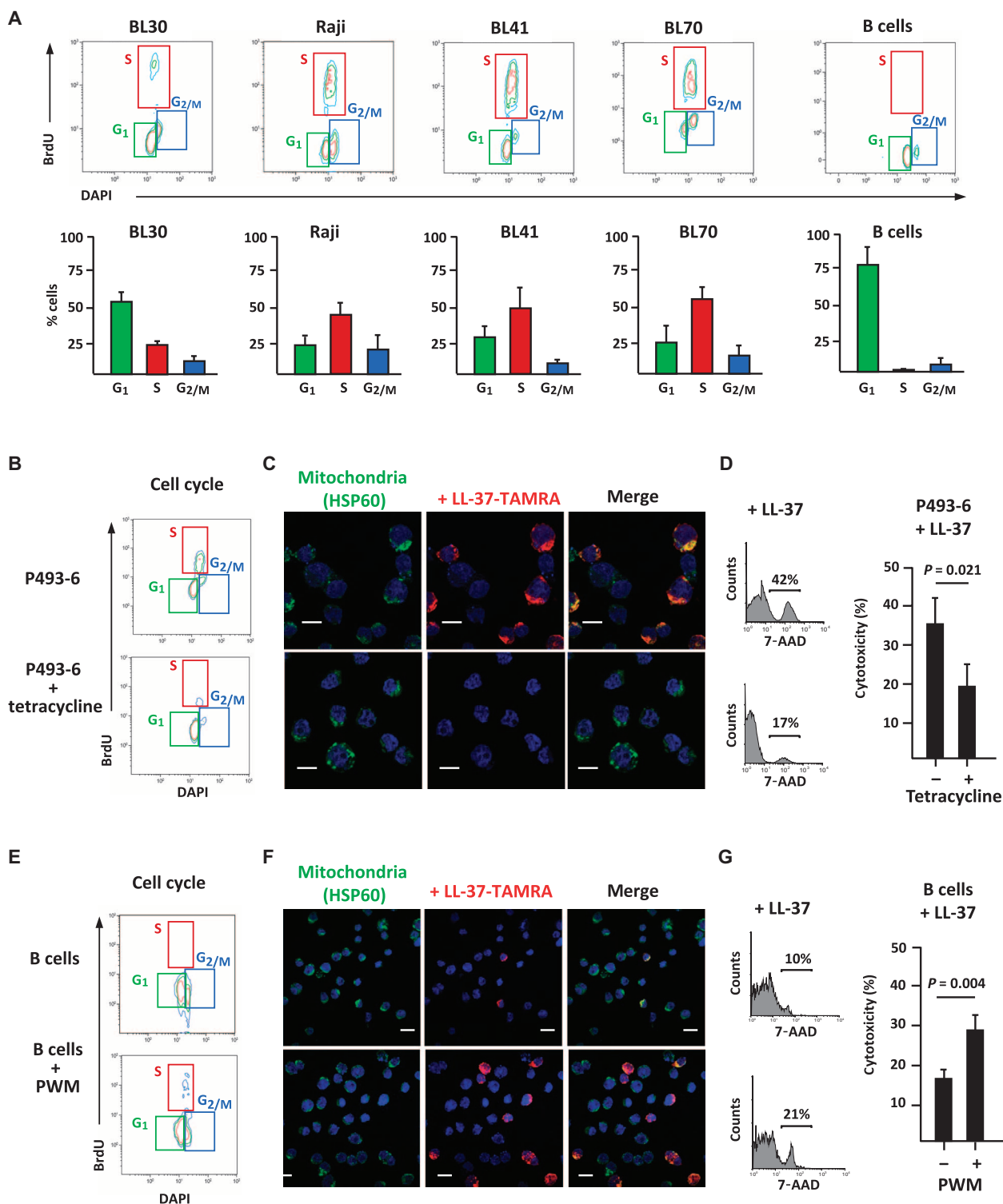
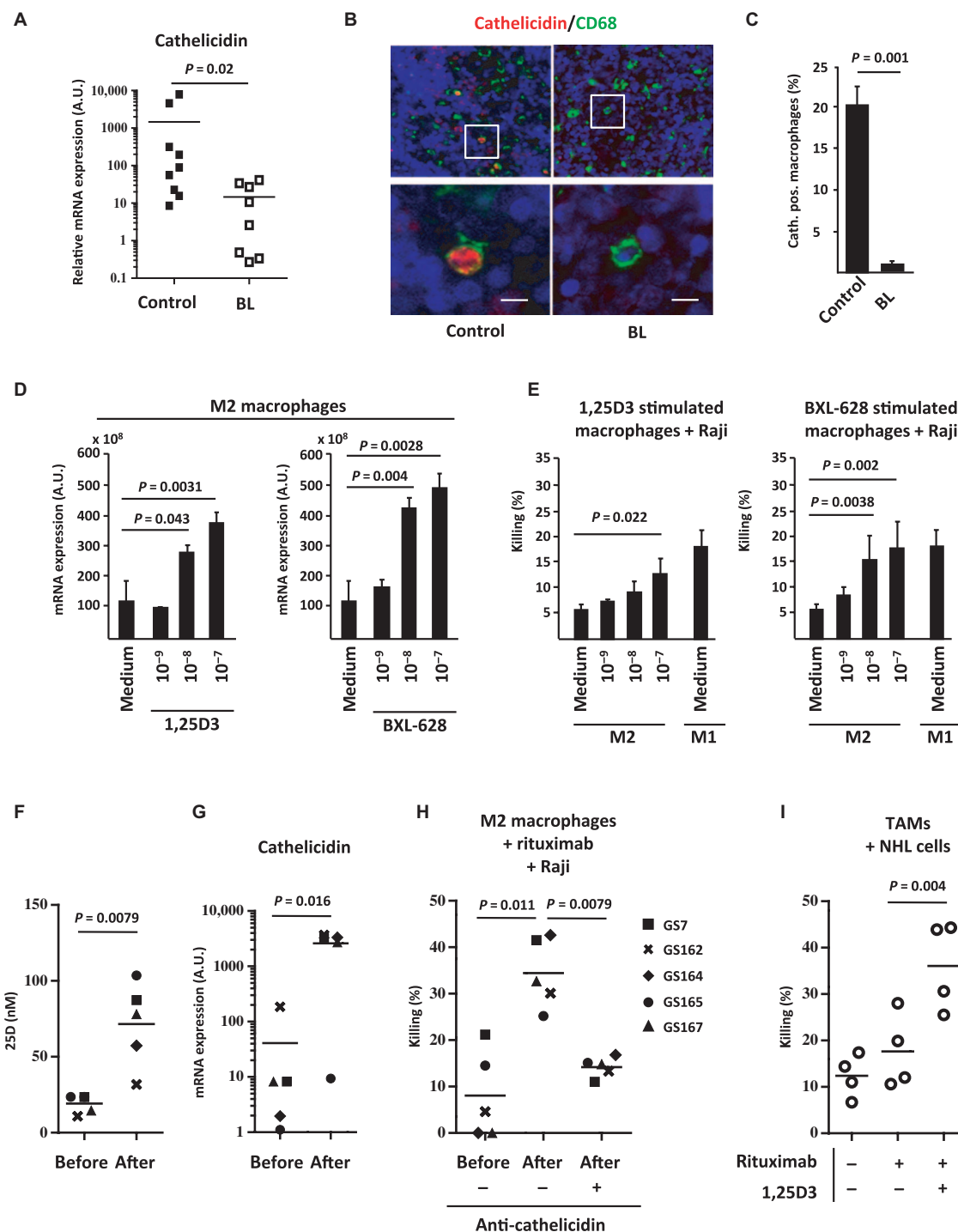


Fig. 5. LL-37 is cytotoxic for proliferating cells. (A) Cell cycle analysis of BL30, Raji, BL41, BL70, and B cells. Cells were analyzed for cell cycle distribution by flow cytometry [BrdU (5-bromo-2'-deoxyuridine) uptake/DAPI (4',6-diamidino-2-phenylindole) fluorescence intensity, G₀/G₁: green, S: red, G₂/M: blue]. (B to D) Cell cycle analysis of P493-6 cells in the presence or absence of tetracycline (10 nM) (B), colocalization of HSP60 (green) with LL-37-TAMRA (red) (C), and viability of B cells after LL-37

treatment (D). Switching off *c-MYC* expression by tetracycline reduces proliferation and renders cells insensitive to LL-37, thereby increasing viability (7-AAD exclusion). (E to G) Cell cycle analysis (E), colocalization of HSP60 (green) with LL-37-TAMRA (red) (F), and viability after LL-37 treatment of PWM-stimulated normal B cells (G). Graphs show the cell viability relative to the untreated control ($n = 5$). Error bars show SEM. Scale bars, 10 μm (C and F).

Fig. 6. Vitamin D increases ADCC of M2 macrophages and TAMs. (A) qPCR analysis of coding RNA for cathelicidin isolated from paraffin-embedded tissue. (B) Immunostaining of macrophages by anti-CD68 (green) and anti-cathelicidin (red) in BL and reactive lymph node specimens ($n = 5$). TAMs in BL do not express cathelicidin. Scale bar, 10 μm . (C) Percentage of cathelicidin⁺ macrophages in reactive lymph nodes and BL tissues. Percentage of cathelicidin⁺ macrophages was calculated from microscopically counted CD68⁺ macrophages (minimum 100 per donor, $n = 5$) in blinded fashion. (D) M2 macrophages were treated for 2 days with 1,25D3 or BXL-628, and *cathelicidin* expression was analyzed by real-time qPCR. (E) M2 macrophages were incubated for 2 days with 1,25D3 and BXL-628 and subsequently cocultured with Raji targets (5:1, 48 hours). Viability of the lymphoma cell lines (gated CD11b⁺) was determined by annexin-V/7-AAD staining and flow cytometry. The graph shows the percentage of Raji cells killed (CD11b⁺ and 7-AAD⁺) calculated relative to the untreated control ($n = 10$). M1 macrophages were used as a positive control. (F) Healthy vitamin D-deficient (serum concentrations <10 ng/ml) volunteers ($n = 5$) were treated with 40,000 IE units of 25-OH vitamin D per week for 6 weeks, and serum vitamin 25D concentrations were measured by a chemiluminescence immunoassay before and after vitamin D therapy. (G) Monocytes were isolated before and after vitamin D supplementation, differentiated to M2 macrophages, and cathelicidin expression was determined by real-time qPCR. (H) Raji cells were incubated for 30 min with rituximab (1 $\mu\text{g}/\text{ml}$) and co-incubated with M2 macrophages (E/T = 5:1) from donors before and after vitamin D supplementation for 24 hours in the presence or absence of a monoclonal anti-cathelicidin antibody (10 $\mu\text{g}/\text{ml}$). Viability of the lymphoma cell line was determined by 7-AAD staining and flow cytometric analysis. The graph shows the average



result of five experiments with different donors ($n = 5$). (I) TAMs and lymphoma cells were isolated by flow cytometry. TAMs were treated for 24 hours with 1,25D3 (10^{-7} M) and subsequently cocultured with autologous lymphoma targets (5:1, 48 hours) with or without rituximab (1 $\mu\text{g}/\text{ml}$). Viability of the lymphoma cells (gated CD11b⁺) was determined by annexin-V/7-AAD staining by flow cytometry. The graph shows percent killing of lymphoma cells calculated relative to the untreated control ($n = 4$). Error bars show SEM. Concentration of 25D in the medium used in (D) to (I) was ≈ 70 nM.

BXL-628 increased the cytotoxicity of M2 macrophages against BL cells (1,25D3: 10^{-7} M, $P < 0.005$; BXL-628: 10^{-8} M, $P < 0.005$; 10^{-7} M, $P = 0.002$), reflecting the expression of cathelicidin (Fig. 6E). Addition of a cathelicidin-specific antibody significantly reduced the cytotoxicity of vitamin D-activated M2 macrophages (fig. S16B, $P = 0.005$) in comparison to a control antibody, suggesting that increased cytotoxicity of M2 macrophages was mainly mediated by cathelicidin. We did not observe direct cytotoxic effects of 1,25D3 or BXL-628 in our experimental settings (fig. S16C). Notably, M1 macrophage toxicity in the presence or absence of 1,25D3 was most effective within 48 to 72 hours of coculture (fig. S17). Within this observation period, we did not observe skewing of M2 macrophages toward an M1 phenotype after 1,25D3 treatment (fig. S18, A to C). In summary, these findings strongly indicate that treatment of M2 macrophages with vitamin D increases their cathelicidin production, thereby triggering their intrinsic tumoricidal activity and promoting antitumor immunity.

Vitamin D increases antibody-dependent cytotoxicity by cathelicidin

Given the key role of macrophages in the antitumor effects of monoclonal antibodies, as well as the finding that vitamin D deficiency negatively affects treatment of high-grade NHL patients with rituximab-containing chemotherapy (19), we speculated that cathelicidin also represents an important effector molecule for rituximab-mediated cytotoxicity and that vitamin D supplementation would enhance ADCC due to an increase of cathelicidin production. To test this hypothesis, we measured antibody-dependent cytotoxic activity of M2 macrophages from healthy individuals before and after vitamin D treatment. After informed consent, we treated volunteers with low-serum vitamin D levels (<25 nM, $n = 5$) with 40,000 IE units of 25-OH vitamin D per week. After 6 weeks of treatment, we observed an increase of vitamin D serum levels (25D) in all five individuals (before: 19.0 ± 2.72 nM versus after: 71.55 ± 12.44 nM, $P = 0.0079$; Fig. 6F). Monocytes were isolated from these donors before and after vitamin D treatment and differentiated to M2 macrophages. Notably, M2 macrophages after vitamin D supplementation displayed a significantly increased cathelicidin mRNA expression in comparison to M2 macrophages before treatment (Fig. 6G; before: 41 ± 32 AU versus after: 2592 ± 595 AU, $P = 0.016$). In addition, treatment with vitamin D significantly up-regulated *VDR* ($P = 0.03$) and *CYP24A1* ($P = 0.06$), whereas *CYP27B1* ($P = 0.02$) expression remained, on average, unchanged (fig. S19). To determine whether vitamin D supplementation also enhances ADCC, we assessed rituximab-mediated cytotoxicity of M2 macrophages isolated before and after vitamin D supplementation. Macrophages were incubated with Raji target cells in the presence of the mAb rituximab (1 μ g/ml), and cytotoxicity was determined by annexin-V and 7-AAD staining. Strikingly, macrophages from donors after vitamin D supplementation killed more efficiently compared to macrophages from the same donors before vitamin D treatment (before: $8.0 \pm 4.2\%$ killing versus after: $34 \pm 3.3\%$ killing, $P = 0.011$; Fig. 6H). Furthermore, the rituximab-induced cytotoxicity of macrophages from vitamin D-supplemented donors was markedly inhibited in the presence of the anti-cathelicidin antibody (anti-cathelicidin: $14 \pm 1\%$ killing, $P = 0.0079$). To further demonstrate the relevance of the finding that vitamin D supplementation enhances ADCC of TAMs, we isolated TAMs and primary lymphoma cells from the bone marrow of patients with DLBCL, the most frequent high-grade B cell lymphoma in humans. TAMs were treated with 1,25D3 and tested for ADCC

against autologous lymphoma cells in the presence of rituximab. Notably, 1,25D3 treatment enhanced rituximab-mediated killing of isolated TAMs in vitro (Fig. 6I; $P = 0.004$). These results support a model in which rituximab-mediated ADCC by macrophages depends in great part on the expression of cathelicidin.

DISCUSSION

In high-grade B cell lymphomas such as BLs, macrophages represent the most prominent stroma cell type and are present in abundance. Despite their intrinsic tumoricidal potential, TAMs of high-grade lymphoma fail to kill malignant cells. Here, we provide one explanation for this paradox by describing a vitamin D-dependent tumoricidal effector mechanism of human macrophages, which is defective in TAMs of BL. We show that (i) M1 macrophages kill proliferating lymphoma cells via secretion of the vitamin D-dependent peptide cathelicidin; (ii) M2 macrophages and M2-like TAMs in BL have a defective vitamin D metabolic pathway, leading to a reduced expression of cathelicidin, and therefore fail to lyse lymphoma cells; (iii) this defect in cytotoxicity can be overcome by VDR agonists; and (iv) treatment of individuals with vitamin D improves ADCC via cathelicidin in vitro.

To study macrophage cytotoxicity, we used an in vitro polarization model in which generated M2 macrophages reflect a variety of features found in BL macrophages ($CD163^{\text{high}}$, $CD206^{\text{high}}$, $iNOS_{\text{low}}$, $BAFF^+$, $IL-10^+$, TNF^+ , and $IL-12^-$). A key finding of our study is that human macrophages are able to directly kill high-grade lymphoma cells by secreting the immune defense peptide cathelicidin. Previously, it has been shown that the absence of the mouse cathelicidin homolog CRAMP in NK cells resulted in increased growth of skin cancers in mice (17). Furthermore, cathelicidin has been shown to be expressed in normal colon mucosa but is down-regulated in colon cancer tissues, where its expression correlates with apoptosis of tumor tissues. Conversely, overexpression of cathelicidin in tumor cells seems to contribute to proliferation and invasion of cancer cells or immune cells (31). However, we found only low levels of cathelicidin in BL tissue and no growth-promoting effect on BL cells. This study highlights the potential role of cathelicidin as a cytotoxic effector molecule of human macrophages in the presence of malignant or proliferating B cells. In comparison to NK cells, which are believed to be key antitumor effector cells, macrophage-mediated tumor cytotoxicity is a relatively slow but equally effective process (25). In this context, it is worth noting that, in contrast to macrophages, NK cells are hardly present within the stroma of aggressive B cell lymphomas (32).

One limitation of our study is the use of an artificial in vitro macrophage polarization model and various BL cell lines as targets to replicate our in vivo findings. Because of the limited availability of fresh lymph node material from patients with high-grade lymphomas, who typically constitute emergency cases, it is difficult to conduct reliable experiments based on primary cells. Furthermore, isolation of macrophages from various tumors by flow cytometric cell sorting or by magnetic bead isolation proves difficult, because tumor-residing macrophages are vulnerable, and their viability after cell sorting is very low. Given these constraints, we isolated primary lymphoma cells and macrophages from infiltrated bone marrow through flow cytometric cell sorting. We used lymphoma-infiltrated bone marrow samples from patients with diffuse large B cell lymphoma as a source of neoplastic B cells and for TAMs. We showed that killing of primary

lymphoma cells by M1 macrophages is identical to what we show with BL cell lines. By contrast, M2 macrophages and autologous TAMs from the patients failed to eliminate primary lymphoma cells.

The finding that cathelicidin affects only proliferating cells is supported by a previous report that antimicrobial peptides lyse mitogen-activated, but not resting, lymphocytes (33). At least two major mechanisms by which cathelicidin affects only proliferating B cells are conceivable. The first mechanism involves cell membrane composition, which is altered during cell division. Cathelicidin binds preferentially to negatively charged membranes (34), and surface exposure of negatively charged phosphatidylserine in the external layers of tumor cells has been found in numerous studies (35). Second, intact mitochondrial function is a prerequisite for proliferating (such as cancer) cells. This cannot just be attributed to mitochondria's role as the powerhouse of cells, because most cancer cells cover their energy demands by glycolysis ("Warburg effect"). Rather, growing evidence suggests that the mitochondrial citric acid cycle intermediates serve as precursors for the synthesis of nucleotides, lipids, and amino acids, which are essential for tumor cell proliferation (36).

In hematopoietic lymphatic malignancies such as BL, infiltration by macrophages is a characteristic morphological hallmark. It has also been shown that macrophage infiltration correlates with poor prognosis in a variety of human tumors (37). Given that macrophages have the ability to eliminate malignant cells, it is puzzling that TAMs are unable to kill lymphoma cells. One potential explanation provided by our findings is that M2 macrophages and TAM from BL patients display virtually no cathelicidin expression. This is likely due to their impaired vitamin D metabolic pathway. Conversely, we show that expression of CYP24A1, which is also regulated by vitamin D, is as high in BL as in reactive lymph node samples, although M2 macrophages and TAMs display a low expression of VDR. Given these findings, it is tempting to speculate that high-grade lymphomas create an environment that results in quick inactivation of active vitamin D3 through CYP24A1 to avoid induction of effector molecules such as cathelicidin. Which regulators of CYP24A1 are responsible for this phenotype is currently unclear and requires further investigation.

Numerous studies have demonstrated that VDR, CYP27B1, and CYP24A1 expression frequently becomes dysregulated in human tumors, highlighting their role in tumor progression (20). Furthermore, case-control studies have found an association between VDR polymorphisms and susceptibility to DLBCL (38). Malignant B cells have the ability to internalize and degrade vitamin D-binding protein (39), suggesting that B cell lymphomas may interfere with vitamin D metabolism and preferentially recruit or foster M2-differentiated macrophages. Here, by linking the vitamin D pathway and TAMs in BL, we showed that macrophages with an M2-like phenotype have a reduced capacity to metabolize vitamin D, which is required for optimal tumor cytotoxicity of cathelicidin.

Several VDR agonists have been developed for the treatment of cancer based on their capacity to inhibit cell growth, promote apoptosis, and favor cell differentiation. Whether VDR agonists stimulate the antitumor immune response remains unclear. Our data show that despite their deregulated vitamin D metabolic pathway, M2 macrophages can be fully activated to express cathelicidin peptide when treated with direct VDR agonists *in vitro*. This leads us to speculate that VDR agonists could be used therapeutically to increase cathelicidin production in TAMs and enhance their cytotoxic activity *in vivo*. It is worth noting that the expression of cathelicidin is also up-regulated by myco-

bacterial antigens, interferon- γ (12), and CD40 ligand (40) in human macrophages. Recent studies also indicate that macrophage tumoricidal activity could be therapeutically enhanced upon administration of live mycobacterium *Bacille Calmette-Guérin* (41), muramyl tripeptide (42), or CD40 agonistic antibodies (2). The identification of cathelicidin as a tumoricidal effector molecule of macrophages provides insight into mechanisms by which bacteria and bacterial cell walls are efficient immunotherapies in bladder cancer (43) and melanoma (44).

Macrophages are critical to the efficacy of anti-CD20 antibodies (4, 45). Recent data support the likely positive effect of TAMs in the clinical response to rituximab (46, 47). In patients with follicular lymphoma treated with chemotherapy alone, higher levels of macrophage infiltration in the lymph node correlate with poor prognosis (37). However, this correlation is reversed in patients treated with rituximab in addition to standard chemotherapy (46, 47). One effector mechanism of rituximab is the activation of ADCC. Because a recent study shows that vitamin D deficiency impairs rituximab treatment in NHL patients (19), we speculated that cathelicidin is also an important effector molecule for rituximab-mediated cytotoxicity. We found that ADCC of macrophages from vitamin D-deficient donors was improved after vitamin D treatment. Furthermore, the enhanced antibody-dependent elimination of lymphoma cells was dependent on cathelicidin expression. These results have important clinical ramifications because recent studies have pointed to the central role of vitamin D in enhancing chemoimmunotherapy efficacy and survival in patients with lymphatic neoplasias. We believe that our study provides the potential mechanism for this surprising observation (48). Another important mechanism by which rituximab kills cancer cells is antibody-dependent phagocytosis (5). In a very recent study, circulating tumor cells were eliminated during antibody therapy by liver macrophages (Kupffer cells) in a mouse model (5). Although TAMs in BL and M2 macrophages also display an increased phagocytic activity and have the ability to phagocytose rituximab-opsonized lymphoma cells (22), several studies indicate that lymphoma cells counteract effective phagocytosis of rituximab via increased CD47 expression (49). However, given that vitamin D treatment also improves phagocytosis (13) and ROS production (14) of macrophages, we expect that vitamin D supplementation during antibody therapy will positively contribute to the effects of rituximab.

Together, our results provide evidence that vitamin D regulates antitumor effector functions of macrophages by inducing the expression of cathelicidin. Strikingly, TAMs that display impaired vitamin D metabolism do not express cathelicidin or exert cytotoxic functions. We believe that our findings will support the initiation of clinical trials by suggesting vitamin D agonists as potential agents to treat NHL in combination with monoclonal anti-CD20 antibodies, as well as provide evidence that maintaining adequate vitamin D serum levels may be beneficial for sustaining antitumor responses.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. M2-like phenotype of TAMs in BL.

Fig. S2. Cytokine profile in lymph node sections.

Fig. S3. Phenotype of generated M1 and M2 macrophages.

Fig. S4. Activation of M1 and M2 macrophages after exposure to Raji targets.

Fig. S5. Analysis of effector molecules secreted by macrophages.

Fig. S6. Expression of TNFR1, CD95, DR4, and DR5 on BL cell lines.

Fig. S7. Phagocytosis of BL cells.

Fig. S8. Dose-dependent cytotoxicity of LL-37 for BL Raji cells.
 Fig. S9. Expression of "LL-37 receptors" on BL cell lines and proliferating B cells.
 Fig. S10. Cytotoxic activity of LL-37 counteracted by overexpression of BCL-2.
 Fig. S11. LL-37 targeting mitochondria of BL cells.
 Fig. S12. Z-stack analysis of colocalization of LL-37 and mitochondria.
 Fig. S13. Colocalization of LL-37 and mitochondria.
 Fig. S14. ROS induced by LL-37 in BL cells.
 Fig. S15. Expression of antiapoptotic BCL-2 family proteins in BL cells.
 Fig. S16. Cathelicidin mediating vitamin D-induced cytotoxicity of M2 macrophages.
 Fig. S17. Time course of macrophage-mediated cytotoxicity.
 Fig. S18. Phenotype of M2 macrophages after vitamin D treatment.
 Fig. S19. *VDR*, *CYP27B1*, and *CYP24A1* expression in vitamin D-treated healthy volunteers.
 Fig. S20. Cell sorting strategy to obtain TAMs and lymphoma cells.
 Reference (50)

REFERENCES AND NOTES

1. J. W. Pollard, Tumour-educated macrophages promote tumour progression and metastasis. *Nat. Rev. Cancer* **4**, 71–78 (2004).
2. G. L. Beatty, E. G. Chiorean, M. P. Fishman, B. Saboury, U. R. Teitelbaum, W. Sun, R. D. Huhn, W. Song, D. Li, L. L. Sharp, D. A. Torigian, P. J. O'Dwyer, R. H. Vonderheide, CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science* **331**, 1612–1616 (2011).
3. S. Jaiswal, M. P. Chao, R. Majeti, I. L. Weissman, Macrophages as mediators of tumor immunosurveillance. *Trends Immunol.* **31**, 212–219 (2010).
4. J. Uchida, Y. Hamaguchi, J. A. Oliver, J. V. Ravetch, J. C. Poe, K. M. Haas, T. F. Tedder, The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. *J. Exp. Med.* **199**, 1659–1669 (2004).
5. N. Gül, L. Babes, K. Siegmund, R. Korthouwer, M. Bögers, R. Braster, G. Vidarsson, T. L. ten Hagen, P. Kubes, M. van Egmond, Macrophages eliminate circulating tumor cells after monoclonal antibody therapy. *J. Clin. Invest.* **124**, 812–823 (2014).
6. T. Keler, P. K. Wallace, L. A. Vitale, C. Russoniello, K. Sundarapandian, R. F. Graziano, Y. M. Deo, Differential effect of cytokine treatment on Fcα receptor I- and Fcγ receptor I-mediated tumor cytotoxicity by monocyte-derived macrophages. *J. Immunol.* **164**, 5746–5752 (2000).
7. M. R. Dinapoli, C. L. Calderon, D. M. Lopez, The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduce expression of the inducible nitric oxide synthase gene. *J. Exp. Med.* **183**, 1323–1329 (1996).
8. A. Sica, T. Schioppa, A. Mantovani, P. Allavena, Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. *Eur. J. Cancer* **42**, 717–727 (2006).
9. P. Sinha, V. K. Clements, S. Ostrand-Rosenberg, Interleukin-13-regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. *Cancer Res.* **65**, 11743–11751 (2005).
10. M. Chittezhath, M. K. Dhillon, J. Y. Lim, D. Laoui, I. N. Shalova, Y. L. Teo, J. Chen, R. Kamaraj, L. Raman, J. Lum, T. P. Thamboo, E. Chiong, F. Zolezzi, H. Yang, J. A. Van Ginderachter, M. Poidinger, A. S. Wong, S. K. Biswas, Molecular profiling reveals a tumor-promoting phenotype of monocytes and macrophages in human cancer progression. *Immunity* **41**, 815–829 (2014).
11. P. T. Liu, S. Stenger, H. Li, L. Wenzel, B. H. Tan, S. R. Krutzik, M. T. Ochoa, J. Schaubert, K. Wu, C. Meinken, D. L. Kamen, M. Wagner, R. Bals, A. Steinmeyer, U. Zügel, R. L. Gallo, D. Eisenberg, M. Hewison, B. W. Hollis, J. S. Adams, B. R. Bloom, R. L. Modlin, Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* **311**, 1770–1773 (2006).
12. M. Fabri, S. Stenger, D. M. Shin, J. M. Yuk, P. T. Liu, S. Realegeno, H. M. Lee, S. R. Krutzik, M. Schenk, P. A. Sieling, R. Teles, D. Montoya, S. S. Iyer, H. Bruns, D. M. Lewinsohn, B. W. Hollis, M. Hewison, J. S. Adams, A. Steinmeyer, U. Zügel, G. Cheng, E. K. Jo, B. R. Bloom, R. L. Modlin, Vitamin D is required for IFN-γ-mediated antimicrobial activity of human macrophages. *Sci. Transl. Med.* **3**, 104ra102 (2011).
13. H. Xu, A. Soruri, R. K. Gieseler, J. H. Peters, 1,25-Dihydroxyvitamin D3 exerts opposing effects to IL-4 on MHC class-II antigen expression, accessory activity, and phagocytosis of human monocytes. *Scand. J. Immunol.* **38**, 535–540 (1993).
14. M. S. Cohen, D. E. Mesler, R. G. Snipes, T. K. Gray, 1,25-Dihydroxyvitamin D3 activates secretion of hydrogen peroxide by human monocytes. *J. Immunol.* **136**, 1049–1053 (1986).
15. A. F. Gombart, N. Borregaard, H. P. Koeffler, Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D₃. *FASEB J.* **19**, 1067–1077 (2005).
16. M. Zanetti, Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* **75**, 39–48 (2004).
17. A. S. Büchau, S. Morizane, J. Trowbridge, J. Schaubert, P. Kotol, J. D. Bui, R. L. Gallo, The host defense peptide cathelicidin is required for NK cell-mediated suppression of tumor growth. *J. Immunol.* **184**, 369–378 (2010).
18. M. T. Drake, M. J. Maurer, B. K. Link, T. M. Habermann, S. M. Ansell, I. N. Micallef, J. L. Kelly, W. R. Macon, G. S. Nowakowski, D. J. Inwards, P. B. Johnston, R. J. Singh, C. Allmer, S. L. Slager, G. J. Weiner, T. E. Witzig, J. R. Cerhan, Vitamin D insufficiency and prognosis in non-Hodgkin's lymphoma. *J. Clin. Oncol.* **28**, 4191–4198 (2010).
19. J. T. Bittenbring, F. Neumann, B. Altmann, M. Achenbach, J. Reichrath, M. Ziepert, J. Geisel, E. Regitz, G. Held, M. Pfreundschuh, Vitamin D deficiency impairs rituximab-mediated cellular cytotoxicity and outcome of patients with diffuse large B-cell lymphoma treated with but not without rituximab. *J. Clin. Oncol.* **32**, 3242–3248 (2014).
20. N. Lopes, B. Sousa, D. Martins, M. Gomes, D. Vieira, L. A. Veronese, F. Milanezi, J. Paredes, J. L. Costa, F. Schmitt, Alterations in vitamin D signalling and metabolic pathways in breast cancer progression: A study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer* **10**, 483 (2010).
21. D. Montoya, D. Cruz, R. M. Teles, D. J. Lee, M. T. Ochoa, S. R. Krutzik, R. Chun, M. Schenk, X. Zhang, B. G. Ferguson, A. E. Burdick, E. N. Sarno, T. H. Rea, M. Hewison, J. S. Adams, G. Cheng, R. L. Modlin, Divergence of macrophage phagocytic and antimicrobial programs in leprosy. *Cell Host Microbe* **6**, 343–353 (2009).
22. C. A. Ogden, J. D. Pound, B. K. Batth, S. Owens, I. Johannessen, K. Wood, C. D. Gregory, Enhanced apoptotic cell clearance capacity and B cell survival factor production by IL-10-activated macrophages: Implications for Burkitt's lymphoma. *J. Immunol.* **174**, 3015–3023 (2005).
23. A. C. Ross, J. E. Manson, S. A. Abrams, J. F. Aloia, P. M. Brannon, S. K. Clinton, R. A. Durazo-Arvizu, J. C. Gallagher, R. L. Gallo, G. Jones, C. S. Kovacs, S. T. Mayne, C. J. Rosen, S. A. Shapses, The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: What clinicians need to know. *J. Clin. Endocrinol. Metab.* **96**, 53–58 (2011).
24. J. Xue, S. V. Schmidt, J. Sander, A. Draffehn, W. Krebs, I. Quester, D. De Nardo, T. D. Gohel, M. Emde, L. Schmidleithner, H. Ganesan, A. Nino-Castro, M. R. Mallmann, L. Labzin, H. Theis, M. Kraut, M. Beyer, E. Latz, T. C. Freeman, T. Ulas, J. L. Schultze, Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* **40**, 274–288 (2014).
25. J. Hasenkamp, A. Borgerding, G. Wulf, M. Uhrberg, W. Jung, S. Dingeldein, L. Truempner, B. Glass, Resistance against natural killer cell cytotoxicity: Analysis of mechanisms. *Scand. J. Immunol.* **64**, 444–449 (2006).
26. C. A. Alvarez-Breckenridge, J. Yu, R. Price, J. Wojton, J. Pradarelli, H. Mao, M. Wei, Y. Wang, S. He, J. Hardcastle, S. A. Fernandez, B. Kaur, S. E. Lawler, E. Vivier, O. Mandelboim, A. Moretta, M. A. Caligiuri, E. A. Chiocca, NK cells impede glioblastoma virotherapy through Nkp30 and Nkp46 natural cytotoxicity receptors. *Nat. Med.* **18**, 1827–1834 (2012).
27. O. E. Sørensen, P. Follin, A. H. Johnsen, J. Calafat, G. S. Tjåbring, P. S. Hiemstra, N. Borregaard, Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* **97**, 3951–3959 (2001).
28. Z. Oren, J. C. Lerman, G. H. Gudmundsson, B. Agerberth, Y. Shai, Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: Relevance to the molecular basis for its non-cell-selective activity. *Biochem. J.* **341** (Pt. 3), 501–513 (1999).
29. K. A. Sochacki, K. J. Barns, R. Bucki, J. C. Weisshaar, Real-time attack on single *Escherichia coli* cells by the human antimicrobial peptide LL-37. *Proc. Natl. Acad. Sci. U.S.A.* **108**, E77–E81 (2011).
30. M. Schuhmacher, M. S. Staege, A. Pajic, A. Polack, U. H. Weidle, G. W. Bornkamm, D. Eick, F. Kohlhuber, Control of cell growth by c-Myc in the absence of cell division. *Curr. Biol.* **9**, 1255–1288 (1999).
31. S. B. Coffelt, F. C. Marini, K. Watson, K. J. Zwezdaryk, J. L. Dembinski, H. L. LaMarca, S. L. Tomchuck, K. Honer zu Bentrup, E. S. Danko, S. L. Henkle, A. B. Scandurro, The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 3806–3811 (2009).
32. S. M. Ansell, M. Stenson, T. M. Habermann, D. F. Jelinek, T. E. Witzig, Cd4+ T-cell immune response to large B-cell non-Hodgkin's lymphoma predicts patient outcome. *J. Clin. Oncol.* **19**, 720–726 (2001).
33. A. Risso, M. Zanetti, R. Gennaro, Cytotoxicity and apoptosis mediated by two peptides of innate immunity. *Cell. Immunol.* **189**, 107–115 (1998).
34. J. B. McPhee, R. E. Hancock, Function and therapeutic potential of host defence peptides. *J. Pept. Sci.* **11**, 677–687 (2005).
35. M. Klähn, M. Zacharias, Transformations in plasma membranes of cancerous cells and resulting consequences for cation insertion studied with molecular dynamics. *Phys. Chem. Chem. Phys.* **15**, 14427–14441 (2013).
36. F. Weinberg, N. S. Chandel, Mitochondrial metabolism and cancer. *Ann. N. Y. Acad. Sci.* **1177**, 66–73 (2009).
37. P. Farinha, H. Masoudi, B. F. Skinnider, K. Shumansky, J. J. Spinelli, K. Gill, R. Klasa, N. Voss, J. M. Connors, R. D. Gascoyne, Analysis of multiple biomarkers shows that lymphoma-associated macrophage (LAM) content is an independent predictor of survival in follicular lymphoma (FL). *Blood* **106**, 2169–2174 (2005).

38. M. P. Purdue, Q. Lan, A. Kricker, C. M. Vajdic, N. Rothman, B. K. Armstrong, Vitamin D receptor gene polymorphisms and risk of non-Hodgkin's lymphoma. *Haematologica* **92**, 1145–1146 (2007).
39. C. Esteban, M. Geuskens, J. M. Ena, Z. Mishal, A. Macho, J. M. Torres, J. Uriel, Receptor-mediated uptake and processing of vitamin D-binding protein in human B-lymphoid cells. *J. Biol. Chem.* **267**, 10177–10183 (1992).
40. G. M. Klug-Micu, S. Stenger, A. Sommer, P. T. Liu, S. R. Krutzik, R. L. Modlin, M. Fabri, CD40 ligand and interferon- γ induce an antimicrobial response against *Mycobacterium tuberculosis* in human monocytes. *Immunology* **139**, 121–128 (2013).
41. K. Pryor, J. Goddard, D. Goldstein, P. Stricker, P. Russell, D. Golovsky, R. Penny, Bacillus Calmette-Guerin (BCG) enhances monocyte- and lymphocyte-mediated bladder tumour cell killing. *Br. J. Cancer* **71**, 801–807 (1995).
42. P. A. Meyers, C. L. Schwartz, M. D. Krailo, J. H. Healey, M. L. Bernstein, D. Betcher, W. S. Ferguson, M. C. Gebhardt, A. M. Goorin, M. Harris, E. Kleinerman, M. P. Link, H. Nadel, M. Nieder, G. P. Siegal, M. A. Weiner, R. J. Wells, R. B. Womer, H. E. Grier; Children's Oncology Group, Osteosarcoma: The addition of muramyl tripeptide to chemotherapy improves overall survival—A report from the Children's Oncology Group. *J. Clin. Oncol.* **26**, 633–638 (2008).
43. A. Morales, D. Eidinger, A. W. Bruce, Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J. Urol.* **116**, 180–183 (1976).
44. D. Morton, F. R. Eilber, R. A. Malmgren, W. C. Wood, Immunological factors which influence response to immunotherapy in malignant melanoma. *Surgery* **68**, 158–163 (1970).
45. G. Cartron, L. Dacheux, G. Salles, P. Solal-Celigny, P. Bardos, P. Colombat, H. Watier, Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc γ R1IIa gene. *Blood* **99**, 754–758 (2002).
46. M. Taskinen, M. L. Karjalainen-Lindsberg, H. Nyman, L. M. Eerola, S. Leppa, A high tumor-associated macrophage content predicts favorable outcome in follicular lymphoma patients treated with rituximab and cyclophosphamide-doxorubicin-vincristine-prednisone. *Clin. Cancer Res.* **13**, 5784–5789 (2007).
47. D. Canonici, G. Salles, N. Mounier, N. Brousse, M. Keuppens, F. Morchhauser, T. Lamy, A. Sonet, M. C. Rousselet, C. Foussard, L. Xerri, High numbers of tumor-associated macrophages have an adverse prognostic value that can be circumvented by rituximab in patients with follicular lymphoma enrolled onto the GELA-GOELAMS FL-2000 trial. *J. Clin. Oncol.* **26**, 440–446 (2008).
48. J. T. Bittenbring, B. Altmann, F. Neumann, M. Achenbach, J. Reichrath, M. Ziepert, J. Geisel, E. Regitz, G. Held, M. Pfreundschuh, 25-OH-vitamin-D deficiency impairs rituximab-mediated cellular cytotoxicity and is associated with an inferior outcome of elderly DLBCL patients treated with rituximab. *Blood* **122**, 1819 (2013).
49. M. P. Chao, A. A. Alizadeh, C. Tang, J. H. Myklebust, B. Varghese, S. Gill, M. Jan, A. C. Cha, C. K. Chan, B. T. Tan, C. Y. Park, F. Zhao, H. E. Kohrt, R. Malumbres, J. Briones, R. D. Gascoyne, I. S. Lossos, R. Levy, I. L. Weissman, R. Majeti, Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. *Cell* **142**, 699–713 (2010).
50. K. Bohmann, G. Hennig, U. Rogel, C. Poremba, B. M. Mueller, P. Fritz, S. Stoerckel, K. L. Schaefer, RNA extraction from archival formalin-fixed paraffin-embedded tissue: A comparison of manual, semiautomated, and fully automated purification methods. *Clin. Chem.* **55**, 1719–1727 (2009).

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Editor's Summary

Vitamin D lends a helping hand

In theory, macrophages and other immune cells should be able to kill tumor cells. However, cancer cells are clearly capable of escaping from immune surveillance, and tumor-associated macrophages usually do not kill them, for reasons that are not yet well understood. A new study by Bruns *et al.* shows that vitamin D can help promote the antitumor activity of macrophages and stimulate their production of cathelicidin, an antimicrobial peptide that can also induce tumor cell death. The results suggest that for cancer patients who are deficient in vitamin D, providing vitamin D supplementation may be helpful in battling the disease and promoting the efficacy of antitumor therapy.

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