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Bisphosphonates Target B Cells to Enhance Humoral Immune Responses

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SUMMARY

Bisphosphonates are a class of drugs that are widely used to inhibit loss of bone mass in patients. We show here that the administration of clinically relevant doses of bisphosphonates in mice increases antibody responses to live and inactive viruses, proteins, haptens, and existing commercial vaccine formulations. Bisphosphonates exert this adjuvant-like activity in the absence of $CD4^+$ and γδ T cells, neutrophils, or dendritic cells, and their effect does not rely on local macrophage depletion, Toll-like receptor signaling, or the inflammasome. Rather, bisphosphonates target directly B cells and enhance B cell expansion and antibody production upon antigen encounter. These data establish bisphosphonates as an additional class of adjuvants that boost humoral immune responses.

INTRODUCTION

Bisphosphonates (BPs) are small-molecule inhibitors of bone resorption that are clinically approved for the treatment of skeletal diseases such as osteoporosis and Paget's disease of bone (Favus, 2010; Scott and Gershon, 1970); these compounds represent a large family of drugs that include first-generation clodronate (CLD) and etidronate (ETD) and nitrogen-containing alendronate (ALD), pamidronate (PMD), zoledronate (ZLD), and neridronate (NRD). Upon liposome encapsulation, BPs like CLD have been widely used to experimentally deplete tissue-resident phagocytes in rodents (Moseman et al., 2012; Van Rooijen and Sanders, 1994). A few studies in BP-treated mice unexpectedly noted increased antigen (Ag)-specific humoral immune responses (Gonzalez et al., 2010; Iannacone et al., 2010; Norton et al., 2011); herein, we set out to systematically dissect the mechanistic basis for this activity.

RESULTS AND DISCUSSION

As the majority of the above-mentioned studies utilized subcutaneously administered BP-encapsulated liposomes prior to local viral challenge, we initially chose the same experimental setup to ask whether liposome encapsulation is required to increase antibody (Ab) responses. To this end, footpads of C57BL/6 mice were injected with PBS, PBS liposomes (PBS-Lip), CLD liposomes (CLD-Lip), or CLD prior to infection in the same footpad with vesicular stomatitis virus (VSV), a prototypic cytopathic virus that induces an early T-independent immunoglobulin M (IgM) response followed by a T-dependent immunoglobulin G (IgG) response (Hangartner et al., 2006). When compared to PBS-injected mice, mice that received CLD exhibited up to 100-fold higher neutralizing antibody (nAb) titers toward VSV (Figure 1A), and this occurred when CLD was administered either prior to or concomitantly with the Ag (Figure S1A). Importantly, free CLD was as effective as CLD-Lip (Figure 1A), it exhibited a dose-dependent effect (Figure S1B), and its adjuvant activity was shared by other BPs that are currently in clinical use, including ETD, PMD, and ALD (Figure 1B).

Subcutaneously administered CLD also increased Ab titers against inactive VSV, soluble proteins (ovalbumin [OVA]), haptens (NP-CGG), and the adjuvant-containing formulation Engerix-B (an approved vaccine against hepatitis B virus) (Figures 1C–1F); similar results were observed when CLD was administered intramuscularly along with the hemoagglutinin/neuroaminidase subunits of the human influenza virus A/NewCaledonia/20/99 (H_1N_1; Figure 1G).

CLD treatment increased both neutralizing IgM and IgG responses against VSV (Figures S1C–S1E) without altering the subtype of Ag-specific IgG induced upon immunization (Figure S1F), and this correlated with the total number of $CD138^+$

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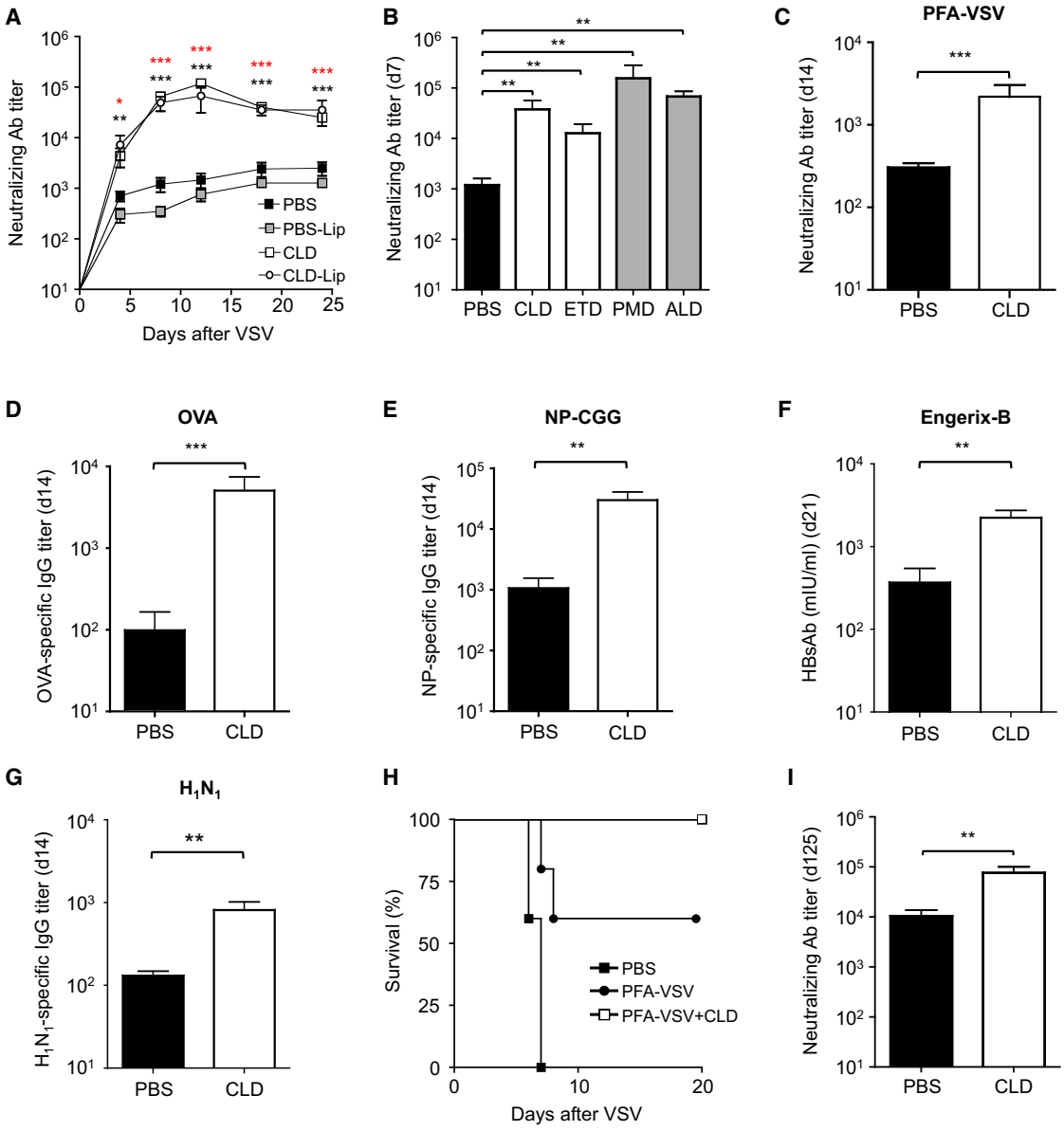

Figure 1. Bisphosphonates Increase Antibody Responses to Live and Inactive Viruses, Proteins, Haptens, and Existing Commercial Vaccine Formulations

(A) VSV nAb titers in the serum of C57BL/6 mice that were footpad injected with PBS, PBS-liposomes (PBS-Lip), clodronate (CLD), or clodronate liposomes (CLD-Lip) prior to VSV infection in the same footpad (see Experimental Procedures for details). n = 5 per group. Black asterisks, PBS versus CLD-Lip; red asterisks, PBS versus CLD. Results are representative of more than ten independent experiments.

(B) VSV nAb titers 7 days p.i. in the serum of C57BL/6 mice that were footpad injected with CLD, etidronate (ETD), pamidronate (PMD), or alendronate (ALD) prior to VSV infection in the same footpad. n = 5 per group; results are representative of three independent experiments.

(C) VSV nAb titers at day 14 postimmunization in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to paraformaldehyde-inactivated VSV (PFA-VSV) immunization in the same footpad. n = 10 per group; results are representative of three independent experiments.

(D) Ovalbumin (OVA)-specific IgG titers at day 14 postimmunization in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to OVA immunization in the same footpad. n = 4 per group; results are representative of three independent experiments.

(E) 4-Hydroxy-3-nitrophenylacetyl (NP)-specific IgG titers at day 14 postimmunization in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to NP-chicken gamma globulin (CGG) immunization in the same footpad. n = 5 per group; results are representative of two independent experiments.

(F) HBsAb titers (mIU/ml) 21 days p.i. in the serum of C57BL/6 mice that were injected in the footpad with PBS or CLD prior to Engerix-B (an approved vaccine against hepatitis B virus) immunization in the same footpad. n = 5 per group; results are representative of five independent experiments.

(G) H_1N_1-specific IgG titers in the serum of C57BL/6 mice that were injected intramuscularly with PBS or CLD prior to intramuscular immunization with H_1N_1. n = 5 per group; results are representative of two independent experiments.

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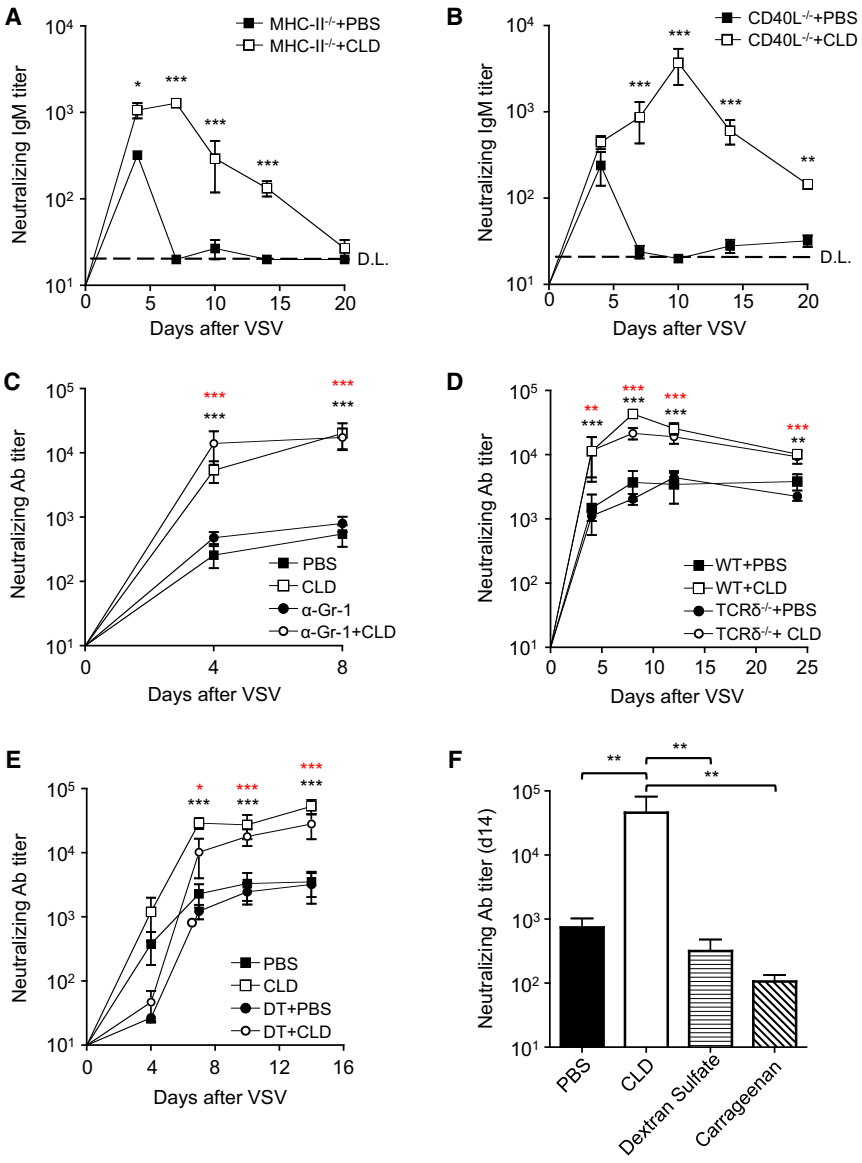

Figure 2. Bisphosphonates Increase Antibody Responses in the Absence of CD4$^+$ and γδ T Cells, Neutrophils, or Dendritic Cells and Their Effect Does Not Require Local Macrophage Depletion

(A) VSV neutralizing IgM titers in the serum of MHC-II$^{-/-}$ mice that were footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 5 per group; results are representative of three independent experiments.

(B) VSV neutralizing IgM titers in the serum of CD40L$^{-/-}$ mice that were footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 5 per group; results are representative of two independent experiments.

(C) VSV nAb titers in the serum of C57BL/6 mice that were injected (or not) with Gr-1 depleting Ab (α–Gr-1; see Experimental Procedures for details) and were subsequently footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 6 per group; black asterisks, PBS versus CLD; red asterisks, αGr-1+PBS versus αGr-1+CLD; results are representative of two independent experiments.

(D) VSV nAb titers in the serum of C57BL/6 (WT) or TCRδ$^{-/-}$ mice that were footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 10 per group; black asterisks, WT+PBS versus WT+CLD; red asterisks, TCRδ$^{-/-}$+PBS versus TCRδ$^{-/-}$+CLD; results are representative of three independent experiments.

(E) VSV nAb titers in the serum of CD11c-DTR-GFP mice that were treated (or not) with diphtheria toxin (see Experimental Procedures for details) and were subsequently footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 6 per group; black asterisks, PBS versus CLD; red asterisks DT+PBS versus DT+CLD. Results are representative of three independent experiments.

(F) VSV nAb titers at day 14 p.i. in the serum of C57BL/6 mice that were footpad injected with PBS, CLD, dextran sulfate or carrageenan prior to VSV infection in the same footpad (see Experimental Procedures for details). n = 3 per group; results are representative of three independent experiments.

Results are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05; D.L., detection limit. See also Figures S2, S3, and S4.

plasma cells recovered from draining LNs (Figure S1G). In combination with inactivated VSV, CLD boosted the partial protection afforded by immunization with inactivated virus alone (Figure 1H), and its adjuvant effect lasted for at least 4 months after a single administration (Figure 1I). CLD treatment also increased neutralizing IgM titers upon VSV infection in MHC-II$^{-/-}$ mice (which lack CD4$^+$ T cells) and in CD40L$^{-/-}$ mice, in which T cell help for B cells is known to be compromised (Renshaw et al., 1994) (Figures 2A and 2B). While these data do not rule out a possible effect of CLD on T cells, they indicate that CLD adjuvant activity can occur independently of CD4$^+$ T cell help and raise mechanistic questions about how this adjuvant effect is mediated. To address this issue, we systematically analyzed cellular and molecular

(H) Kaplan-Meier survival curves of C57BL/6 mice injected in the right footpad with PBS, 10^6 pfu equivalents of PFA-inactivated VSV (PFA-VSV), or 10^6 pfu equivalents of PFA-inactivated VSV and clodronate (PFA-VSV+CLD) 3 weeks prior to infection in the left footpad with 3.5×10^8 pfu of VSV. n = 12 per group; PFA-VSV+CLD versus PFA-VSV: p < 0.05, log-rank (Mantel-Cox) test. Results are representative of two independent experiments.

(I) VSV nAb titers 125 days p.i. in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to VSV infection in the same footpad. n = 5 per group; results are representative of two independent experiments.

Results are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05. See also Figure S1.

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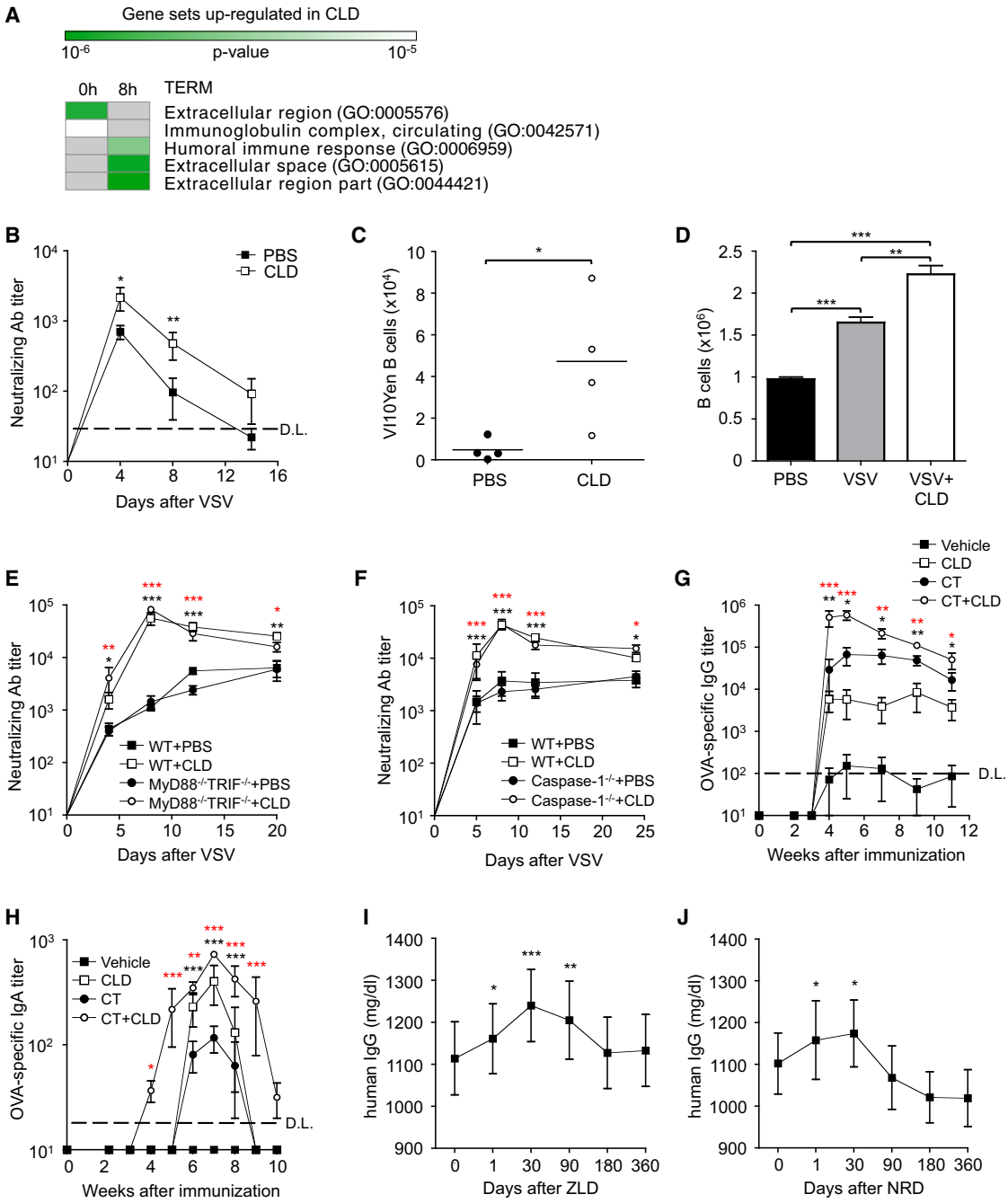

Figure 3. Bisphosphonates Directly Target B Cells and Enhance B Cell Expansion and Antibody Production upon Antigen Encounter Independently of Toll-like Receptor Signaling or the Inflammasome

(A) Gene enrichment analysis of clodronate-dependent genes from microarray measurements of whole lymph node. Shown are the Gene Ontology (GO) terms (rows) statistically enriched among the 156 genes with at least a 2-fold increase in expression at 0 and 8 hr (columns) after VSV infection in clodronate (CLD)-treated mice compared to control (PBS). Enriched GO term categories include BP (biological process) and CC (cellular component). Green: p value < 10^{-6}; white: p value < 10^{-4}; gray: no enrichment. See Experimental Procedures for details.

(B) VSV nAb titers in the serum of D_HLMP2A mice that were intravenously transferred with PBS- or CLD-treated VI10Yen B cells 24 hr prior to footpad VSV infection. n = 6 per group; results are representative of two independent experiments.

(C) Quantification of the total number of VI10Yen B cells recovered from the spleen of the mice described in (B) 8 days after VSV.

(D) Quantification of the total number of cells recovered 48 hr upon in vitro culture of purified VI10Yen B cells with PBS, VSV, or 10 nM clodronate and VSV.

(E) VSV nAb titers in the serum of C57BL/6 (WT) or MyD88$^{-/-}$/Trif$^{-/-}$ mice that were footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 4 per group; black asterisks, WT + PBS versus WT + CLD; red asterisks MyD88$^{-/-}$/Trif$^{-/-}$+PBS versus MyD88$^{-/-}$/Trif$^{-/-}$+CLD; results are representative of three independent experiments.

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changes induced by BPs at the site of injection and at the level of the draining LN.

First, we examined the BP injection site. In agreement with previously published data (Iannacone et al., 2010; Norton et al., 2011), footpad injection of CLD alone induced a local inflammatory infiltrate composed mostly of Gr-1$^+$ neutrophils and inflammatory monocytes (Figure S2A). Elimination of this infiltrate by systemic anti-Gr-1 treatment (Figures S2A and S2B), however, did not prevent CLD from increasing nAb titers upon VSV infection (Figure 2C), indicating that CLD adjuvant activity does not require Gr-1$^+$ leukocytes.

Next, we considered the role of two additional footpad-resident cell types: γδ T cells and conventional dendritic cells (DCs). γδ T cells can provide help to B cells (Pao et al., 1996; Wen et al., 1996) and are reportedly activated by CLD (Schilbach et al., 2001) or ALD (Thompson et al., 2010). Footpad CLD or ALD injection did not increase the local number of γδ T cells (Figure S2C), and VSV infection of TCRδ$^{-/-}$ mice (Itohara et al., 1993) revealed that CLD and ALD adjuvant activity is maintained in the absence of these cells (Figures 2D and S2D). We then reasoned that BPs might act on DCs, because DCs can present Ag to B cells (Qi et al., 2006) and most known adjuvants are thought to function by activating DCs (Coffman et al., 2010). Incubating DCs with CLD in vitro did not alter expression of activation markers, such as CD86 and CD40 (Figures S3A and S3B), and the systemic elimination of these cells in vivo (Figure S3C) did not significantly inhibit CLD adjuvant activity. Indeed, CLD administration in DC-depleted mice challenged with VSV resulted in nAb titers that, by day 7 postinfection (p.i.), were comparable to those observed in DC-competent animals subjected to the same procedures (Figure 2E). It is worth noting that, independently of CLD treatment, DC depletion caused a transient reduction in nAb titers at day 4 p.i., consistent with a role of DC in early B cell activation (Qi et al., 2006; Scandella et al., 2007). Overall, these data indicate that CLD adjuvant activity does not require conventional DCs.

We next analyzed the draining LN. The only quantitative change in LN cellular composition detectable upon CLD treatment was the depletion of CD169$^+$ macrophages lining the subcapsular and medullary sinuses (Figures S4A and S4B). We found it surprising that BPs increase Ab responses despite depleting subcapsular sinus macrophages, because these cells were recently identified as critical Ag-presenting cells for B cell responses (Carrasco and Batista, 2007; Junt et al., 2007; Phan et al., 2007). To clarify these seemingly contradictory observations, we sought alternative strategies to deplete LN macrophages independently of BP administration. These strategies (footpad dextran sulfate or carrageenan injections in wild-type [WT] mice or footpad diphtheria toxin injection in CD11c-DTR mice) effectively depleted LN macrophages (Figure S4C; Iannacone et al., 2010), but they failed to increase nAb titers upon VSV infection (Figures 2F and S4D). This indicates that LN macrophage depletion per se does not increase humoral immune responses and suggests that CLD adjuvant activity does not rely on LN macrophages.

To investigate the molecular basis for CLD adjuvant effect, we next performed genome-wide mRNA profile analysis of draining LNs from mice treated with either PBS or CLD and sacrificed before (0 hr) or 8 hr after VSV infection (Figure S5; Table S1). This analysis showed that a relatively small number of genes were regulated by CLD treatment (Figures 3A, S6, S7, S8, S9, and S10; Table S1). Most of the downregulated genes upon CLD treatment were macrophage specific (Figures S6, S7, and S8; Table S1), in line with the observation that CLD treatment depleted LN macrophages (Figure S4). CLD treatment also downregulated type I interferon (IFN-I) genes induced upon VSV infection (Figures S7 and S8; Table S1). This is consistent with previously published data showing that LN subcapsular sinus macrophages are a major source of IFN-I during this infection and their depletion inhibits IFN-I gene expression (Iannacone et al., 2010). Reduced IFN-I gene expression could have also resulted from plasmacytoid DC (pDC) dysfunction, as these cells represent an additional source of IFN-I in this system and they are not depleted upon BP treatment (Iannacone et al., 2010). The CLD-dependent reduced expression of the pDC-specific marker Siglec-H (Blasius et al., 2006) is also suggestive of pDC dysfunction (Figures S6 and S7; Table S1). It is noteworthy, however, that depletion of pDCs (which reduced IFN-I by ~50%; Iannacone et al., 2010) failed to increase Ab responses (Figure S11A), suggesting that CLD adjuvant activity does not rely on pDCs. We also noted that some natural killer (NK) cell-specific transcripts were reduced upon CLD treatment (Figures S6 and S7; Table S1). Depleting these cells did not alter the capacity of CLD to increase nAb responses upon VSV infection (Figure S11B), arguing against a role for NK cells in the adjuvant activity of BPs.

Next, we examined genes that were upregulated by CLD treatment. CLD induced several neutrophil-specific transcripts (Figures 3A, S9, and S10; Table S1), in keeping with the ability of BPs to recruit and activate these cells (Norton et al., 2011; 2012). Data previously described in Figure 2C, however, ruled out a role for neutrophils in BP adjuvant activity. Of note, CLD upregulated a number of B cell-specific transcripts and gene sets associated with B cell function (Figures 3A, S9,

(F) VSV nAb titers in the serum of C57BL/6 (WT) or caspase-1$^{-/-}$ mice that were injected in the footpad with PBS or CLD immediately prior to VSV infection in the same footpad. n = 10 per group; black asterisks, WT+PBS versus WT+CLD; red asterisks, caspase-1$^{-/-}$+PBS versus caspase-1$^{-/-}$+CLD; results are representative of three independent experiments.

(G) Ovalbumin (OVA)-specific IgG titers in the serum of C57BL/6 mice that were immunized orally with OVA alone (vehicle), OVA + CLD (CLD), OVA + cholera toxin (CT), or OVA + cholera toxin + CLD (CT + CLD). n = 5 per group; results are representative of two independent experiments.

(H) OVA-specific IgA titers in the feces of the mice described in (G).

(I) Total IgG in the sera of patients immediately prior to or at the indicated time points after a single intravenous infusion of zoledronate (ZLD) is shown. n = 11; see Table S2 for patient characteristics.

(J) Total IgG in the sera of patients immediately prior to or at the indicated time points after a single intravenous infusion of neridronate (NRD). n = 9; see Table S2 for patient characteristics.

Results are expressed as mean ± SEM. ***p < 0.001; **p < 0.01; *p < 0.05. D.L., detection limit. See also Figures S5, S6, S7, S8, S9, S10, and S11.

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and S10; Table S1). When compared to controls, these B cell-related transcripts were higher in CLD-treated animals before (Figure S9) and after (Figure S10) VSV infection, suggesting that CLD might directly impact B cells. To directly test this possibility, we isolated B cells from VI10Yen mice (which express a VSV-specific B cell receptor), and treated them with PBS or CLD in vitro prior to adoptive transfer into D_HLMP2A mice (which are devoid of surface-expressed and secreted Abs). In this experimental setup - where Abs can be produced only by the transferred B cells—VSV infection led to nAb titers that were higher in mice that received CLD-treated, rather than PBS-treated, VI10Yen B cells (Figure 3B); this correlated with the capacity of CLD to promote Ag-specific B cell expansion both in vivo and in vitro (Figures 3C and 3D). Taken together, these results indicate that CLD directly targets B cells to enhance their expansion and their Ab production upon Ag encounter. Although the molecular pathways by which CLD and other BPs impact B cell function remain to be determined, it is worth noting that CLD adjuvanticity did not involve the two known signaling pathways through which most other adjuvants are thought to act (Coffman et al., 2010) (the Toll-like receptors and the inflammasome pathway, which rely on Myd88/TRIF and caspase-1 for signaling, respectively). Indeed, VSV nAb titers in CLD-treated Myd88$^{-/-}$/TRIF$^{-/-}$ and caspase-1$^{-/-}$ mice were indistinguishable from those of similarly treated WT mice (Figures 3E and 3F).

Since BPs are often taken orally by patients, we next sought to evaluate whether these small molecules act as adjuvants upon oral immunization in mice. As expected (Pierre et al., 1992), the oral administration of OVA alone failed to induce a detectable OVA-specific Ab response (Figures 3G and 3H). By contrast, the concomitant oral administration of OVA and CLD induced OVA-specific serum IgG and fecal immunoglobulin A (IgA) responses (Figures 3G and 3H). Furthermore, CLD was able to increase the OVA-specific Ab response afforded when OVA was administered together with the mucosal adjuvant cholera toxin (Pierre et al., 1992) (Figures 3G and 3H). These data indicate that CLD is an effective oral adjuvant capable of increasing both systemic and mucosal Ab responses.

Finally, we tested whether the nitrogen-containing BPs ZLD and NRD increase Ab responses in a cohort of patients affected by osteoporosis or Paget disease of bone (Table S2). Notably, a transient but significant increase in total serum IgG levels was detected for up to 3 months upon a single intravenous infusion of BPs (Figures 3I and 3J). Although the impact of BPs on Ag-specific Ab responses remains to be determined, this observation suggests that these drugs enhance B cell responses in humans.

In conclusion, these data establish BPs as adjuvants that target B cells to increase humoral immune responses. Translational evaluation of these compounds in humans should be relatively straightforward, because BPs, unlike all other adjuvants, are already widely used in the clinic as stand-alone drugs with excellent safety profiles. Thus, BPs could be readily combined with both existing and newly developed vaccines, especially in settings where immune responses to a vaccine alone are weak or where Ags are in short supply.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and Balb/c mice were purchased from Charles River or The Jackson Laboratory. CD11c- GFP-DTR [B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J] mice were purchased from The Jackson Laboratory. MHC-II$^{-/-}$ (B6.129-H2-Ab1^{tm1Doi}/DoiOrl) mice were provided by P. Dellabona (San Raffaele Scientific Institute). TCR$\delta^{-/-}$ (B6.129P2-Tcrd^{tm1Mom}/J) mice were provided by W. Havran (The Scripps Research Institute). MyD88$^{-/-}$/Trif$^{Lps2/Lps2}$ mice were provided by B. Beutler (The Scripps Research Institute). Caspase-1$^{-/-}$ (B6.129S2-Casp1^{tm1sesh}) and CD40L$^{-/-}$ (B6.129S2-*Cd40^{tm1Imx}*/J) mice were obtained through the Swiss Immunological Mutant Mouse Repository (Zurich, Switzerland). D_HLMP2A mice (Casola et al., 2004) were originally provided by K. Rajewsky (Harvard Medical School) and bred five generations against C57BL/6 mice. VI10Yen mice (Hangartner et al., 2003) were originally provided by R. M. Zinkernagel and H. Hengartner. Bone marrow chimeras were generated by irradiation of C57BL/6 mice with 1,300 rad in split doses and reconstitution with CD11c-DTR-GFP bone marrow; mice were allowed to reconstitute for at least 8 weeks prior to use. Mice were housed under specific pathogen-free conditions and used at 6–8 weeks of age. All experimental animal procedures were approved by the Institutional Animal Committees of San Raffaele Scientific Institute, Harvard Medical School, and The Scripps Research Institute.

Bisphosphonate Treatment

CLD, ALD, PMD, and ETD were obtained from Sigma, dissolved in PBS, and injected in the footpad in a volume of 20 μl. The injected amount for each bisphosphonate was 2 mg, unless otherwise indicated. In the experiments described in Figure 1A, 30 μl of clodronate liposomes (CLD-Lip) or PBS liposomes (PBS-Lip, both provided by N. Van Rooijen) were injected in the footpad 7 days before antigen administration.

Infections and Immunizations

Mice were infected with 10^4 plaque-forming units (pfu) of VSV serotype Indiana (VSV-IND), 10^4 pfu of VSV serotype New Jersey (VSV-NJ), or 10^6 pfu of VSVeGFP (Iannacone et al., 2010). Alternatively, mice were immunized with 10^6 pfu equivalent of PFA-inactivated VSV (Bachmann et al., 1993). Viruses were dissolved in 20 μl of PBS and injected into the footpad. In survival experiments, mice were challenged with 3.5×10^8 pfu of VSV in the left footpad 3 weeks after PFA-VSV immunization in the right footpad. Other antigens used for footpad immunization experiments included 4-hydroxy-3-nitrophenyl-chicken gamma globulin (NP-CGG, 50 μg/dose; Biosearch Technologies), ovalbumin (OVA, 100 μg/dose; Sigma), and Engerix-B (20 μl/dose given twice 2 weeks apart; GlaxoSmithKline). Hemoagglutinin/neuroaminidase subunits from the human influenza virus A/NewCaledonia/20/99 (H_1N_1, 3 μg/dose; provided by P. Dellabona) was injected in the anterior tibialis muscle.

For experiments involving oral immunization, mice were given vehicle or 10 mg of CLD 24 hr prior to receiving 1 mg of OVA with or without 10 μg of cholera toxin. All treatments were administered via gavage in 0.2 ml of antacid buffer (3% sodium bicarbonate) on days 0, 7, and 21. For the measurement of mucosal IgA, fresh fecal pellets were collected and immediately frozen at −20°C. Before analysis, fecal pellets were weighed and dissolved in protease inhibitor solution at a concentration of 200 μg/ml, as described previously (Lauterslager et al., 2001).

All infectious work was performed in designated BL-2 workspaces in accordance with institutional guidelines. Mice were retro-orbitally bled at the indicated time points for Ag-specific Abs and measured by endpoint ELISA (Galli et al., 2007; Tonti et al., 2012), VSV neutralization assay (Iannacone et al., 2010), or with an HBsAb detection kit (Diagnostic Bioprobes) in accordance with the manufacturer's instructions. For determination of VSV-neutralizing IgG titers, sera were incubated with equal volumes of 0.1 M 2-mercaptoethanol in PBS for 1 hr at room temperature before dilution (Scott and Gershon, 1970).

Confocal Microscopy

Confocal microscopy analysis of popliteal LNs was performed as described previously (Moseman et al., 2012). Sections were stained with eFluor450-conjugated anti-B220 (RA3-6B2, eBioscience), FITC-conjugated anti-CD169

(3D6.112, AbD Serotec), and Alexa Fluor 647-conjugated anti-TCRβ (H57-597, BioLegend).

In Vivo Depletion of Neutrophils, DCs, LN Macrophages, pDCs, and NK Cells

Neutrophils were depleted by intraperitoneal injection of 100 μg of Gr-1 Abs (RB6-8C5, BioXCell) every 2 days, beginning 3 days prior to immunization.

DCs were depleted from CD11c-GFP-DTR → C57BL/6 bone marrow chimeric mice by injecting diphtheria toxin (DT, Sigma) in the footpad (50 ng) and in the peritoneum (500 ng) every 2 days, starting the day prior to immunization.

Popliteal LN macrophages were depleted by injecting 2 mg of carrageenan (Sigma) in the footpad every 2 days, starting 5 days prior to immunization. Alternatively, LN macrophages were depleted by footpad injection of 1 mg of dextran sulfate (Sigma) 5 days prior to immunization. Serum from CD11c-DTR-GFP mice (described in Figure S3 in Iannacone et al., 2010), where LN macrophages are depleted by a single footpad injection of DT (4 ng) 6 days before VSV infection, were assessed for VSV neutralizing Ab titers 4 days after VSV infection.

Serum from the mice described in Figures 3J and S8b of Iannacone et al. (2010) (where pDCs were depleted by intravenous injection of 500 μg of PDCA-1 depleting Ab [JF05-1C2.4.1, Miltenyi Biotec] 24 hr prior to VSV infection) were assessed for VSV neutralizing Ab titers 7 days after VSV infection.

NK cells were depleted by intravenous injection of Asialo-GM1 Abs (Cedarlane) every 2 days beginning 3 days prior to VSV infection, as described previously (Tonti et al., 2012).

All depletions were confirmed by flow cytometry and/or confocal microscopy.

Tissue Digestion and Flow Cytometry

Single-cell suspensions of LNs, spleens, and footpads were generated as described in Iannacone et al. (2010). All flow cytometry analyses were performed in fluorescence-activated cell sorting buffer containing PBS with 2 mM EDTA and 2% FBS on a FACS CANTO (BD Pharmingen) and analyzed with FlowJo software (Tree Star).

Abs used included FITC-conjugated anti-CD169 (3D6.112, AbD Serotec), Alexa Fluor 488-conjugated and PE-Cy7-conjugated anti-B220 (RA3-6B2, BioLegend), PE-conjugated anti-TCRδ (GL3, BioLegend), and anti-CD86 (GL-1, BioLegend), PE-Cy7-conjugated anti-CD45 (30-F11, BioLegend), PB-conjugated anti-Ly6C (HK1.4, BioLegend), APC-Cy7-conjugated anti-F4/80 (BM8, BioLegend), Alexa Fluor 647-conjugated anti-CD8 (53-6.7, BioLegend), biotinylated anti-CD11b (M1/70, BioLegend), PE-conjugated anti-CD138 (281-2, BioLegend), PE-Cy7-conjugated anti-CD3 (145-2C11, BioLegend), PE-Cy5-conjugated anti-NK1.1 (PK136, BioLegend), PerCP-Cy5.5-conjugated anti-Gr1 (RB6-8C5), APC-conjugated anti-CD40 (HM40-3, BD Pharmingen), Alexa Fluor 488-conjugated anti-igG (A-11023, Invitrogen), PE-Cy7-conjugated anti-CD11c (N418, eBioscience), eFluor450-conjugated anti-CD4 (RM4-5, eBioscience). The idiotypic Ab 35.61 for detection of the Vl10 BCR in Vl10Yen mice (Hangartner et al., 2003) was produced from hybridoma supernatants in accordance with standard methods.

Isolation and Activation of DCs and B Cells

Splenic DCs were isolated from C57BL/6 mice that were injected subcutaneously 11–14 days earlier with 4×10^6 Flt3-ligand-secreting B16 tumor cells, as described previously (Cavanagh et al., 2005). $CD11c^+$ DCs were purified by positive selection with anti-CD11c microbeads (>95% $CD11c^+$, Miltenyi) and cultured in the presence of 1 μg/ml LPS (*E. coli* 0.26:B6, Sigma) or with the indicated concentrations of CLD for 48 hr prior to flow cytometry analysis.

Naive B cells from spleens of Vl10Yen mice were negatively selected by magnetic isolation with CD43 beads (Miltenyi) as described previously (Junt et al., 2007). The purity was 98% as determined by CD19 surface staining. B cells (10^6 cells/ml) were cultured in RPMI 1640 media (Lonza) supplemented with 10% fetal bovine serum, 50 mM 2-ME (Sigma), 10 mM HEPES (Lonza), 2 mM L-glutamine (Lonza), and 100 U/ml penicillin/streptomycin with or without 10 nM CLD. After 4 hr, Vl10Yen B cells were harvested, washed three times, and either intravenously injected into D_HLMP2A recipients (10^7 cells/mouse) 24 hr prior to footpad VSV infection (Figures 3B and 3C) or cultured for 48 hr in the presence of VSV at a multiplicity of infection of 1 (Figure 3D).

Gene Expression Profiling

Whole lymph nodes were lysed in 600 μl of QIAzol (QIAGEN) reagent using the TissueLyser II. Total RNA was extracted following the miRNeasy kit's procedure (QIAGEN), and sample quality was tested on a 2100 Bioanalyzer (Agilent). RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real time quantitative PCR reactions were performed on the LightCycler 480 system (Roche) with FastStart Universal SYBR Green Master Mix (Roche). Every reaction was run in triplicate and GAPDH levels were used as an endogenous control for normalization. For oligonucleotide microarray hybridization, 1 μg of RNA were labeled, fragmented, and hybridized to an Affymetrix Mouse Genome 430A 2.0 Array. After scanning, the expression value for each gene was calculated with RMA (Robust Multi-Array) normalization using R. The average intensity difference values were normalized across the sample set. Probe sets that were absent in all samples according to Affymetrix flags were removed. All values below 40 were floored to 40. Only probe sets that changed in two biological duplicates by 2-fold or more were analyzed further in this study: (1) regulated genes were hierarchically clustered using the software Gene-E (http://www.broadinstitute.org/cancer/software/GENE-E/), and (2) functional enrichment of GO terms (BP, biological processes; CC, cellular components) and KEGG pathway enrichment analysis were performed using DAVID (http://david.abcc.ncifcrf.gov) (Huang et al., 2009).

Patients

Total serum IgG levels were assessed in 20 patients affected by osteoporosis or Paget disease of bone (age range 52–78 years; Table S2) that received a single intravenous injection of 5 mg zoledronate (Aclasta, Novartis Pharmaceuticals) or 200 mg neridronate (Nerixia, Abiogen Pharma). Serum samples were obtained before and 1, 30, 90, 180, and 360 days after BP infusion. IgG levels were quantified from stored samples by an immunodiffusion technique using NOR Partigen immunoplates purchased from Siemens (Siemens Healthcare Diagnostics). All experiments involving human subjects were approved by the institutional review board of the University of Siena and performed after obtaining informed consent from all subjects.

Statistical Analyses

Results are expressed as mean ± SEM. All statistical analyses were performed in Prism (GraphPad Software). Means between two groups were compared with a two-tailed t test. Means among three or more groups were compared with one-way or two-way analysis of variance with Bonferroni's post hoc test. Kaplan-Meier survival curves were compared with the log-rank (Mantel-Cox) test.

ACCESSION NUMBERS

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE50403.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eleven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.09.004.

AUTHOR CONTRIBUTIONS

E.T., L.G.G., U.H.v.A., and M.I. designed the study; E.T., N.J.d.O, G.G., E.A.M., P.D.L., A.A., S.S., M.D.G., L.S., and M.I. performed experiments; E.T. and M.I. analyzed the data; N.C. performed and analyzed the microarray data; L.G. performed measurements in human subjects; G.S. gave conceptual advice; and E.T., L.G.G., U.H.v.A. and M.I. wrote the manuscript.

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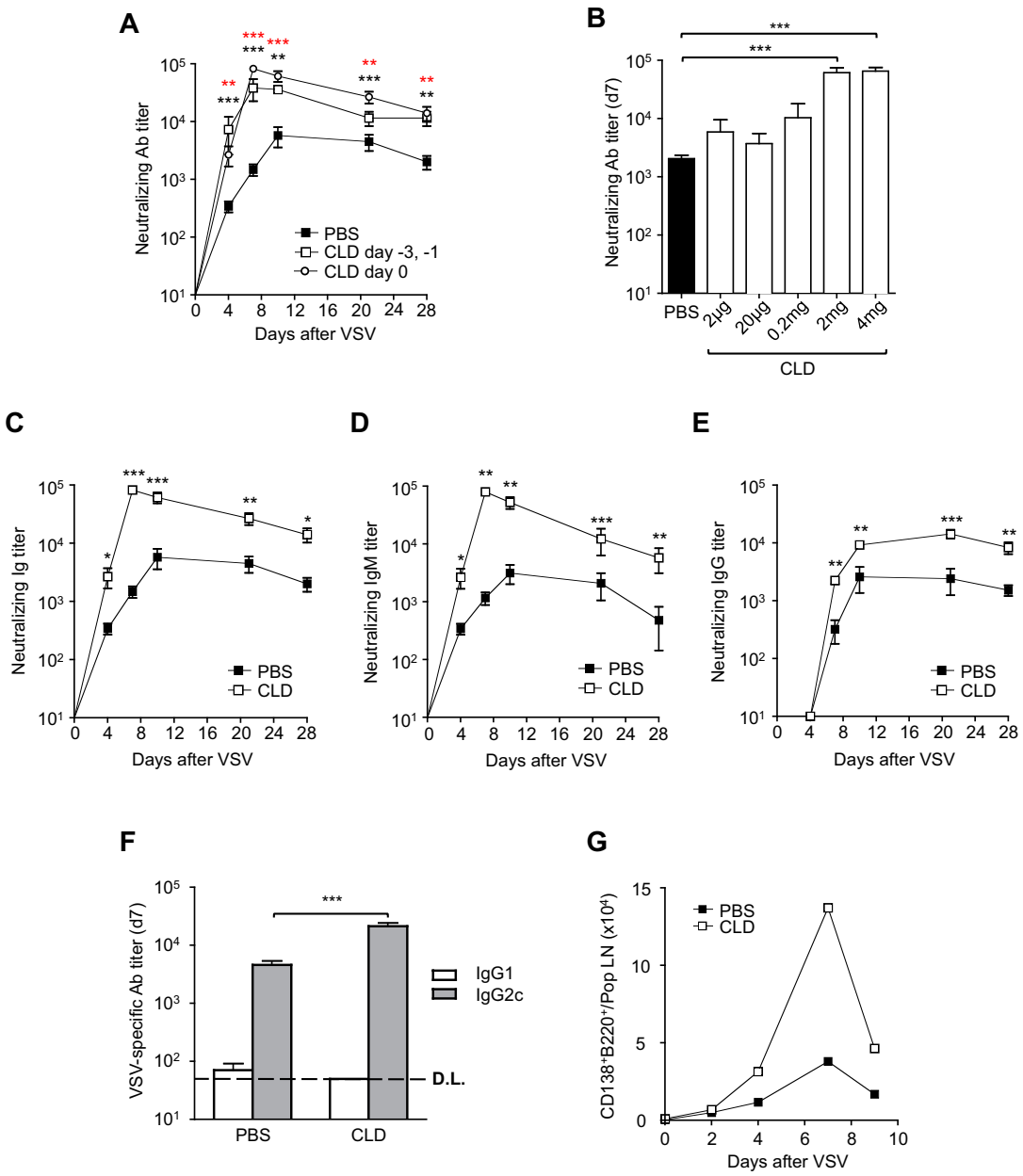

Figure S1. Bisphosphonates Increase Antibody Responses to Live and Inactive Viruses, Proteins, Haptens and Existing Commercial Vaccine Formulations, Related to Figure 1

(A) VSV nAb titers in the serum of C57BL/6 mice that were footpad injected with CLD 3 days and 1 day (day −3, −1) or immediately (day 0) prior to VSV infection in the same footpad. n = 5 per group. Black asterisks, PBS versus CLD day −3, −1; red asterisks PBS versus CLD day 0; results are representative of 3 independent experiments.

(B) VSV nAb titers 7 days p.i. in the serum of C57BL/6 mice that were footpad injected with PBS or with the indicated doses of CLD prior to VSV infection in the same footpad. n = 5 per group; results are representative of 2 independent experiments.

(C) VSV nAb titers in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to infection in the same footpad with VSV. n = 5 per group; results are representative of 3 independent experiments.

(D) Neutralizing IgM titers in the serum of the same mice described in (a).

(E) Neutralizing IgG titers of the same mice described in (a).

(F) VSV-specific IgG1 and IgG2c titers 7 day p.i. in the serum of C57BL/6 mice that were footpad injected with PBS or CLD prior to VSV infection in the same footpad. n = 5 per group; results are representative of 2 independent experiments.

(G) Total number of CD138$^+$ cells recovered from draining popliteal lymph nodes (Pop LN) that were pooled from 3 C57BL/6 mice footpad injected with PBS or CLD prior to VSV infection in the same footpad. Results are representative of 2 independent experiments.

Results are expressed as mean ± SEM. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$; D.L.: detection limit.

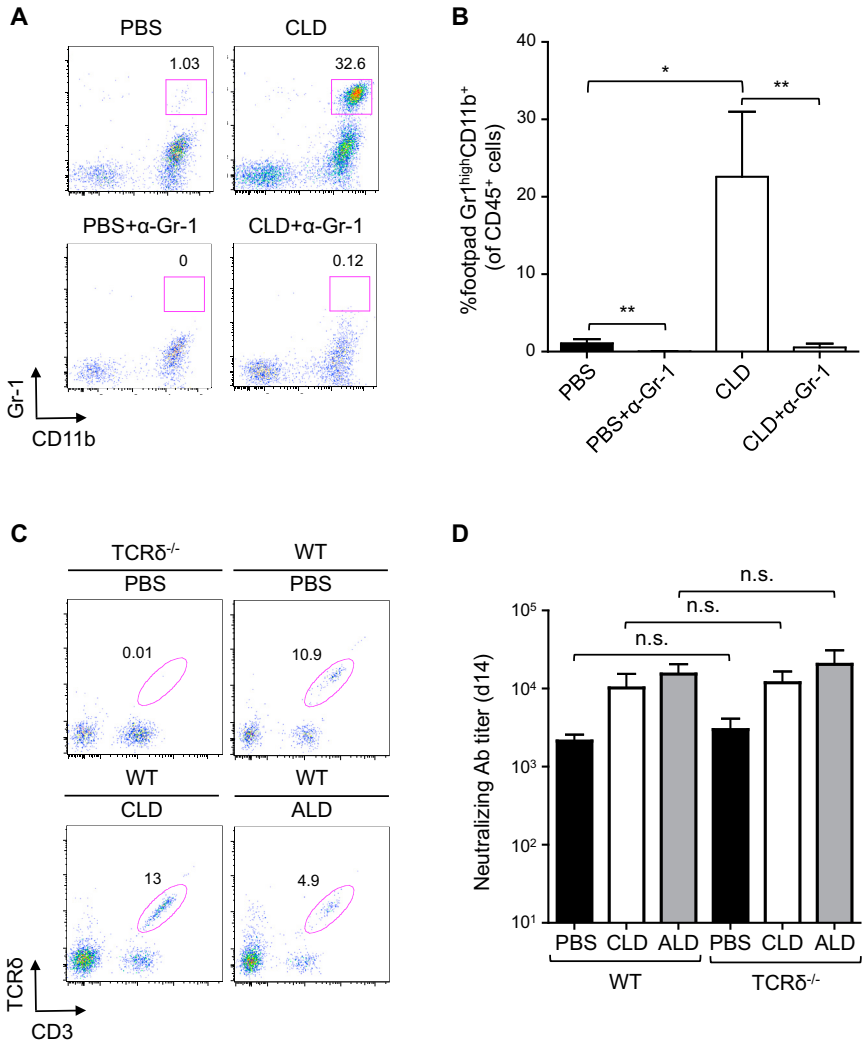

Figure S2. Neutrophils and γδ T Cells Are Not Required for Bisphosphonate Adjuvant Activity, Related to Figures 2C and 2D

(A and B) Representative FACS plots (A) and quantification (B) of the number of Gr-1^{high}CD11b^{+} cells present in digested footpads from the mice described in Figure 2C. Numbers show the percentages of CD45^{+} cells within each gate. n = 4 per group; results are representative of 2 independent experiments.

(C) Representative FACS plots of digested footpads from PBS-injected TCRδ$^{-/-}$ mice or PBS-, CLD- or alendronate (ALD)-injected C57BL/6 (WT) mice. Numbers show the percentages of CD45^{+} cells within each gate. Results are representative of 2 independent experiments (n = 3 per experiment).

(D) VSV nAb titers 14 days p.i. in the serum of C57BL/6 (WT) or TCRδ$^{-/-}$ mice that were footpad injected with PBS, CLD or ALD prior to VSV infection in the same footpad. n = 5 per group; results are representative of 3 independent experiments.

**: $p < 0.01$; *: $p < 0.05$; n.s.: non statistically significant.

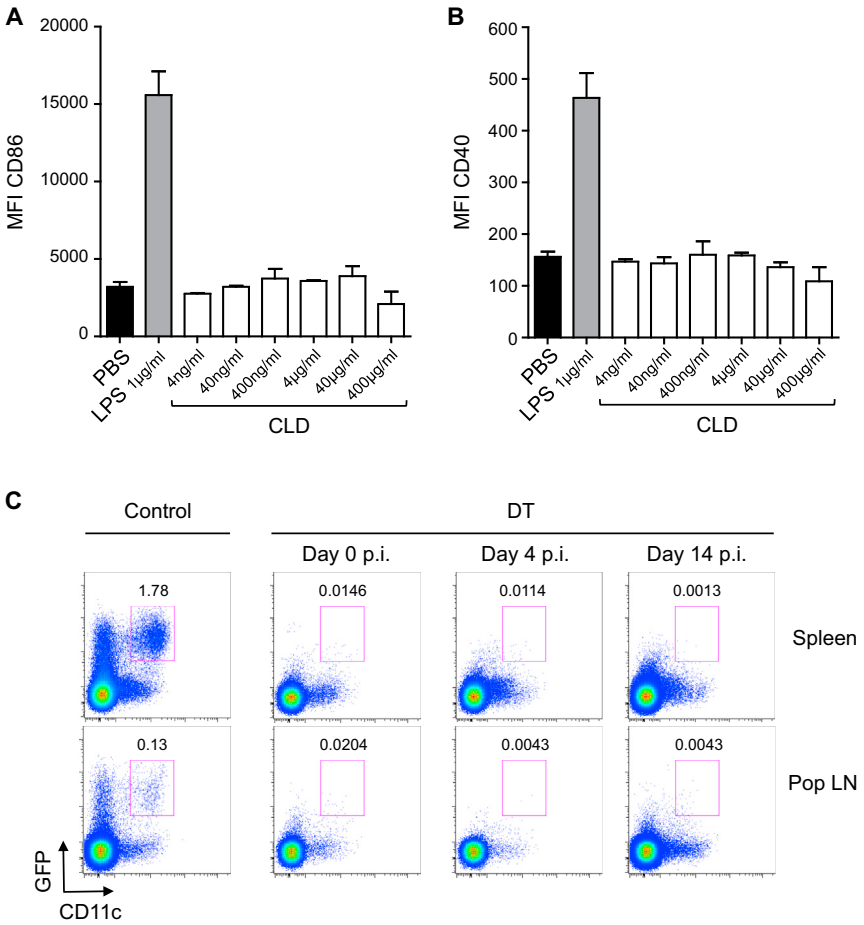

Figure S3. Bisphosphonate Adjuvant Activity Does Not Require Conventional Dendritic Cells, Related to Figure 2E

(A and B) CD86 (A) or CD40 (B) expression on purified CD11c$^+$ DC that were cultured in the presence of 1 μg/ml LPS or with the indicated concentrations of CLD for 48 hr prior to flow cytometry analysis.

(C) Representative FACS plots of digested spleens or popliteal lymph nodes (Pop LN) from CD11c-GFP-DTR → C57BL/6 bone marrow chimeric mice that were left untreated (control) or injected with diphtheria toxin (DT). Numbers show the percentage of cells within each gate. Results are representative of 2 independent experiments (n = 3 per experiment). Results are expressed as mean ± SEM.

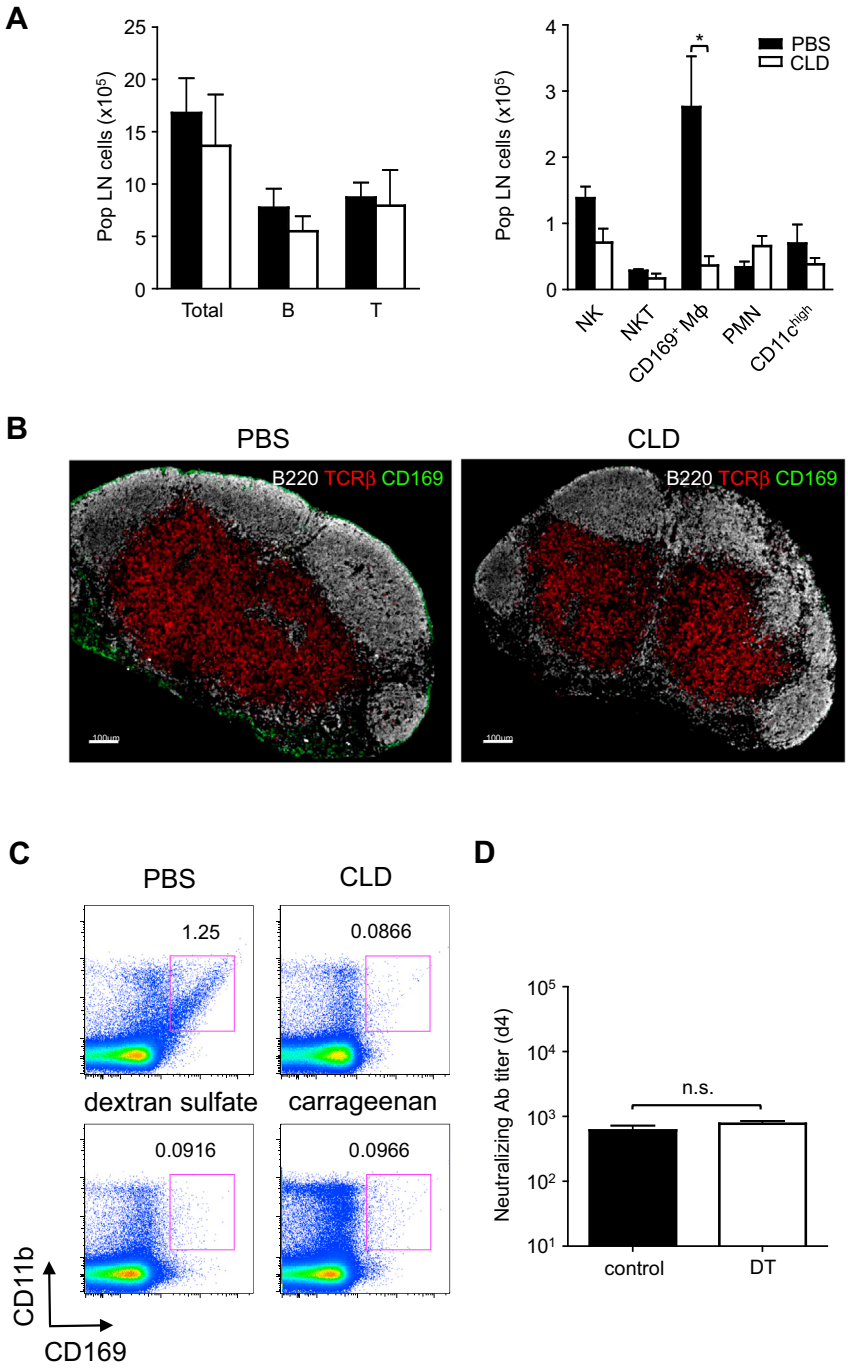

Figure S4. Lymph Node Macrophage Depletion Per Se Does Not Increase Humoral Immune Responses, Related to Figure 2F

(A) Cellular subset frequency in popliteal lymph nodes (Pop LN) from C57BL/6 mice that were treated with PBS or CLD. n = 3 per group; results are representative of 3 independent experiments.

(B) Representative confocal micrographs of Pop LN from C57BL/6 mice that were treated with PBS or CLD. Sections were stained with anti-B220 (gray), anti-TCRβ (red) and anti-CD169 (green) to identify B cells, T cells and macrophages, respectively. Scale bars represent 100 μm. Results are representative of 2 independent experiments (n = 3 per experiment).

(C) Representative FACS plots of digested popliteal lymph nodes from C57BL/6 mice that were treated with PBS, CLD, dextran sulfate or carrageenan. Numbers show the percentage of cells within each gate. Results are representative of 2 independent experiments (n = 3 per experiment).

(D) VSV nAb titers 4 days p.i. in the serum of CD11c-GFP-DTR mice that were left untreated (control) or were depleted of $CD169^+$ LN macrophages through a single footpad injection of diphtheria toxin (DT) 6 days prior to VSV infection. These mice were described in Figure S3 from ref. (Iannacone et al., 2010). Results are expressed as mean ± SEM. *: $p < 0.05$; n.s.: non statistically significant.

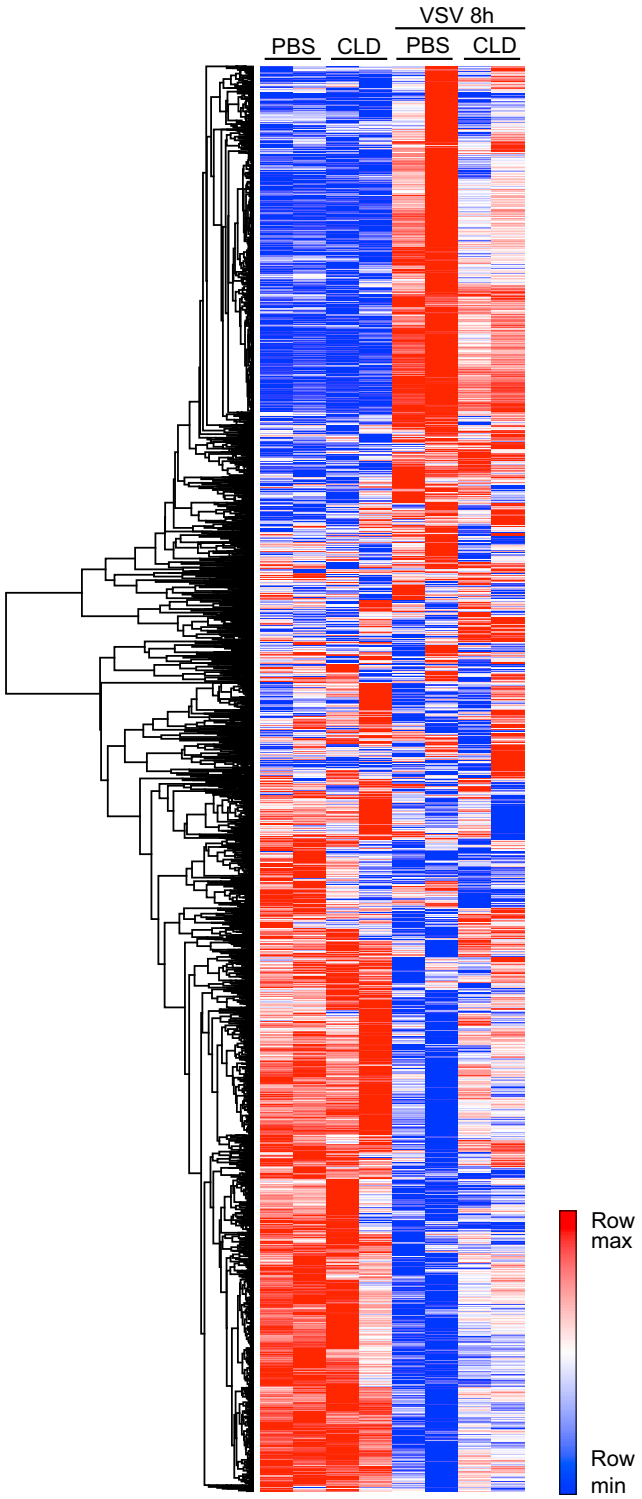

Figure S5. mRNA Expression Profiles of Differentially Expressed Genes in Whole Lymph Node, Related to Figure 3A

Shown are expression profiles for 4761 differentially expressed genes (rows) at 0 and 8 hr after VSV infection in mice treated with CLD or PBS as control (columns). Shown are genes with at least a 2-fold change in expression between treatments in both duplicate arrays (each column corresponds to a replicate). Values from multiple probe sets targeting the same gene were collapsed and gene-expression profiles were hierarchically clustered. Results are expressed as mean ± SEM.

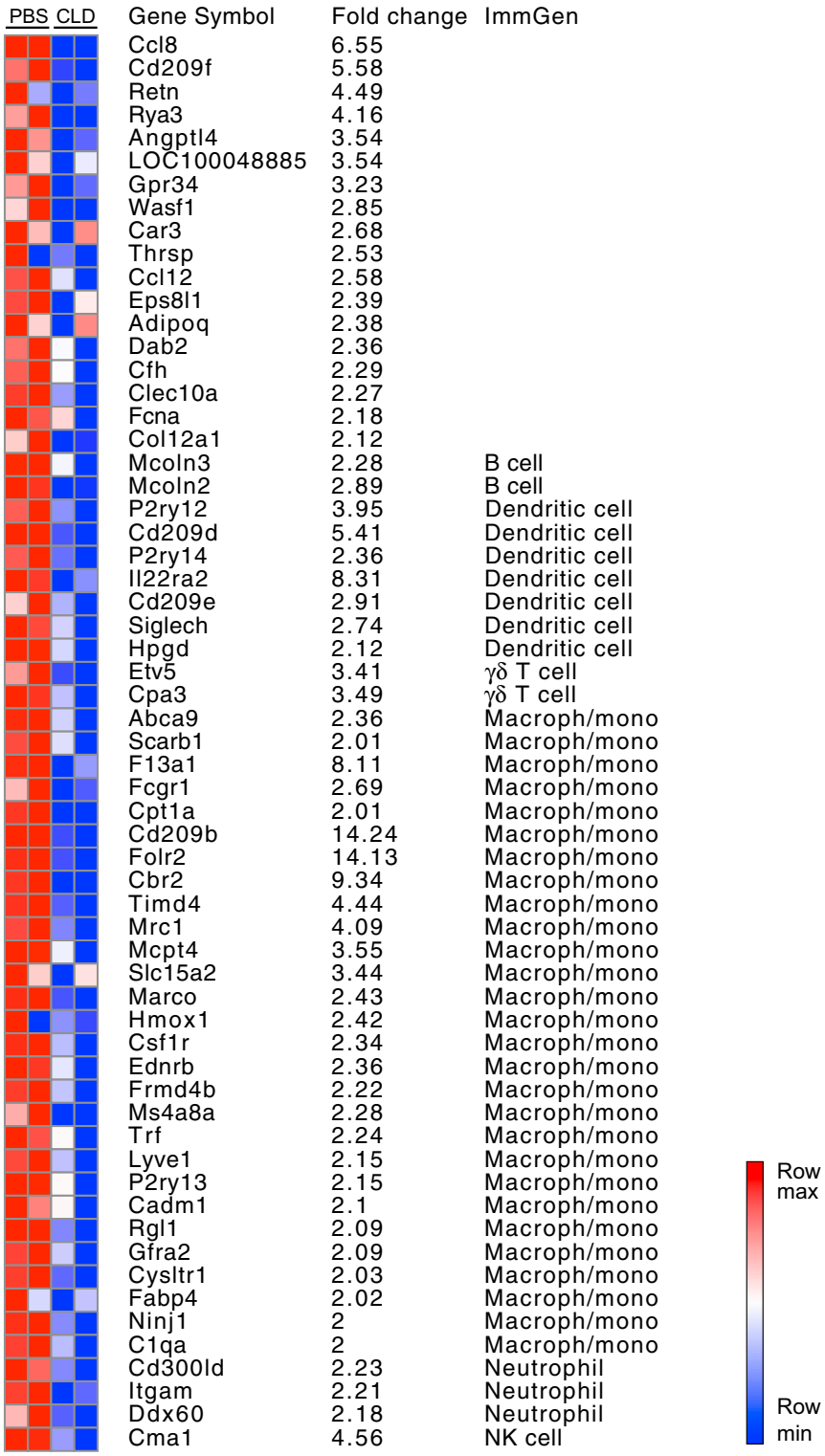

Figure S6. mRNA Expression Profiles of Genes Decreased upon CLD Treatment in Whole Lymph Node before Infection, Related to Figure 3A

Shown are expression profiles for 62 differentially expressed genes (rows) with at least a 2-fold decrease in expression in CLD-treated mice compared to controls (PBS). The right-most column indicates the cell types in which each gene is specifically expressed based on the Immunological Genome (ImmGen) database. Results are expressed as mean ± SEM.

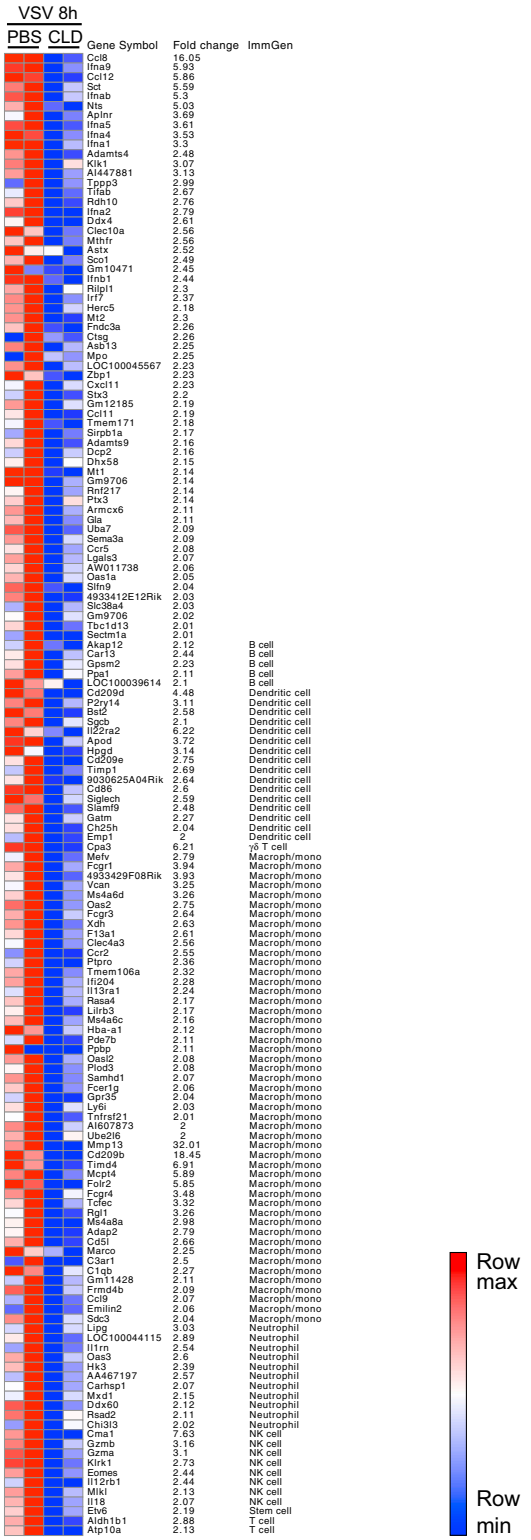

Figure S7. mRNA Expression Profiles of Genes Decreased upon CLD Treatment in Whole Lymph Node after Infection, Related to Figure 3A

Shown are expression profiles for 155 differentially expressed genes (rows) with at least a 2-fold decrease in expression in CLD-treated mice compared to controls (PBS) at 8 hr after VSV infection. The right-most column indicates the cell types in which each gene is specifically expressed based on the Immunological Genome (ImmGen) database. Results are expressed as mean ± SEM.

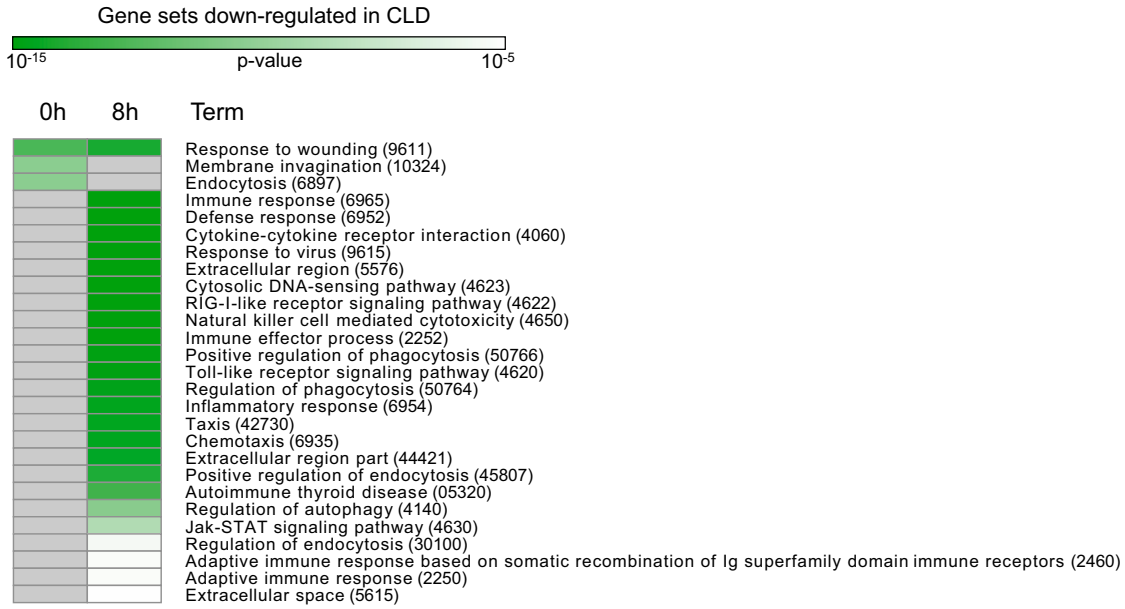

Figure S8. Gene Enrichment Analysis of CLD-Dependent Genes from Microarray Measurements of Whole Lymph Node, Related to Figure 3A

Shown are the Gene Ontology (GO) terms and canonical pathways (KEGG database) (rows) statistically enriched among the 217 genes with at least a 2-fold decrease in expression at 0 and 8 hr (columns) after VSV infection in CLD-treated mice compared to control (PBS). Enriched GO term categories include BP: biological process, and CC: cellular component. Green: p value < 10^{-15}; white: p value < 10^{-4}; gray: no enrichment. Results are expressed as mean ± SEM.

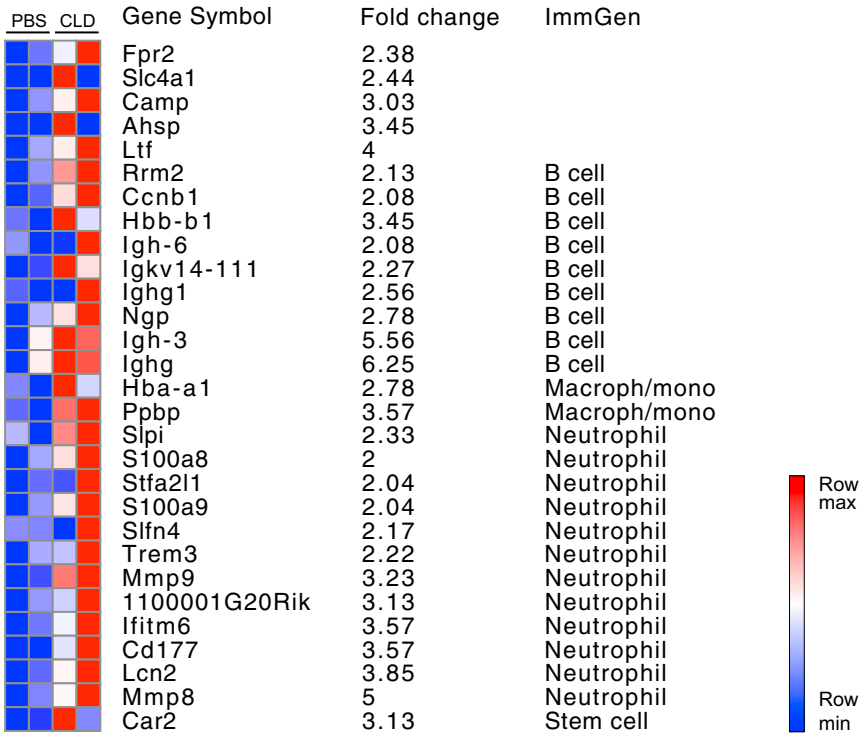

Figure S9. mRNA Expression Profiles of Genes Increased upon CLD Treatment in Whole Lymph Node before Infection, Related to Figure 3A

Shown are expression profiles for 29 differentially expressed genes (rows) with at least a 2-fold increase in expression in CLD-treated mice compared to controls (PBS). The right-most column indicates the cell types in which each gene is specifically expressed based on the Immunological Genome (ImmGen) database. Results are expressed as mean ± SEM.

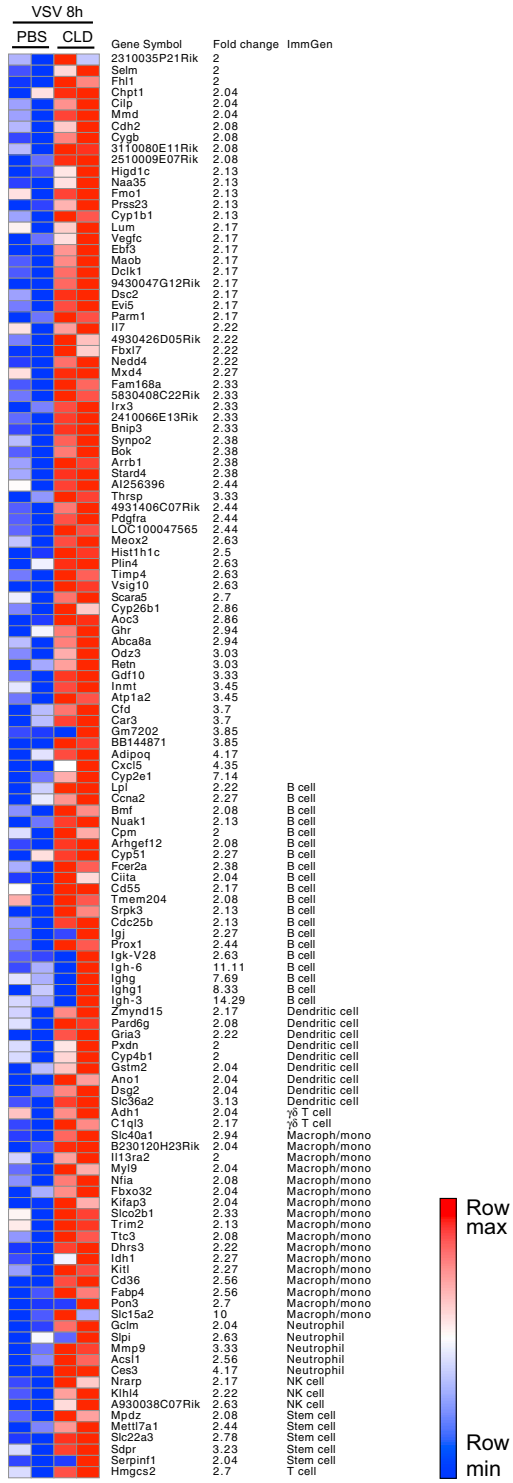

Figure S10. mRNA Expression Profiles of Genes Increased upon CLD Treatment in Whole Lymph Node after Infection, Related to Figure 3A

Shown are expression profiles for 127 differentially expressed genes (rows) with at least a 2-fold increase in expression in CLD-treated mice compared to controls (PBS) at 8 hr after VSV infection. The right-most column indicates the cell types in which each gene is specifically expressed based on the Immunological Genome (ImmGen) database. Results are expressed as mean ± SEM.

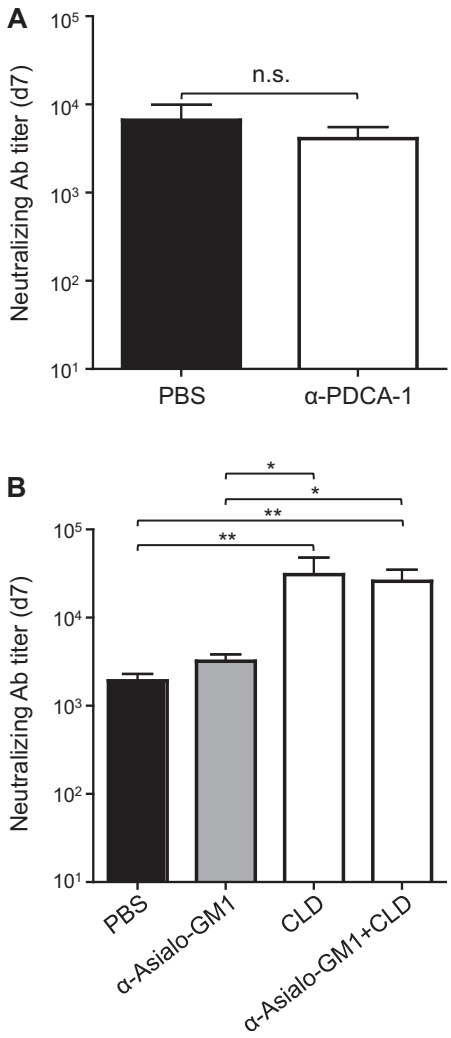

Figure S11. CLD Increases Ab Responses Independently of pDCs or NK Cells, Related to Figure 3A

(A) VSV nAb titers 7 days p.i. in the serum of C57BL/6 mice that received PBS or were depleted of pDCs via anti-PDCA-1 (α−PDCA1) injection 24 hr prior to VSV infection. These mice were described in Figure 3j and Suppl. Figure 8b from ref. (Iannacone et al., 2010).

(B) VSV nAb titers 7 days p.i. in the serum of C57BL/6 mice that were injected (or not) with anti-Asialo-GM1 depleting Ab (α−Asialo-GM1, see Methods for details) and were subsequently footpad injected with PBS or CLD immediately prior to VSV infection in the same footpad. n = 5; results are representative of 2 independent experiments.

Results are expressed as mean ± SEM. **: $p < 0.01$; *: $p < 0.05$; n.s.: non statistically significant.