

TLR9 and IL-1R1 Promote Mobilization of Pulmonary Dendritic Cells during Beryllium Sensitization

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Metal-induced hypersensitivity is driven by dendritic cells (DCs) that migrate from the site of exposure to the lymph nodes, up-regulate costimulatory molecules, and initiate metal-specific CD4⁺ T cell responses. Chronic beryllium disease (CBD), a life-threatening metal-induced hypersensitivity, is driven by beryllium-specific CD4⁺ Th1 cells that expand in the lung-draining lymph nodes (LDLNs) after beryllium exposure (sensitization phase) and are recruited back to the lung, where they orchestrate granulomatous lung disease (elicitation phase). To understand more about how beryllium exposures impact DC function during sensitization, we examined the early events in the lung and LDLNs after pulmonary exposure to different physiochemical forms of beryllium. Exposure to soluble or crystalline forms of beryllium induced alveolar macrophage death/release of IL-1 α and DNA, enhanced migration of CD80^{hi} DCs to the LDLNs, and sensitized HLA-DP2 transgenic mice after single low-dose exposures, whereas exposures to insoluble particulate forms of beryllium did not. IL-1 α and DNA released by alveolar macrophages up-regulated CD80 on immature BMDC via IL-1R1 and TLR9, respectively. Intrapulmonary exposure of mice to IL-1R and TLR9 agonists without beryllium was sufficient to drive accumulation of CD80^{hi} DCs in the LDLNs, whereas blocking both pathways prevented accumulation of CD80^{hi} DCs in the LDLNs of beryllium-exposed mice. Thus, in contrast to particulate forms of beryllium, which are poor sensitizers, soluble or crystalline forms of beryllium promote death of alveolar macrophages and their release of IL-1 α and DNA, which act as damage-associated molecular pattern molecules to enhance DC function during beryllium sensitization. *The Journal of Immunology*, 2018, 201: 2232–2243.

Metal hypersensitivity reactions are generated against a variety of metals. Clinical manifestations of metal sensitization vary depending on the metal and site of exposure, ranging from skin rashes to the development of pulmonary granulomatous lung disease, as occurs in chronic beryllium disease (CBD). CBD occurs in genetically susceptible workers who are exposed to beryllium by inhaling fumes or airborne particulates. Sensitization occurs when beryllium-reactive CD4⁺ T cells expand,

circulate to the lung, and secrete IFN- γ . These cells orchestrate pulmonary granulomas in chronic stages of disease (1–3). In HLA-DP2⁺ patients, beryllium ions coordinate changes in the HLA-DP2/self-peptide complex. This effectively results in the generation of a neoantigen that is presented to naive CD4⁺ T cells (4–6).

During sensitization, expansion of CD4⁺ Th1 cells that recognize the beryllium-altered HLA-DP2/peptide complex (beryllium-specific CD4⁺ T cells) occurs before clinical development of granulomas that characterizes CBD. Beryllium sensitization is required but does not always lead to chronic lung inflammation/CBD (7–9). Different physiochemical forms of beryllium may impact rates of sensitization (10–12). Development of a mouse model for CBD has provided the opportunity to study CBD pathogenesis, and we used it in this study to study how different forms of beryllium impact sensitization. This model uses mice expressing the human HLA-DP2 gene under control of the MHC class II promoter (HLA-DP2 transgenic [Tg] mice) (13). These mice express HLA-DP2 in dendritic cells (DCs), B cells, and macrophages (13). After pulmonary beryllium exposure, HLA-DP2 Tg mice become sensitized as detected by CD4⁺ T cells in the spleen, lung-draining lymph nodes (LDLNs), and lung that secrete IFN- γ and IL-2 upon beryllium restimulation *in vitro* (13). In addition, a subset of CD4⁺ T cells in the lungs of beryllium-exposed HLA-DP2 Tg mice recognize the same HLA-DP2/peptide/Be²⁺ epitopes as beryllium-specific CD4⁺ T cells derived from the lungs of humans with CBD (6, 13–15). After long-term exposure to beryllium, HLA-DP2 Tg mice develop features of CBD, including pulmonary mononuclear infiltrates and granulomatous inflammation (13). Thus, this animal model recapitulates the major features of the human disease.

Acute inflammation, as opposed to chronic granulomatous inflammation in CBD, can occur immediately after Be(OH)₂ exposure, is not dependent upon HLA-DP2 or CD4⁺ T cells, and involves signaling through innate pattern recognition receptors

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BALF, BAL fluid; BMDC, bone marrow–derived DC; CBD, chronic beryllium disease; cDC, conventional DC; cRPMI, complete RPMI 1640; DAMP, damage-associated molecular pattern molecule; DC, dendritic cell; DPBS, Dulbecco's PBS; GB, gnotobiotic; i.t., intratracheal; KO, knockout; LDLN, lung-draining lymph node; LN, lymph node; SN, culture supernatant; SPF, specific pathogen-free; Tg, transgenic; WT, wild-type.

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(16). Thus, analysis of acute inflammation provides insights into innate receptor pathways that could be involved in early phases of beryllium sensitization. The effects of innate receptor mediated activation and mobilization of DCs play a central role in the sensitization phase of metal hypersensitivity (17, 18). Metal exposures that engage innate pattern recognition receptors or induce cellular death and the release of damage-associated molecular pattern molecules (DAMPs) are associated with higher rates of sensitization and disease after low dose exposures (19, 20). Under steady-state conditions, presentation of foreign Ags by DCs to naive T cells is tolerogenic (21); however, activated DCs presenting neoantigens together with strong costimulatory signals, such as CD80, drive expansion of effector memory T cells with the capacity to migrate back to the site of exposure (22). Innate receptors upregulate expression of costimulatory molecules on the plasma membranes of DCs and accelerate migration of DCs to the draining lymph nodes (LNs) where naive T cells scan for foreign Ag (22, 23). Naive CD4⁺ T cells licensed by DCs, expand and differentiate into effector cells that can home to inflamed tissues and orchestrate chronic inflammation (24). Thus, determining the roles that innate receptors play during beryllium sensitization will define early immunological mechanisms in CBD pathogenesis.

We previously showed that exposure of mice to Be(OH)₂ drives release of IL-1 α and DNA into the airways and IL-1R/MyD88-dependent neutrophil recruitment. Be(OH)₂ exposure drives upregulated expression of CD80 on migratory DCs in the lung and LDLNs, which is reduced in MyD88 knockout (KO) mice but is not affected in TLR9KO or IL-1RKO mice (16). The presence of CD80^{hi} DCs in the LDLN is associated with enhanced CD4⁺ T cell responses, but the receptors driving MyD88-dependent upregulation of CD80 on DCs following beryllium exposure are not known.

In this study, we expand these findings to determine if these mechanisms are initiated by exposures to different physiochemical forms of beryllium. We first set out to determine whether beryllium exposure promoted death of alveolar macrophages as a potential early source of IL-1 α and DNA. We show that soluble and partially soluble crystalline forms of beryllium [BeSO₄ and Be(OH)₂] were toxic to alveolar macrophages, leading to release of DNA and IL-1 α , whereas insoluble particulate forms of beryllium (BeO or Be metal powder) did not. Then we examined how different forms of beryllium impacted cell death and the release of DNA and IL-1 α in vivo. Pulmonary exposure of mice to BeSO₄ or Be(OH)₂ resulted in release of IL-1 α and DNA into the airways, accumulation of CD80^{hi} DCs in the LDLNs, and sensitization of HLA-DP2 Tg mice after a single exposure, whereas exposure to insoluble particulate forms of beryllium (BeO and Be metal powder) mediated none of these effects. We then assessed how IL-1 α and DNA from alveolar macrophages impacted DCs. We found that the DNA and IL-1 released from dying alveolar macrophages engage TLR9 and IL-1RI, respectively, to drive upregulation of CD80 expression on DCs in vitro. We show that intrapulmonary exposure of mice to IL-1RI and TLR9 agonists in the absence of beryllium is sufficient to promote accumulation of CD80^{hi} DCs in the LDLNs and exposure to either agonist alone upregulates CD80 on DCs. Furthermore, blocking both the TLR9 and IL-1RI pathways impairs accumulation of CD80^{hi} DCs in the LDLNs of Be(OH)₂-exposed mice. Together, these data show that exposure to certain physiochemical forms of beryllium may drive hypersensitivity at low doses. The data suggest that this effect is related to their toxic effects on alveolar macrophages and the release of IL-1 α and DNA, which act as DAMPs by engaging IL-1RI and TLR9 and driving upregulation of CD80 and migration of pulmonary DCs to the LDLNs.

Materials and Methods

Mice and treatments

Wild-type (WT) and MyD88KO C57BL/6J (B6) mice were purchased from The Jackson Laboratory and housed in the Office of Laboratory Animal Resources vivarium at the University of Colorado Anschutz Medical Campus. TLR9KO B6 mice were kindly provided by J. Poole with permission from Dr. S. Akira and bred at the vivarium. HLA-DP2 Tg congenic B6 mice were provided by A. Fontenot (13) and housed/bred at the vivarium. Gnotobiotic (GB) B6 mice were bred and maintained within the Gnotobiotic Facility at the University of Colorado. Mice were housed, bred, and treated in accordance with Institutional Animal Care and Use Committee protocol standards at the University of Colorado Anschutz Medical Campus. Male and female mice (8–12 wk of age) were used in our experiments. For intratracheal (i.t.) instillations, mice were removed from isoflurane anesthesia and supported on a table at 45°C. While depressing the tongue, 50 μ l of DPBS ^{+/−} beryllium, IL-1 α , or CpG ODN were administered to the tracheal opening and observed for inhalation during breathing. In some experiments, mice were treated with 200 μ g mouse anti-mouse IL-1 α Ab in Dulbecco's PBS (DPBS) (XBiotech, Austin, TX) or 100 mg/kg sIL-1R antagonist by i.p. injection 30–45 min prior to DPBS or beryllium instillations. In some experiments, mice were treated with 10 ng rIL-1 α or 10 μ g CpG in PBS by i.t. instillation.

Reagents and Abs

BeO 1877 was purchased and characterized by National Institute of Standards and Technology. Be metal powder –325 mesh, 99+% was purchased from Alfa Aesar. BeSO₄ solution was provided kindly by Dr. L. Maier at National Jewish Health. Be(OH)₂ was precipitated from BeSO₄ solution and characterized as previously described (16). All stocks were prepared using EndoGrade water (Hyglos) or DPBS (HyClone) and tested for endotoxin activity <0.1 EU/ml with the Endpoint Chromogenic *Limulus* Amebocyte assay (Lonza). Prior to use, Be(OH)₂, BeO, and Be stocks were placed in a sonicating water bath for 15 min to disperse particles. Mouse anti-mouse IL-1 α was a kind gift of XBiotech. Soluble IL-1R antagonist was kindly provided by Dr. C. Dinarello. Functional-grade low endotoxin rIL-1 α was purchased from eBioscience, functional-grade low endotoxin anti-IL-1RI (JAMA-1) was purchased from Bio X Cell, mouse genomic DNA was purchased from Promega, and Vaccigrade LE CpG type C ODN (5'-TCGTCGTTTTCGGCGC:GCGCCG-3' [22 mer]) was purchased from InvivoGen. The following mAbs against mouse targets were used for flow cytometry: anti-CD11c (N418), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-IA_b (AF6.120.1), anti-CD4 (GK1.5), anti-Ly6G (1A8-Ly6G), anti-CD103 (2E7), anti-CD80 (16.10A1), anti-CD86 (GL-1), anti-CD8, (all from eBioscience); anti-CD16/CD32 (2.4G2), anti-CD44 (IM7), and anti-Siglec-F (E250-4440) (from BD Biosciences). Fixable Viability Dye eFluor 506 (eBioscience) was used to assess viability. Collagenase D and DNase I (both from Roche) were used for LN digestion, and collagenase from *Clostridium histolyticum* (Sigma-Aldrich) was used for lung digestion. ELISA kits for mouse IL-1 β and IL-1 α and ELISPOT kits for IL-2 and IFN- γ were purchased from eBioscience and used according to manufacturer instructions. The AEC Substrate set (BD Biosciences) and ELISPOT plates (EMD Millipore) were used for ELISPOT assays. GM-CSF was purchased from PeproTech. Lipofectamine 2000 and Opti-MEM media were purchased from Thermo Fisher Scientific. Mitochondrial DNA extraction kits were purchased from BioVision and used per manufacturer instructions. DNA stocks (extracted and purchased) were brought to final concentration of 0.9% NaCl using 9% sterile saline in EndoGrade water (DNA vehicle).

Tissue collection and processing

Bronchoalveolar lavage (BAL) was harvested with three washes of 1 ml DPBS and harvested into 15-ml conical tubes containing 1 ml RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), 10 mM HEPES, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin (all from Invitrogen), referred to as complete RPMI (cRPMI). The upper right mediastinal LDLNs were dissected into cRPMI and teased apart with 18-gauge needles. Lungs were flushed by placing an incision in the left atrium of the heart followed by infusion of 10 ml DPBS through the lung vasculature via insertion of a 25-gauge needle into the right ventricle of the heart. Flushed lungs were dissected into cRPMI, chopped into 3-mm slices using razor blades, and transferred into 15-ml tubes containing 3 ml cRPMI with 1 mg/ml collagenase and incubated 30 min at 37°C. Remaining tissue fragments were disrupted by serial passage through 16- and 18-gauge needles (five to six times each). Lung digests were pelleted, and red cells were lysed for 2 min in ammonium chloride red cell lysis buffer. Each digest was brought up to 15 ml with

DPBS, and the cells were strained through a 70- μ m filter (Falcon). Cells were pelleted and resuspended in cRPMI media. LNs were teased apart and digested with 1 mg/ml collagenase D and 100 μ g/ml DNase in cRPMI (RPMI 1640 [Invitrogen] containing 0.2 U/ml penicillin, 0.2 μ g/ml streptomycin, 0.6 μ g/ml L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate [Invitrogen], and 10% FCS) for 30 min at 37°C, 5% CO₂. An equal volume 100 mM EDTA in DPBS (pH 7.4) was added to each digest, and cells were washed with DPBS. As a source of APCs in ELISPOT assays, spleens from naive B6 HLA-DP2 Tg mice were harvested into DPBS and pressed through 70- μ m cell strainers. RBCs were lysed, and cells were resuspended in cRPMI. Viable cells were counted on a hemocytometer using trypan blue.

ELISPOTS

Lung CD4⁺ T cells were purified using the mouse CD4⁺ T cell positive selection kit and LS columns (Miltenyi Biotec) according to manufacturer instructions. B cells from three to five naive HLA-DP2 Tg spleens were purified using CD19 positive selection kit (Miltenyi Biotec). 10⁵ CD4⁺ T cells were cocultured with 10⁵ B cells in cRPMI media plus or minus 100 μ M BeSO₄ on coated and blocked IL-2 or IFN- γ ELISPOT plates overnight and the number of spots was determined as previously described (13) using ImmunoSpot Reader and software (CTL).

Ex vivo analysis of DNA and cytokines in the BAL fluid

BAL cells were pelleted, and BAL fluid (BALF) was separated from the cells. For cytokine analysis, BALF was concentrated 10-fold using 3 K Amicon concentrators. Concentrated BALF was tested for IL-1 α and IL-1 β by ELISA. DNA in unconcentrated BALF was quantified using the HS dsDNA Qubit Assay (Invitrogen). Cell-free media (for in vitro experiments) and PBS used to harvest BAL (for in vivo experiments) were used as blanks in determining DNA concentration in all experimental samples.

In vitro assays for alveolar macrophage death

For alveolar macrophage assays, BAL cells were harvested and pooled from naive B6 mice and purified by plastic adherence. Alveolar macrophages were stimulated in complete DMEM (cDMEM) media (DMEM high glucose with L-glutamine and sodium pyruvate, supplemented with 0.2 U/ml penicillin, 0.2 μ g/ml streptomycin, 0.6 μ g/ml L-glutamine, 10 mM HEPES [all from Invitrogen], and 10% FCS [heat inactivated; Hyclone]) plus or minus indicated concentrations of BeSO₄, Be(OH)₂, BeO, or Be metal powder in a final volume of 200 μ l/well. After culture, cells were observed by microscopy, plates were spun, and supernatants were harvested for DNA and IL-1 α analysis, and cells were analyzed by flow cytometry.

Generation of bone marrow-derived DC and bone marrow-derived DC activation assays

Bone marrow-derived DCs (BMDC) were generated as previously described (25) by culture of bone marrow cells from WT, MyD88KO, or TLR9KO in GM-CSF/cRPMI media. After culture, BMDC were counted and resuspended to 5 $\times$ 10⁶ cells/ml in media with 5 ng/ml GM-CSF, and 100 μ l was added to the wells of a 96-well plate. In some wells, 10 μ g/ml anti-IL-1RI was added to the cells. For assessing the DAMP activity in BALF from Be-exposed mice, cohorts of 10 mice were exposed i.t. to either PBS or PBS containing Be(OH)₂. Eighteen hours later, mice were sacrificed and BAL were obtained as described above in PBS, and BAL cells were removed by centrifugation at 400 $\times$ g and BALF were concentrated 10-fold using Amicon 3K concentrators. For assessing the DAMP activity of factors released from alveolar macrophages, supernatants were collected from alveolar macrophages cultured 18 h in media $\pm$ 50 μ g/ml Be(OH)₂ as described above. BALF and culture supernatants (SN) were spun at 10,000 rpm in the microfuge to remove beryllium crystals and debris. The soluble fractions were retained and tested for the presence of DNA. To assess whether the soluble fractions were toxic to macrophages (and thus contained residual soluble beryllium), 1 $\times$ 10⁵ alveolar macrophages per well were stimulated for 18 h with 50 μ l of each fluid, and viability was assessed by flow cytometry. To assess DAMP activity in the soluble fractions, WT, TLR9KO, and MyD88KO BMDC plated as described above in cRPMI media $\pm$ 50 μ l of BALF from PBS or Be-treated mice or with 50 μ l SN from media or Be(OH)₂-treated alveolar macrophages. In some experiments, DNA was extracted from the BALF or SN using the mitochondrial DNA extraction kit from BioVision per manufacturer instructions. DNA was brought to a 0.9% saline concentration, quantified, and normalized so that 35 ng would be delivered to BMDC in lipofectamine at a 1:2 ratio of ng of DNA/nl of Lipofectamine 2000. In these assays, BMDC were plated in 100 μ l Opti-MEM media

containing 5 ng/ml GM-CSF without antibiotics or FCS. The liposomal DNA from the test groups, CpG DNA, mouse genomic DNA, or DNA vehicle controls were added to each well and the BMDC were cultured for 6 h, after which FCS (final concentration 10%) was added to the cells for the remaining 18 h of culture. BMDC were stimulated in triplicate in each experiment and were incubated for a total of 24 h at 37°C, 5% CO₂. After culture, BMDC were stained and analyzed by flow cytometry.

Flow cytometry

To assess viability, alveolar macrophages or BAL cells were stained with eFluor 506 Fixable Viability Dye. BAL cells were stained with the following mAb: FcBlock, PE anti-Siglec, FITC anti-Ly6G, PerCP-Cy5.5 anti-CD11b, eFluor anti-F4/80, and PE-Cy7 anti-CD11c in flow wash (DPBS containing 10 mg/ml BSA, 0.1 mg/ml NaN₃). LDLN were stained with the following mAbs: FcBlock, PE-Cy7 anti-CD11c, PE anti-CD80, PerCP-Cy5.5 anti-CD11b, eFluor 450 anti-IA β , and allophycocyanin anti-CD103. LN cells from HLA-DP2-sensitized mice were labeled with 20 μ M PE-labeled plexinA4/HLA-DP2/Be²⁺ tetramers (6) in a volume of 25 μ l cRPMI media containing FcBlock and normal mouse serum for 2.5 h at 37°C, 5% CO₂. Cells were then stained with the following Abs on ice for 30 min: AlexaFluor700 anti-CD4, PerCP-Cy5.5 anti-CD44, eFluor 450 Dump (anti-IA β , -F4/80, -B220, -CD8 α). BMDC were stained with the following Abs for 30 min: FcBlock, PE-Cy7 anti-CD11c, PE anti-CD80, PerCP-Cy5.5 anti-CD11b, eFluor 450 anti-IA β , and allophycocyanin CD86. Cells were washed with flow wash and analyzed on a Canto II flow cytometer using FlowJo software (Tree Star).

Statistical analysis

A one-way ANOVA and Bonferroni posttest was used to test for differences for normally distributed data, and a Mann-Whitney *U* test was used to test for differences for data without a normal distribution. Data were analyzed using Prism GraphPad Software.

Results

BeSO₄ and Be(OH)₂ induce death of alveolar macrophages and their release of IL-1 α and DNA into the airways, whereas BeO and Be metal powder do not

Our previous study showed that pulmonary exposure of mice to Be(OH)₂ resulted in a drop in alveolar macrophage numbers in the BAL after 6 h, followed by a rapid recovery in alveolar macrophage numbers by 18 h (16). Due to autofluorescence, the phenotype of the dying cells could not be reliably assessed by flow cytometry. Additionally, during inflammation, alveolar macrophages may alter their adhesion molecules and be more difficult to recover with BAL. Thus, we could not conclude in this previous study whether alveolar macrophages were dying after Be(OH)₂ exposure. To test this, we isolated and cultured alveolar macrophages with media plus or minus Be(OH)₂ for 6, 12, or 18 h and assessed cell death and concentrations of DNA and IL-1 α in the SN. Be(OH)₂ enhanced death of alveolar macrophages 12–18 h after exposure compared with alveolar macrophages cultured in media alone (Fig. 1A). Similarly, DNA and IL-1 α concentrations were elevated in the supernatants 12–18 h after exposure to Be(OH)₂. These data indicate that alveolar macrophages die after exposure to Be(OH)₂ and subsequently release their cellular contents, including DNA and IL-1 α (Fig. 1B).

To extend this analysis to other forms of beryllium, we incubated alveolar macrophages with media alone or media containing soluble BeSO₄, partially soluble/crystalline Be(OH)₂, or two different forms of insoluble beryllium (BeO or Be metal powder), (Table I) and assessed death and release of IL-1 α or DNA 18 h later. BeSO₄, Be(OH)₂, and BeO induced higher rates of alveolar macrophage death compared with media stimulation (Fig. 1C). Furthermore, BeSO₄ and Be(OH)₂ were the only forms of beryllium that induced the release of DNA and IL-1 α into the supernatants compared with alveolar macrophages cultured in media alone (Fig. 1D).

To determine the impact of different forms of beryllium on cell death and release of DNA and IL-1 α in vivo, we exposed mice i.t.

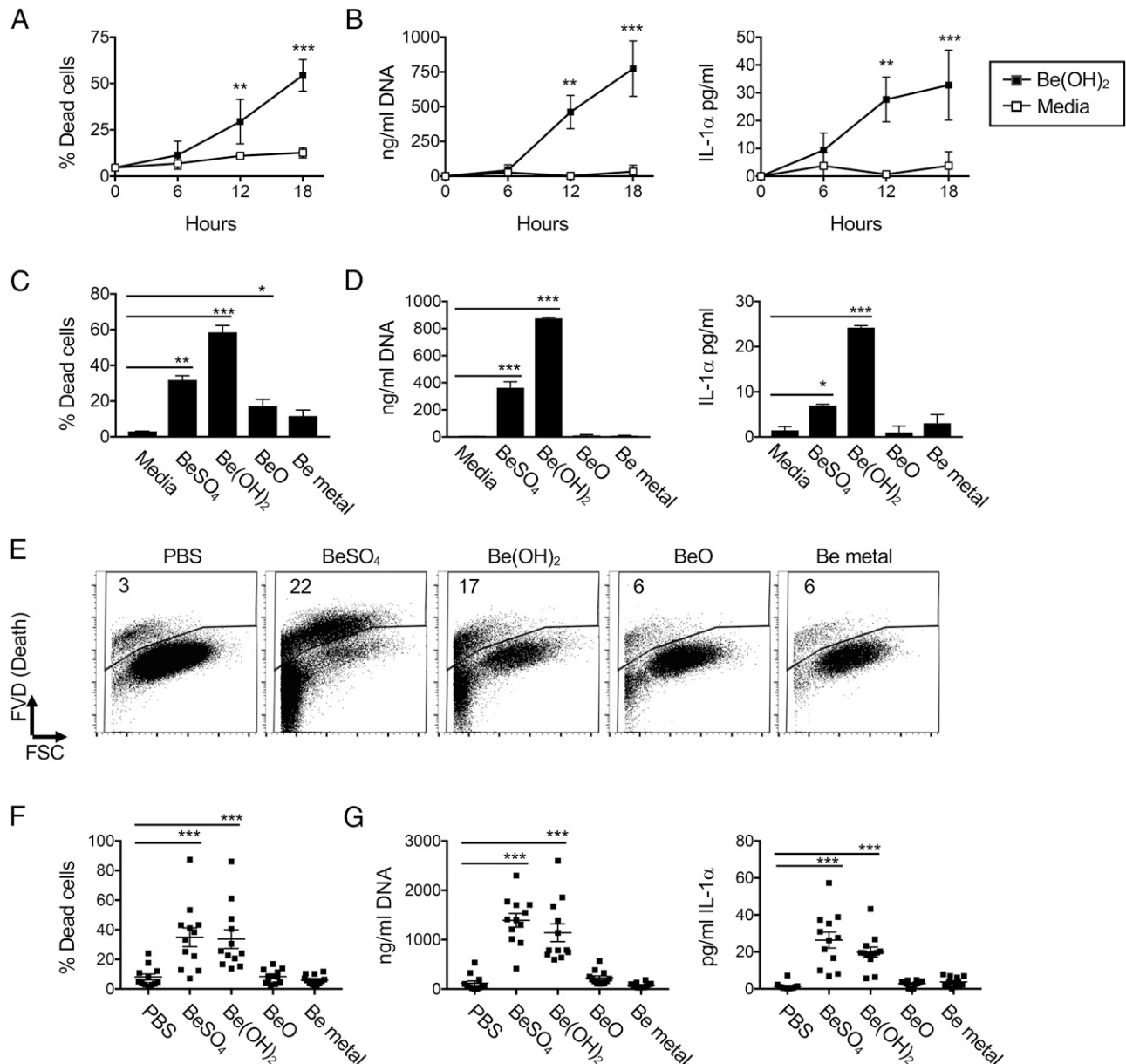


FIGURE 1. BeSO₄ and Be(OH)₂ induce DNA and IL-1α release from alveolar macrophages upon cell death, whereas BeO and Be metal do not. (A–D) Analysis of alveolar macrophage death and release of DNA and IL-1α into SN after culture in media plus or minus beryllium. (A) Percentage cell death and (B) concentrations of DNA and IL-1α in the supernatants of alveolar macrophages cultured in media alone (open squares) or with 50 μg/ml Be(OH)₂ (solid squares). Data were combined from three independent experiments performed on separate days with three separate cohorts of alveolar macrophage donors. Dots on graphs represent mean of the three experimental values from each treatment group (calculated first within each experiment as an average of triplicate measures for each group). Error bars indicate SD of the experimental means to indicate the variation between the three experiments. (C) Percentage cell death. (D) DNA and IL-1α concentrations in the supernatants of alveolar macrophages cultured for 18 h with media ± 50 μg/ml BeSO₄, Be(OH)₂, BeO, or Be metal powder. Data are combined from two independently performed experiments performed on separate days with two separate cohorts of alveolar macrophage donors. Bars on graphs represent means and bars indicate SD as described for (A) and (B). (E–G) Impact of intrapulmonary exposure to different forms of beryllium on cell death and the presence of DNA and IL-1α in the BALF 18 h later. (E) Flow plots show gating strategy for dead cells in a representative example from each treatment group. (F) Percentage dead cells and (G) DNA and IL-1α in the BALF from mice treated 18 h previously with PBS ± 80 μg BeSO₄, Be(OH)₂, BeO, or Be metal powder. Data in (F) and (G) are combined from three independent experiments (*n* = 12 mice per group altogether). Dots on graphs indicate values for individual mice, bars indicate overall mean values and error bars indicate SEM. A one-way ANOVA was performed to determine significant differences between means of each treatment compared with media or PBS-treated controls. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

to PBS alone plus or minus BeSO₄, Be(OH)₂, BeO, or Be metal powder and assessed cell death in the BAL and the presence of DNA and IL-1α in the BALF 18 h later. Pulmonary exposure of mice to either BeSO₄ or Be(OH)₂ led to an increased percentage of dead cells in the BAL compared with exposure to PBS, but exposure to BeO or Be metal did not (Fig. 1E, 1F). Similarly, exposure of mice to BeSO₄ or Be(OH)₂ induced elevated levels of

DNA and IL-1α in the BALF compared with PBS controls, whereas exposure to BeO or Be metal powder did not (Fig. 1G). We detected no IL-1β in the BALF of mice exposed to any form of beryllium, even after concentrating the BALF 10-fold (data not shown). These data show that the physiochemical form of beryllium has profound effects on alveolar macrophage viability and the release of IL-1α and DNA in vitro and in vivo.

Table I. Beryllium forms used in this study

Formula	Chemical Properties	Potential Exposure
BeSO ₄ (Be ²⁺) _{aq}	Clear solution. Solubility 2×10^{-1} mol/kg ^a	Processing: fumes, furnace work, chemical purification
Be(OH) ₂	Crystals 0.2–20 μm. ^b Amorphous, white gel. ^c Solubility 5×10^{-7} mol/kg ^a	Processing: chemical purification. Soluble form (BeOH ⁺) is the main form that Be ²⁺ ions take in physiological solutions
BeO (NIST standard reference)	Particles 0.12 ± 1.5 μm. Light white powder. Insoluble ^d	Processing, machining, ceramics
Be	Particles <44 μm. Gray powder. Insoluble ^d	Mining, processing, machining

BeSO₄ is soluble whereas Be(OH)₂ is a solid amorphous crystalline particle in water at neutral pH with low solubility. Be(OH)₂ is more soluble in lysosomes at pH 4.5 because of its amphoteric chemistry, and BeOH⁺ ions are the main form that Be ions take in physiological solutions (29). BeO and Be metal powder are both insoluble with limited solubility at lysosomal pH (soluble dissociation rate $\sim 10^{-7}$ mmol/kg per day at pH 4.5).

^aSolubility in aqueous solution at pH 7.4, 25°C.

^bParticle size determined by microscopy.

^cSolubility of amphoteric Be(OH)₂ increases in acid solutions.

^dParticle size determined by manufacturer.

NIST, National Institute of Standards and Technology.

Intrapulmonary exposure to BeSO₄ or Be(OH)₂ leads to accumulation of neutrophils in the airways

We previously showed that neutrophils accumulated in the airways 18 h after pulmonary exposure of mice to Be(OH)₂ and that this acute inflammation was IL-1R dependent (16). To determine whether the different levels in IL-1α released after exposure to different forms of beryllium resulted in higher numbers of neutrophils, we exposed mice i.t. to PBS, BeSO₄, Be(OH)₂, BeO, or Be metal powder and analyzed the cells in the BAL 18 h later (See gating strategy in Fig. 2A). Total numbers of cells in the BAL were increased in mice exposed to BeSO₄ or Be(OH)₂ compared with PBS-treated control mice, but not in those exposed to BeO or Be metal powder (Fig. 2B). We previously showed that exposure to Be(OH)₂ results in a drop in alveolar macrophages 6–12 h after pulmonary instillation, but that total numbers of these cells recover by 18 h (16), consistent with the data in Fig. 2C for Be(OH)₂. Total alveolar macrophage numbers were similar in the BAL 18 h after i.t. exposure of mice to PBS, BeSO₄, Be(OH)₂, or BeO (Fig. 2C). In contrast, the total number of alveolar macrophages in the BAL of Be metal-exposed mice was increased compared with PBS-treated controls (Fig. 2C). Neutrophil numbers were increased in both BeSO₄- and Be(OH)₂-exposed mice compared with PBS controls, but not in mice exposed to BeO or Be metal powder (Fig. 2D). Eosinophils and recruited monocyte/macrophage populations were only increased in mice exposed to Be(OH)₂ (Fig. 2E, 2F).

Treatment of Be(OH)₂-exposed mice with an anti-IL-1α Ab prevented neutrophil recruitment (Fig. 2G). The effect of anti-IL-1α Ab treatment on neutrophil accumulation was comparable to the effects of treatment with soluble human IL-1R antagonist (anakinra), which impairs both IL-1α and IL-1β activity through the IL-1R1 (Fig. 2G). Together, these data show that pulmonary exposure to BeSO₄ and Be(OH)₂ induce release of IL-1α, which drives neutrophil accumulation. In contrast, BeO or Be metal do not induce release of DNA or IL-1α, nor do they induce an acute inflammatory response.

Be(SO)₄ and Be(OH)₂ sensitize B6 HLA-DP2 Tg mice after single low-dose exposures, whereas BeO and Be metal do not

HLA-DP2 Tg mice mount a CD4⁺ T cell response against plexinA4 peptide presented in beryllium-loaded HLA-DP2 molecules after seven exposures of BeO over 21 d (6). Because of workplace controls, beryllium workers are no longer exposed to high doses of beryllium. Yet beryllium sensitization can still occur at very low doses. To determine whether different forms of beryllium drive expansion of beryllium-specific CD4⁺ T cells after a single

exposure, we exposed B6 HLA-DP2 Tg to a single dose of PBS with or without BeSO₄, Be(OH)₂, BeO, or Be metal and analyzed the presence of Be-specific CD4⁺ T cells in the lungs 14 d later using HLA-DP2/plexinA4/Be²⁺ tetramers (Fig. 3A). Total cell numbers and total number of CD4⁺ T cells were similar in the lungs of mice in each treatment group (data not shown). However, total numbers of HLA-DP2/plexinA4/Be tetramer⁺ CD44^{hi} CD4⁺ T cells were elevated in lungs of mice exposed to BeSO₄ or Be(OH)₂ but not in lungs of mice exposed to BeO or Be metal (Fig. 3B). In accordance with this, lung CD4⁺ T cells from BeSO₄- and Be(OH)₂-exposed mice secreted IFN-γ in response to beryllium restimulation, whereas CD4⁺ T cells from BeO or Be metal-exposed mice did not (Fig. 3C). Thus, analysis of lung cells from mice using the similar assays used to assess beryllium sensitization in BAL samples from beryllium workers, showed that BeSO₄ and Be(OH)₂ were potent beryllium sensitizers as compared with BeO or Be metal powder. Furthermore, a single exposure to 500 ng Be(OH)₂ per mouse was sufficient to detect beryllium sensitization in HLA-DP2 Tg mice as detected by ELISPOT, the most sensitive measure of beryllium sensitization (Fig. 3D).

Pulmonary DCs upregulate CD80 and are mobilized to the LDLNs after exposure to BeSO₄ or Be(OH)₂

To test whether different forms of beryllium impact DC function, we exposed mice i.t. to PBS plus or minus BeSO₄, Be(OH)₂, BeO, or Be metal powder and assessed the accumulation of migratory CD11b^{hi} and CD103^{hi} DCs in the LDLNs by flow cytometry (Fig. 4A, gating strategy). The percentage of migratory DCs in the LDLNs was enhanced after exposure to either BeSO₄ or Be(OH)₂, and there was an increased percentage of CD11b^{hi} DCs in these mice compared with PBS controls (Fig. 4A). These effects were not observed in the LDLNs of mice exposed to BeO or Be metal powder. The total number of migratory DCs was enhanced 48 h after exposure to either BeSO₄ or Be(OH)₂ compared with PBS controls, but not in mice exposed to BeO or Be metal (Fig. 4B). Upregulation of CD80 on DCs is associated with enhanced CD4⁺ responses in Be(OH)₂-exposed mice (16). Exposure to either BeSO₄ or Be(OH)₂ led to upregulation of this costimulatory molecule on DCs in the LDLN, whereas exposure to BeO or Be metal did not (Fig. 4B). To determine if exposure to Be(OH)₂ at low doses had similar effects, we exposed mice to 500 ng, 5 μg, or 50 μg Be(OH)₂ and assessed concentrations of DNA and IL-1α in the BALF and DC expression of CD80 in the LDLN 48 h later. Pulmonary exposure of mice to doses of Be(OH)₂ as low as 500 ng/mouse resulted in elevated levels of DNA in the BALF (Fig. 4C). There was a positive correlation between the

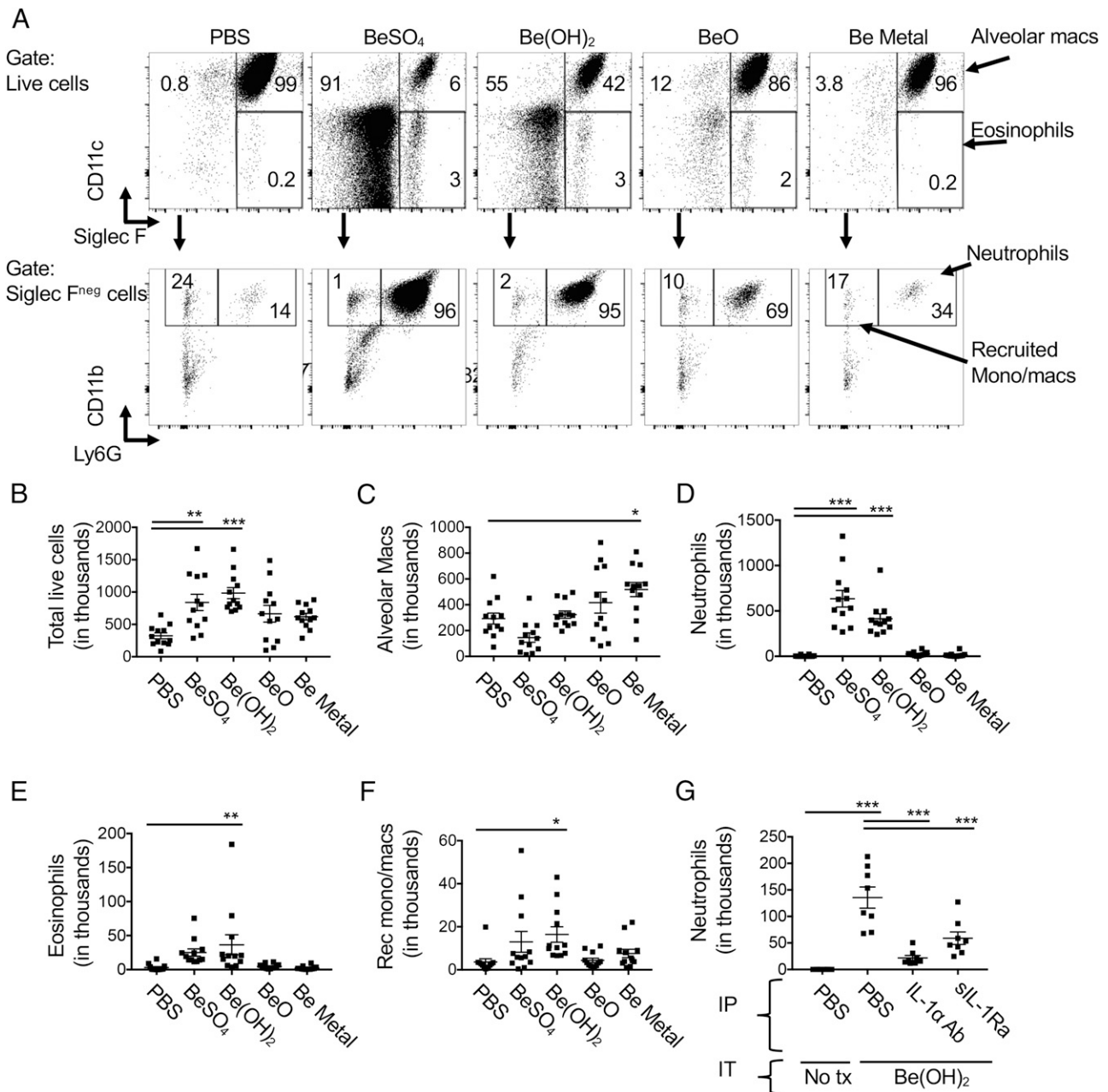


FIGURE 2. Exposure of mice to BeSO₄ or Be(OH)₂ induces release of IL-1α and DNA and acute inflammation, whereas exposure to BeO or Be metal particles does not. Flow cytometry analysis of BAL 18 h after i.t. exposure to PBS ± 80 μg BeSO₄, Be(OH)₂, BeO, or Be powder. **(A)** Gating strategy is shown on viable BAL cells for analysis of alveolar macrophages (SiglecF^{hi} CD11c^{hi}) and eosinophils (SiglecF^{hi} CD11c^{lo}) (upper row). SiglecF^{neg} cells (second row) were then analyzed for the presence of neutrophils (Ly6G^{hi} CD11b^{hi}) and recruited monocyte/macrophages (Ly6G^{lo} CD11b^{hi}). Numbers on graphs indicate percent of parent population in indicated gates for each representative sample in each treatment group. **(B)** The total number of viable BAL cells in PBS or beryllium-treated mice is shown as determined by cell counts. Total alveolar macrophages **(C)**, neutrophils **(D)**, eosinophils **(E)**, and inflammatory monocyte/macrophages **(F)** were determined using total cell counts and percentages from flow cytometric analysis. Data in **(B)–(F)** are combined data from three independent experiments ($n = 12$ mice per treatment group). Data in **(G)** are combined from two independent experiments ($n = 8$ mice per treatment group). Points on graphs indicate values for individual mice, bars indicate means, and error bars indicate SEM. A one-way ANOVA was used to test for differences between treatment groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

concentration of DNA released in the BALF and upregulation of CD80 on DCs in the LDLNs (Fig. 4D).

Be(OH)₂ exposure promotes migration of activated DCs to the LDLN in GB mice

Exposure to Be(OH)₂ enhances the mobilization of CD80^{hi} pulmonary DCs to the LDLNs, which peaks 48 h after exposure and is MyD88KO dependent (16). We hypothesized that this was due to the release of DAMPs that engaged TLRs or the receptors of the

IL-1 family that drive MyD88-dependent activation. However, it is also possible that exposure to Be(OH)₂ may promote entry of bacteria and the engagement of TLRs by bacterial products. To test this, we treated regularly housed mice (specific pathogen-free [SPF]) or GB mice with PBS or Be(OH)₂ and examined the presence of CD80^{hi} conventional DCs (cDCs) in the LDLN 48 h later. SPF and GB mice exposed to Be(OH)₂ exhibited similar accumulations of CD11b^{hi} and CD103^{hi} DCs in the LDLNs and increased expression of CD80 (Fig. 5), indicating that bacterial

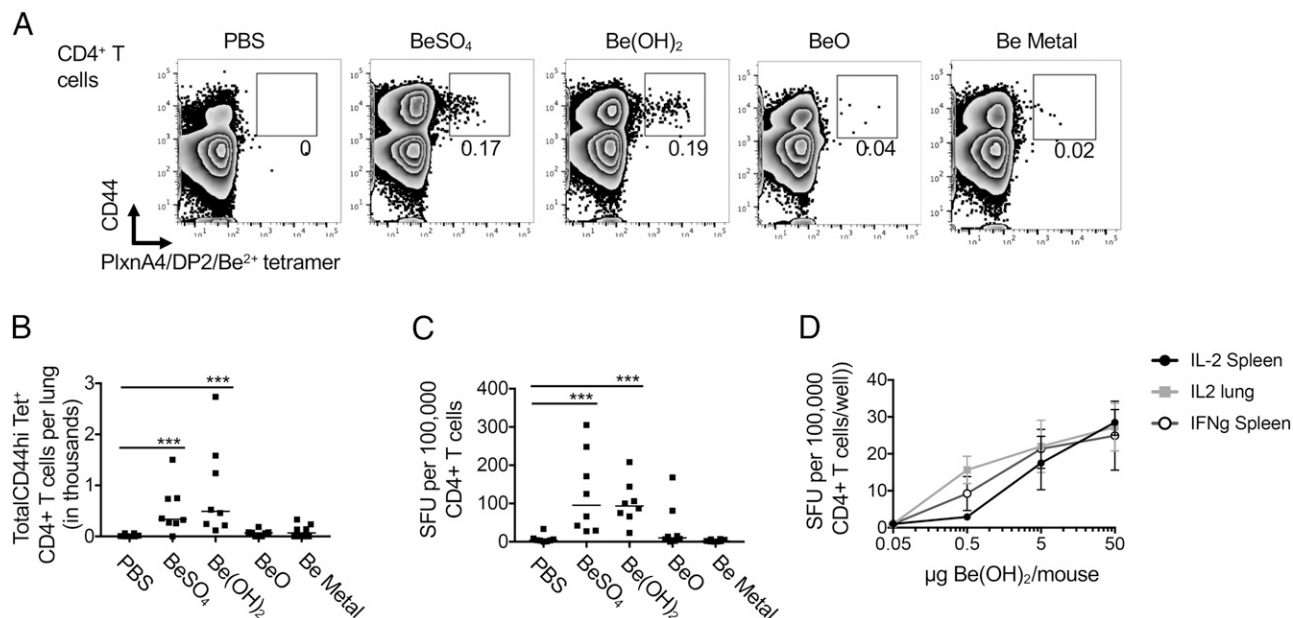


FIGURE 3. Exposure of HLA-DP2 Tg mice to a single dose of BeSO₄ or Be(OH)₂ induces accumulation of beryllium-specific CD4⁺ Th1 cells in the lung (sensitization). B6 HLA-DP2 Tg mice were treated i.t. with PBS ^{+/−} BeSO₄, Be(OH)₂, BeO, or Be metal powder, and lungs were harvested 14 d later to assess beryllium sensitization by tetramer staining and ELISPOT. **(A)** CD4⁺ T cells (CD3⁺ F480/MHCII/CD11b/B220^{neg}) from lungs of mice in each treatment group were analyzed for CD44 expression and positive staining with HLA-DP2/plexinA4/Be²⁺ tetramers as shown for a representative mouse in each treatment group. Numbers on graphs are the percentages of CD4⁺ T cells that are CD44^{hi} HLA-DP2/plexinA4/Be²⁺ tetramer⁺ for each sample shown. **(B)** Total CD4⁺ CD44^{hi} Tet⁺ cells per lung are shown for each treatment group based on total lung counts for each mouse, CD4⁺ T cell percentages, and the percentage of CD4⁺ T cells that were CD44^{hi} Tet⁺. **(C)** Be-specific CD4⁺ T cell IFN-γ production in the lung as detected by ELISPOT. Each dot represents the mean number of IFN-γ spot forming units (SFUs) per 200,000 CD4⁺ T cells in the presence of Be²⁺ stimulation normalized to media stimulated controls for an individual mouse. **(D)** B6 HLA-DP2 Tg mice were exposed to indicated doses of Be(OH)₂, and whole lung or spleen cells were analyzed for IL-2 or IFN-γ-producing cells. For (B) and (C), points on scatter plots indicate values of individual mice. Data are combined from two independent experiments (*n* = 8 mice per group) and lines indicate median values. In (D), points on graphs indicate means (*n* = 8 mice per dose) and bars indicate SD. A Mann-Whitney *U* test was used to test for differences between groups in (B) and (C). ****p* < 0.001 between indicated groups.

pathogen-associated molecular patterns are not responsible for these effects.

DNA and IL-1α released by alveolar macrophages upregulate CD80 on DCs

To assess the impact of IL-1α and DNA released into the airways following beryllium exposure, we developed an *in vitro* assay to test their effects on the upregulation of CD80 on DCs. We first purified DCs from spleens and lungs of naive WT B6 mice; however, processing of the organs led to spontaneous activation of the DCs after *in vitro* culture in media alone, masking any effects of TLR9-mediated activation (Supplemental Fig. 1). Therefore, we generated BMDC (25) as a source of large numbers of immature DCs. BMDC upregulated MHC class II, CD80, and CD86 in response to CpG (Supplemental Fig. 2). We first wanted to test whether IL-1α and/or DNA in the BALF of Be(OH)₂-exposed mice (Be-BALF) could directly activate BMDC. Cell-free soluble BALF from PBS (PBS-BALF) or Be(OH)₂-exposed mice (Be-BALF) were not toxic to alveolar macrophages (Supplemental Fig. 3). Be-BALF upregulated CD80 on WT BMDC, compared with BMDC cultured with PBS-BALF or media alone (Fig. 6A, black bars). The DAMP activity in the BALF from Be-exposed mice was reduced in WT BMDC in the presence of an IL-1R1 neutralizing Ab (black hatched bars) and in TLR9KO BMDC (solid gray bars), suggesting both receptors induce upregulation of CD80 in response to Be-BALF. Accordingly, Be-BALF did not induce any upregulation of CD80 in TLR9KO DCs treated with the IL-1R1 Ab or in MyD88KO DC (Fig. 6A gray hatched bars or white bars). In contrast, anti-IL-1RI had no effect on the upregulation of CD80 on WT BMDC

stimulated with CpG ODN, which, as expected, was entirely TLR9 dependent (Fig. 6A).

To confirm that DNA in Be-BALF could activate TLR9, we extracted DNA from the BALF samples. Vertebrate DNA can activate TLR9 when delivered to endosomes, a process that can be influenced by factors in the tissue (26). To determine if isolated DNA could activate TLR9, DNA (35 ng/well) was delivered to BMDC by liposomes (27). DNA extracted from Be-BALF induced a higher degree of TLR9 dependent upregulation of CD80 on BMDC compared with equivalent amounts of DNA extracted from PBS-BALF or of genomic DNA from unexposed mice (Fig. 6B). The effects of Be-BALF DNA was comparable to equivalent amounts of CpG ODN (Fig. 6B). DNA released into the BALF fluid may include DNA from other cells, including neutrophils (16); therefore, we tested whether DNA released from Be-exposed alveolar macrophages could promote TLR9 dependent upregulation of CD80 on BMDC. DNA released from Be-exposed alveolar macrophages upregulated CD80 via TLR9 on BMDC compared with vehicle controls, equivalent amounts of DNA extracted from media stimulated alveolar macrophages or equivalent amounts of genomic DNA from naive mice (Fig. 6B). Together these data indicate that DNA and IL-1α released by alveolar macrophages after exposure to toxic forms of beryllium act as DAMPs to promote upregulation of CD80 on DCs via TLR9 and IL-1RI, respectively.

Engagement of IL-1RI and TLR9 in the lung is sufficient to promote accumulation of CD80^{hi} DCs in the LDLNs

To test whether engagement of IL-1RI, TLR9, or both receptors promotes effects on DCs *in vivo*, we treated mice i.t. to agonists

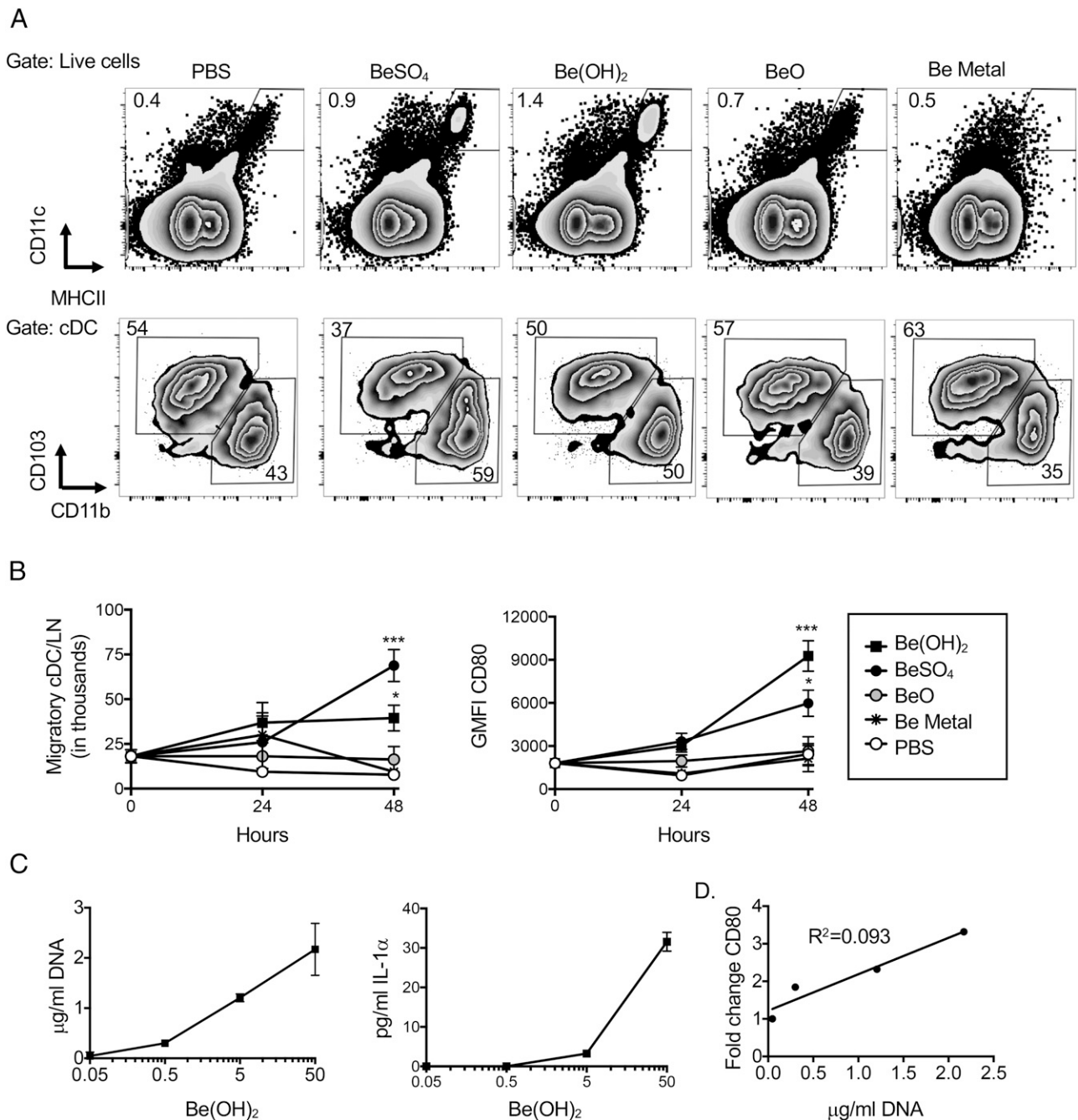
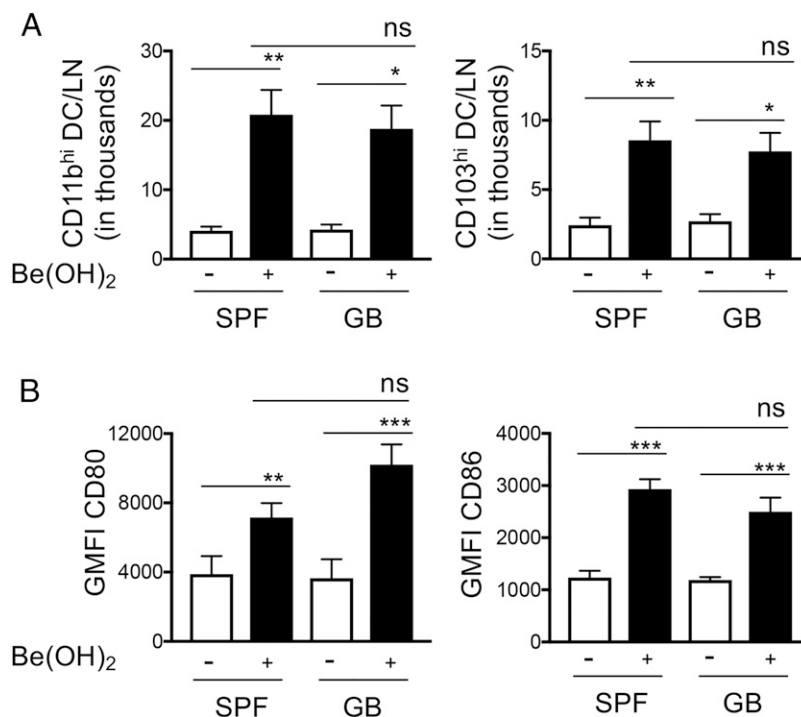


FIGURE 4. Accumulation of CD80^{hi} DCs in the LDLN after exposure to physiochemical forms of beryllium that induce DAMP release. Flow cytometry analysis of cDCs in the LDLN after i.t. exposure of mice to PBS ^{+/−} BeSO₄, Be(OH)₂, BeO, or Be metal powder. **(A)** Gating strategy for LN cells analyzed for the presence of migratory (CD11c^{hi}MHCII^{hi}) DCs, and CD11b^{hi} and CD103^{hi} subsets for a representative example in each treatment group. **(B)** Total number of migratory cDCs and geometric mean fluorescence intensity (GMFI) of staining for CD80 on migratory DCs 24–48 h after exposure to different forms of beryllium. **(C and D)** Mice were treated i.t. with indicated doses of Be(OH)₂ and BALF and LDLN DCs analyzed 48 h later. **(C)** Concentrations of DNA and IL-1α in the BALF are shown. **(D)** Association between concentration of DNA in the BALF and change in CD80 expression on DCs was assessed. Data in **(B)** are combined data from two independent experiments (*n* = 8 per treatment group). Data in **(C)** are combined from three independent experiments (*n* = 9 per treatment group). Points on line graphs indicate mean values and error bars indicate SEM. A one-way ANOVA was used to test for differences between treatment groups. **p* < 0.05, ****p* < 0.001 between indicated groups or between the indicated group and PBS control on line graphs.

of either receptor (rIL-1α or CpG) or a combination of both rIL-1α and CpG and analyzed DCs in the LDLN 48 h later. Treatment of mice with either rIL-1α or DNA alone was not sufficient to enhance the numbers of CD11b^{hi} or CD103^{hi} DCs in the LDLNs 48 h later (Fig. 7A). Treatment with both agonists, however, promoted accumulation of CD11b^{hi} and CD103^{hi} DC in the LDLNs (Fig. 7B). In contrast, rIL-1α or CpG DNA treatment alone were each sufficient to induce upregulation of

CD80 on CD11b^{hi} and CD103^{hi} DCs (Fig. 5B). These data suggest that the TLR9 and IL-1RI receptor pathways can synergize to drive migration of DCs from the lung to the LDLN to enhance priming of naive T cells, but engagement of either receptor alone is sufficient to promote upregulation of CD80 on LN DCs. These effects on CD80 mirror those of BMDC exposed to IL-1α and DNA released from alveolar macrophages in vitro (Fig. 6).

FIGURE 5. Accumulation migratory DC in the LDLNs and their upregulation of CD80 and CD86 is similar in Be(OH)₂-exposed SPF housed and GB mice. CD11b^{hi} and CD103^{hi} DCs in the LDLNs were analyzed by flow cytometry as described in Fig. 4A. WT B6 mice housed in SPF room or bred and housed in the GB core facility were treated with PBS (open bars) or PBS + 80 µg Be(OH)₂ (solid black bars). **(A)** Total DC/LN in thousands and **(B)** Geometric mean fluorescence intensity (GMFI) of staining for CD80 and CD86 on migratory DC. Data are combined from two separate experiments (*n* = 8 mice per treatment group), bars indicate mean values, and error bars indicate SEM. A one-way ANOVA was used to test for differences between treatment groups. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 between indicated groups.



Neutralization of both the DNA/TLR9 and IL-1α/IL-1R pathways impaired accumulation of CD80^{hi} immunogenic DCs in the LDLN of Be(OH)₂-exposed mice

We previously showed that upregulation of CD80 on DCs in beryllium-exposed mice was MyD88 dependent but was not impaired in Be(OH)₂-exposed IL-1RI KO mice or TLR9KO mice (16). However, our in vitro and in vivo experiments (Figs. 6, 7A, 7B) suggest that these signals are redundant in upregulating CD80 on DCs. Thus, we decided to test the effects of neutralizing both TLR9 and IL-1R pathways on DC migration and CD80 expression in Be(OH)₂-exposed mice. We treated TLR9KO mice with an anti-IL-1α and analyzed the presence of CD80^{hi} DCs in the LDLNs 48 h after i.t. exposure to Be(OH)₂.

The accumulation of DCs in the LDLNs following Be(OH)₂ exposure was partially reduced in TLR9KO mice but was further reduced to background levels in TLR9KO mice treated with anti-IL-1α (Fig. 7C). In addition, Be(OH)₂-induced upregulation of CD80 was reduced in TLR9KO mice treated with anti-IL-1α compared with WT mice, albeit not to background levels (Fig. 7D). These data suggest that IL-1α and DNA are redundant in driving MyD88-dependent upregulation of CD80 on CD11b^{hi} and CD103^{hi} DCs.

Together, these data show that soluble and crystalline forms of beryllium induce death of alveolar macrophages, whereas highly insoluble particulates (BeO and Be metal) do not. This death is associated with the release of IL-1α and DNA, which promote acute inflammation in the lung and drive migration of CD80^{hi} DCs to the LDLN via TLR9 and IL-1R mediated engagement of MyD88. Thus, in contrast to particulate forms of beryllium, which are poor sensitizers, soluble or crystalline forms of beryllium promote death of alveolar macrophages and their release of IL-1α and DNA, which act as DAMPs to enhance DC function during beryllium sensitization.

Discussion

In this study, we show that soluble and crystalline forms of beryllium are highly toxic to alveolar macrophages. Furthermore, the

death of these cells has a profound impact on the mobilization of immunogenic DCs in vivo. We show that pulmonary exposures of mice to BeSO₄ and Be(OH)₂ drive release of DNA and IL-1α into the lung and neutrophil influx whereas exposures to BeO and Be metal powder promote limited infiltration of inflammatory cells. The massive neutrophil influx that followed exposure to BeSO₄ and Be(OH)₂ was driven by IL-1α, suggesting the low levels of IL-1α have potent biological effects in vivo, as has been reported elsewhere (28). Our previous study showed that neutrophils themselves contribute to the presence of DNA and IL-1α in the airways 18 h following Be(OH)₂ exposure (16). Although both neutrophils and alveolar macrophages are implicated in DAMP release after exposure to Be(OH)₂, neutrophils require IL-1α to reach the airways, and thus, alveolar macrophages are the likely candidate as the early source of IL-1α. Prevention of neutrophil accumulation in Be(OH)₂-exposed mice has no effect on Be(OH)₂-induced mobilization of activated DCs to the LDLNs (16), suggesting that the release of DAMPs by alveolar macrophages and other cells is sufficient to impact DCs.

In vitro, alveolar macrophages exposed to Be(OH)₂ release higher levels of DAMPs than those exposed to BeSO₄. Conversely, we observed similar levels of DAMPs in BeSO₄ and Be(OH)₂-exposed mice. Be²⁺ ions can interact with hydroxide ions, phosphate ions, and organic molecules in different solutions and biological fluids (29, 30). Therefore, we cannot rule out the possibility that BeSO₄ is in a different physiochemical form before entry into alveolar macrophages in our in vitro versus in vivo experiments. In addition, unlike in vitro experiments, delivery of BeSO₄ and Be(OH)₂ to the deeper small airways of the lung may not be equivalent. In vivo, the depletion of alveolar macrophages is not apparent in the BAL after 18 h. Previous work showed that after exposure to Be(OH)₂, macrophages depletion occurred after 6 h but not at 18 h (16). Alveolar macrophages can self-renew (resident alveolar macrophages) or be recruited to the lung during inflammation (recruited alveolar macrophages) (31–33). The differing results in the total number of alveolar macrophages between in vitro and in vivo experiments (Fig. 1C versus Fig. 2C) are likely due to renewal of the alveolar macrophage pool in vivo

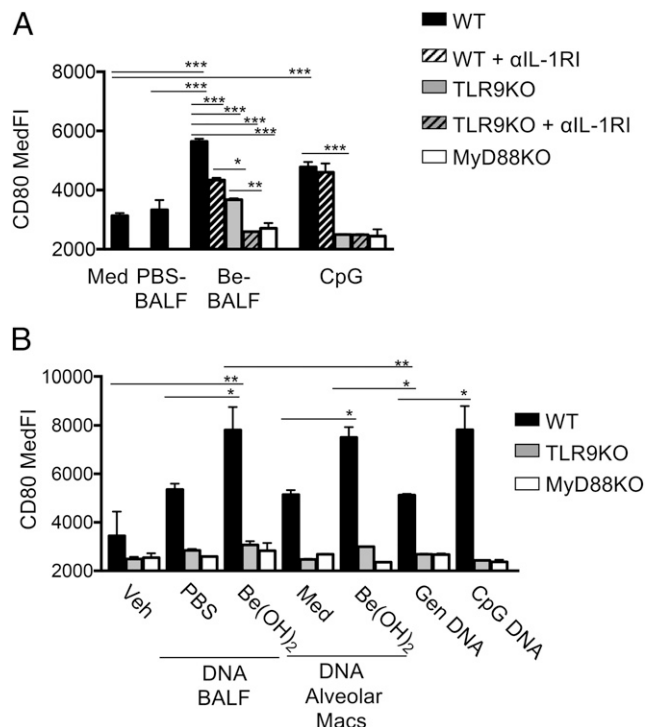


FIGURE 6. DNA and IL-1 α released by dying alveolar macrophages upregulate CD80 on BMDC. **(A)** WT, TLR9KO, or MyD88KO BMDC were incubated with media, 50 μ l soluble BALF from PBS-exposed mice (PBS-BALF), 50 μ l soluble BALF from Be(OH)₂-exposed mice (Be-BALF), or 10 μ g/ml CpG ODN for 24 h. In some cultures, a blocking Ab against the IL-1RI was used to block IL-1 signaling. BMDC were gated as described in Supplemental Fig. 2 and the *Materials and Methods* and stimulated for 24 h and CD80 median fluorescence intensity (MedFI) was assessed by flow cytometry. **(B)** WT, TLR9KO, and MyD88KO BMDC were stimulated with 35 ng/well DNA extracted from PBS-BALF or Be-BALF, 35 ng DNA extracted from supernatants from media or Be(OH)₂ stimulated alveolar macrophages, 35 ng genomic DNA from untreated mice, or 35 ng CpG ODN. DNA was delivered to DC internally using lipofectamine as described in *Materials and Methods*. After 24 h, DC CD80 expression was analyzed as described in (A). Data are combined from three independent experiments for (A) and (B). We used three separate cohorts of PBS- or Be-BALF for (A) and for the DNA sources in (B) and three separate alveolar macrophage cultures for (B). BMDC in each independent experiment were grown from separate donor mice. Bars on graphs indicate mean experimental values ($n = 3$ values), which were determined in each experiment as the average value between triplicate stimulations. Error bars indicate SD of the experimental means to show interexperimental variability. One-way ANOVA was performed to test for differences between indicated treatments. In (B), $p < 0.001$ between WT and TLR9KO DCs and WT and MyD88KO DCs for all treatment groups except vehicle controls. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ between select groups.

by inflammatory monocytes, and lack of renewal of alveolar macrophages in vitro. The in vitro experiments confirm that alveolar macrophages died and released DAMPs after exposure to BeSO₄ and Be(OH)₂.

Studies of other amphoteric metal salts such as Al(OH)₃ (a common vaccine adjuvant) and crystalline particles suggest that phagocytosis of such substances causes cellular stress in macrophages. Phagocytosis of Al(OH)₃ by peritoneal macrophages are unable to maintain the integrity of their lysosomal membranes, which leads to cellular stress, assembly of the NLRP3 inflammasome, activation of caspase-1, and release of IL-1 β (34). However, phagocytosis of Al(OH)₃ particles by alveolar macrophages induced death and release of IL-1 α and DNA (35). In

contrast to Be(OH)₂, IL-1 α (but not DNA) appears to play a role in the adjuvant effects of Al(OH)₃ after pulmonary exposure (35), whereas IL-1R and MyD88 are not required for the adjuvant effects of Al(OH)₃ via other routes of administration (36, 37). However, the effect of IL-1 α release in the lung is limited to driving BALT formation and enhanced Ab production and may not occur at other tissue sites (35) and redundancy between IL-1R1 and TLR9 has not been examined in this model. Interestingly, aluminum and beryllium salts both drive DAMP release and both act as adjuvants for adaptive immunity but drive qualitatively different CD4⁺ T cell responses. Be(OH)₂ drives expansion of Th1 cells, whereas Al(OH)₃ drives expansion of Th2 cells, enhanced IgE production, and eosinophil-dominated inflammation. TLR9 activation in the lung increases IL-12 release (38), a cytokine that is required to drive Th1 responses and likely involved in the adjuvant effects of beryllium, whereas Al(OH)₃ appears to inhibit IL-12 release (39). Perhaps the manner in which these two insoluble metal salts impact innate receptor engagement and cytokine release determines their impact on CD4⁺ T cells. In addition to IL-1 α and DNA, other DAMPs may contribute to the effects on DCs after beryllium exposure, particularly because the role of IL-1 α in DC activation was not elucidated until we examined its role in TLR9KO mice. Thus, it is possible that other DAMPs not detected in this study contributed to the MyD88-dependent effects of beryllium exposure on pulmonary DCs.

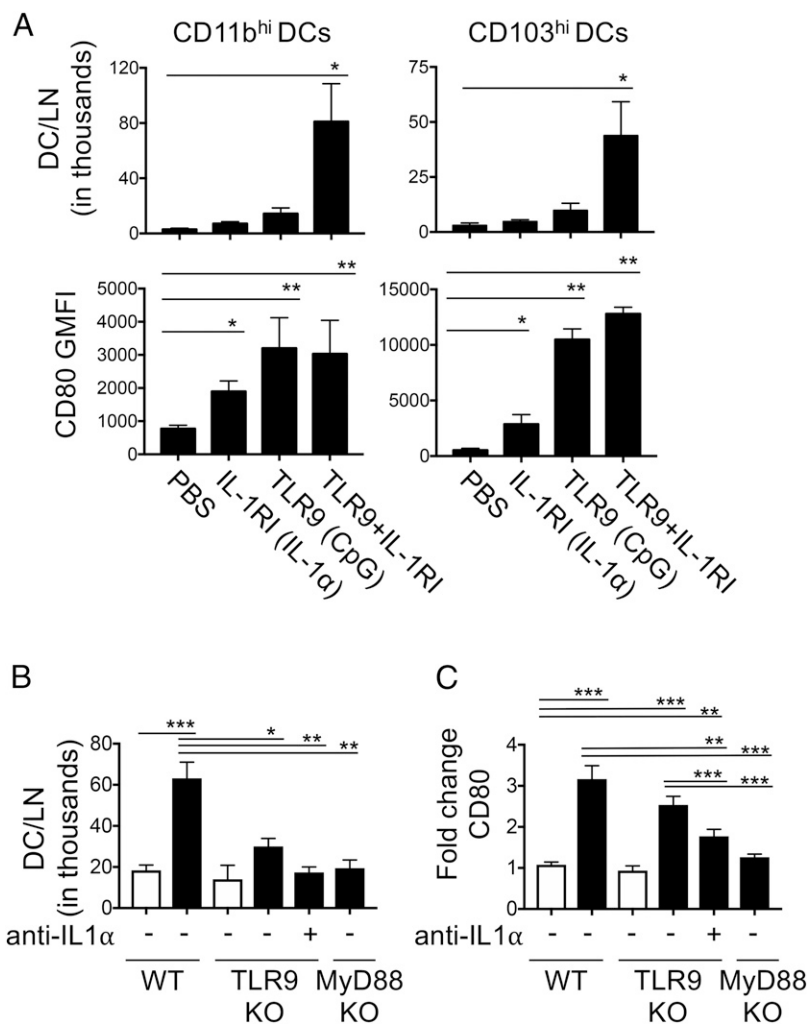
Comparison of sensitization in B6 HLA-DP2 Tg mice to different forms of beryllium showed that BeSO₄ and Be(OH)₂ could rapidly sensitize mice with equal genetic predisposition compared with mice exposed to BeO or Be metal. Treatment of HLA-DP2 Tg mice with multiple doses of BeO sensitizes HLA-DP2 Tg mice and induces the expansion of beryllium-specific CD4⁺ T cells (13). Whether these high-dose exposures to insoluble particles create overload of macrophages or activation of innate pathways in the lung over time are currently under study. We found that DCs in mice exposed to BeSO₄ and Be(OH)₂ migrate at a higher rate to the LDLN than in mice exposed to BeO or Be metal and that these DCs express high levels of CD80. Upregulation of CD80 on DCs in the LNs in Be(OH)₂-exposed mice is MyD88 dependent but not altered in IL-1RKO or TLR9KO mice and associated with enhanced priming of CD4⁺ T cells to bystander Ag (16).

In this study we show that the effects of beryllium exposure on DCs is not due to microbial products. Furthermore, we show that BALF from Be-exposed mice and SN from Be exposed alveolar macrophages promoted upregulation of CD80 on BMDC via IL-1R and TLR9. DNA extracted from the airways of Be(OH)₂-exposed mice or Be(OH)₂-exposed alveolar macrophages can induce upregulation of CD80 in DCs via TLR9 after liposomal delivery and is more potent at activating TLR9 than mouse genomic DNA, similar to CpG ODN. It is unclear if the structure of the DNA or presence of associated histones or other factors such as HMGB1 explains this difference; however, these questions are currently being studied.

Our data show that IL-1 α and DNA together enhanced migration of DCs to the LDLNs in the absence of beryllium, and each was sufficient to upregulate CD80, suggesting redundancy. We showed that inhibiting both TLR9 and IL-1 α /IL-1R1 activity in the same mice reduced upregulation of CD80 and that migration of DCs was reduced to background levels. Thus, cooperation of these pathways promotes optimal migration of immunogenic DCs to the LDLNs.

Together, the data presented here suggest that alveolar macrophages that phagocytose toxic substances like BeSO₄ and Be(OH)₂ and release DNA and IL-1 α can have potent effects on pulmonary DCs via the cooperative effects of IL-1R1 and TLR9. In this way,

FIGURE 7. Engagement of TLR9 and IL-1R1 in vivo promotes accumulation of CD80^{hi} DCs in the LDLNs. **(A)** CD11b^{hi} and CD103^{hi} DCs in the LDLNs were analyzed by flow cytometry as described in Fig. 4A. **(A)** WT B6 mice were treated i.t. with PBS +/- 10 ng rIL-1 α (IL-1R1 agonist), 10 μ g CpG type C DNA (DNA, TLR9 agonist), or 10 ng rIL-1 α + 10 μ g CpG DNA. Total DC/LN in thousands and geometric mean fluorescence intensity (GMFI) of staining for CD80 are shown for each DC subset. **(B)** and **(C)** WT and TLR9KO B6 mice were treated with nothing or 200 μ g anti-IL-1 α i.p., and 30 min later the mice were treated i.t. with PBS (open bars) or PBS with 80 μ g Be(OH)₂ (solid black bars). Migratory DCs in the LDLNs were analyzed by flow cytometry as described in Fig. 4A. **(B)** Migratory DCs that accumulated in the LDLN and **(C)** fold change in CD80 expression versus PBS-treated WT mice are shown for WT, TLR9KO, TLR9KO anti-IL-1 α -treated and MyD88KO mice exposed to PBS (open bars) or 80 μ g Be(OH)₂ (solid black bars). Data in **(A)** and **(B)** are combined from three independent experiments ($n = 15$ mice per treatment group). Bars on graphs indicate mean values and error bars indicate SEM. Data in **(B)** and **(C)** are combined from four independent experiments ($n = 16$ per treatment group), and bars on graphs indicate means and error bars indicate SEM. A one-way ANOVA was used to test for differences between treatment groups, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for select comparisons.



soluble and crystalline forms of beryllium may be particularly dangerous as they not only act as a source of antigenic signals to expand beryllium specific CD4⁺ T cells but also promote DAMP release that enhances the adaptive immune response via DCs.

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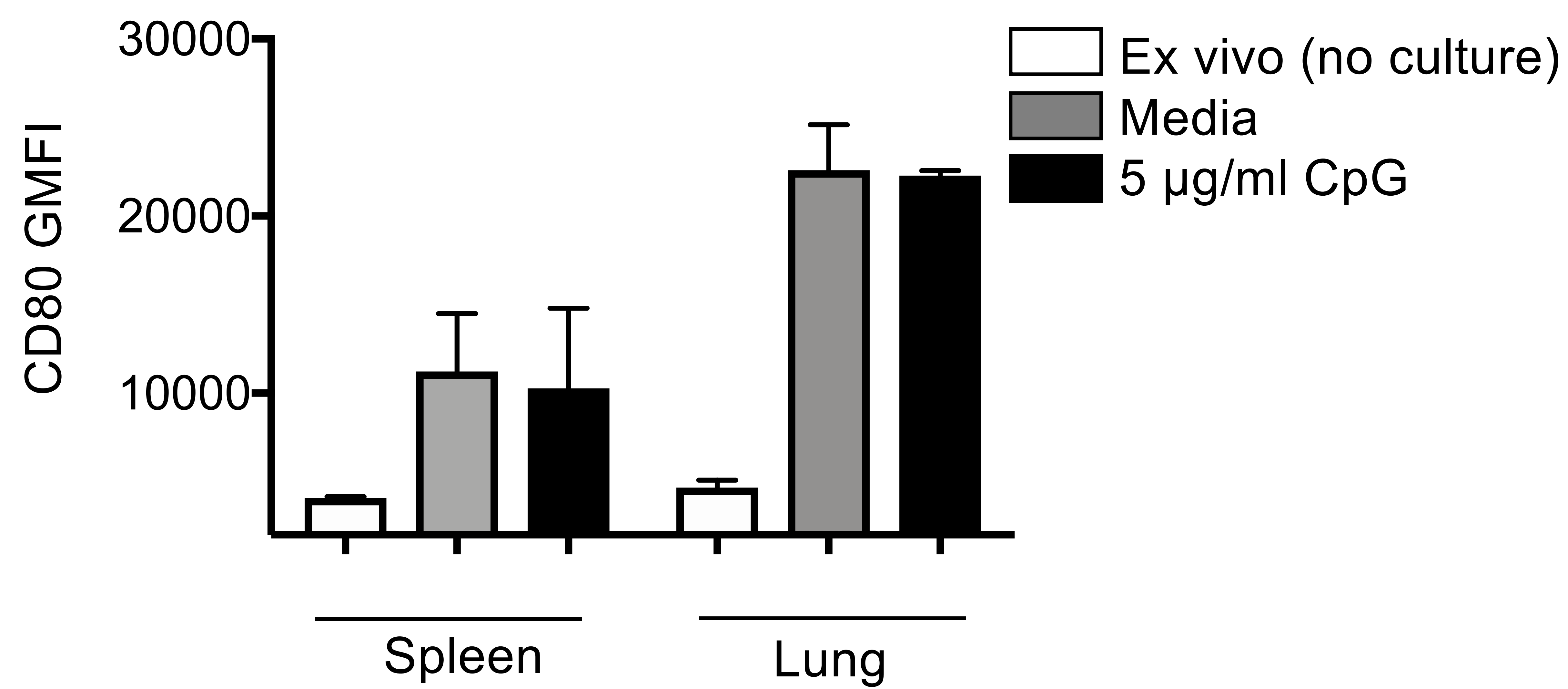
Disclosures

The authors have no financial conflicts of interest.

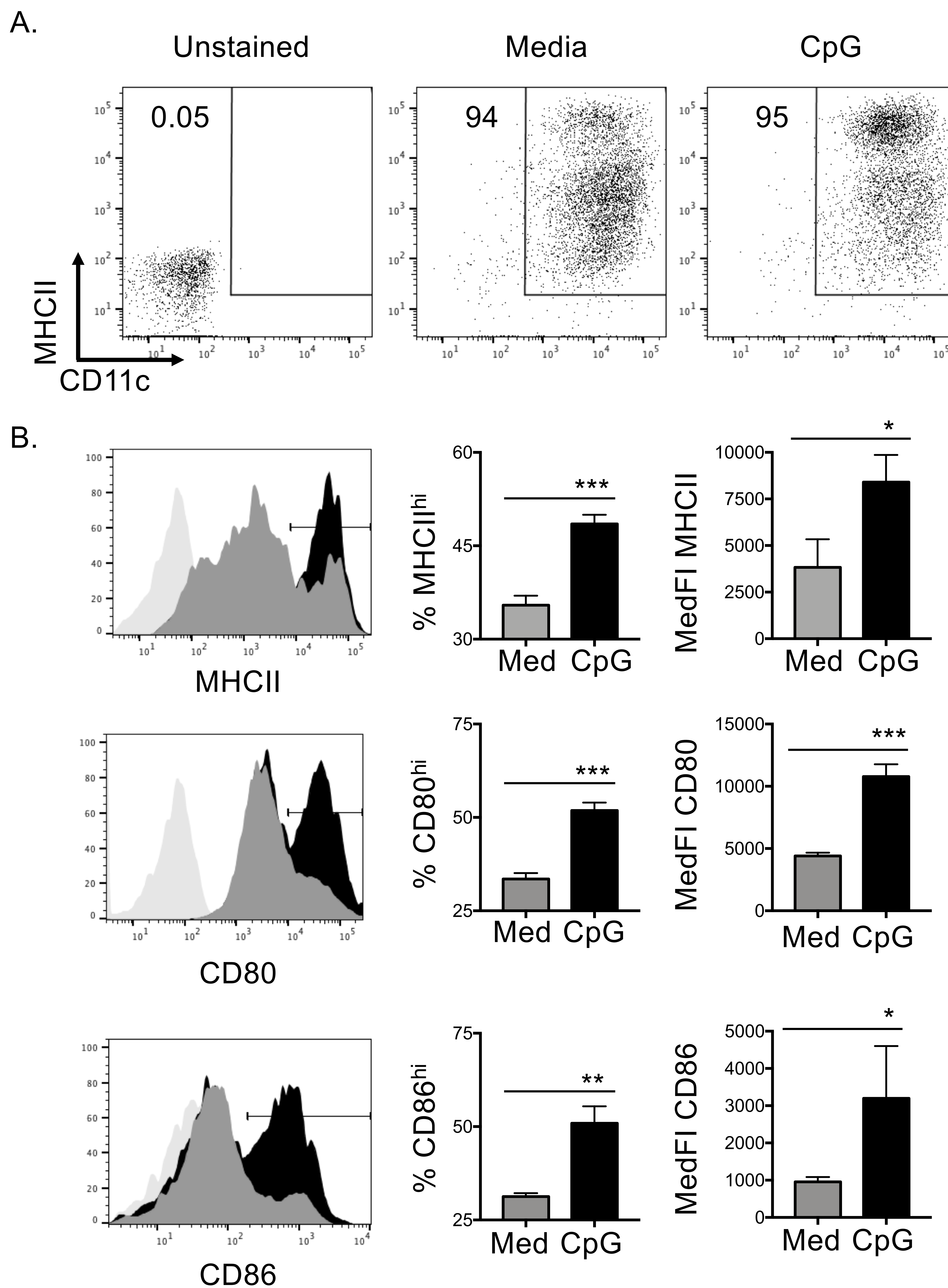
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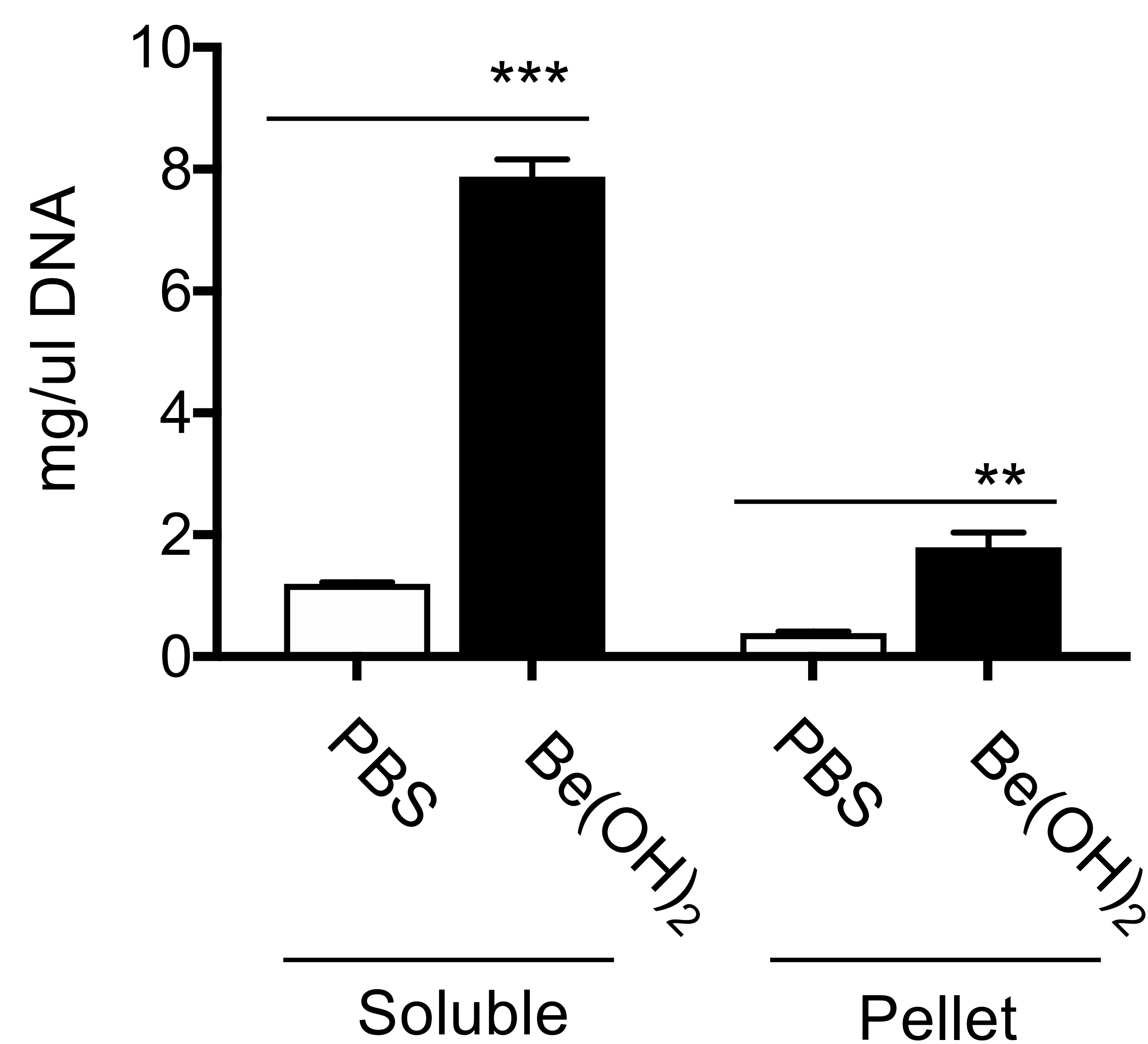


Supplemental figure 1. Activation of lung and splenic DCs after culture. Spleen and lungs from WT B6 mice were harvested and processed into single cell suspension and cultured in media +/- 5 µg/ml CpG ODN for 24hr. DCs were analyzed for the expression of CD80 on cells incubated on ice overnight (ex vivo, white bars), in media alone (grey bars) or with 5µg/ml CpG ODN (black bars) for 24hr.

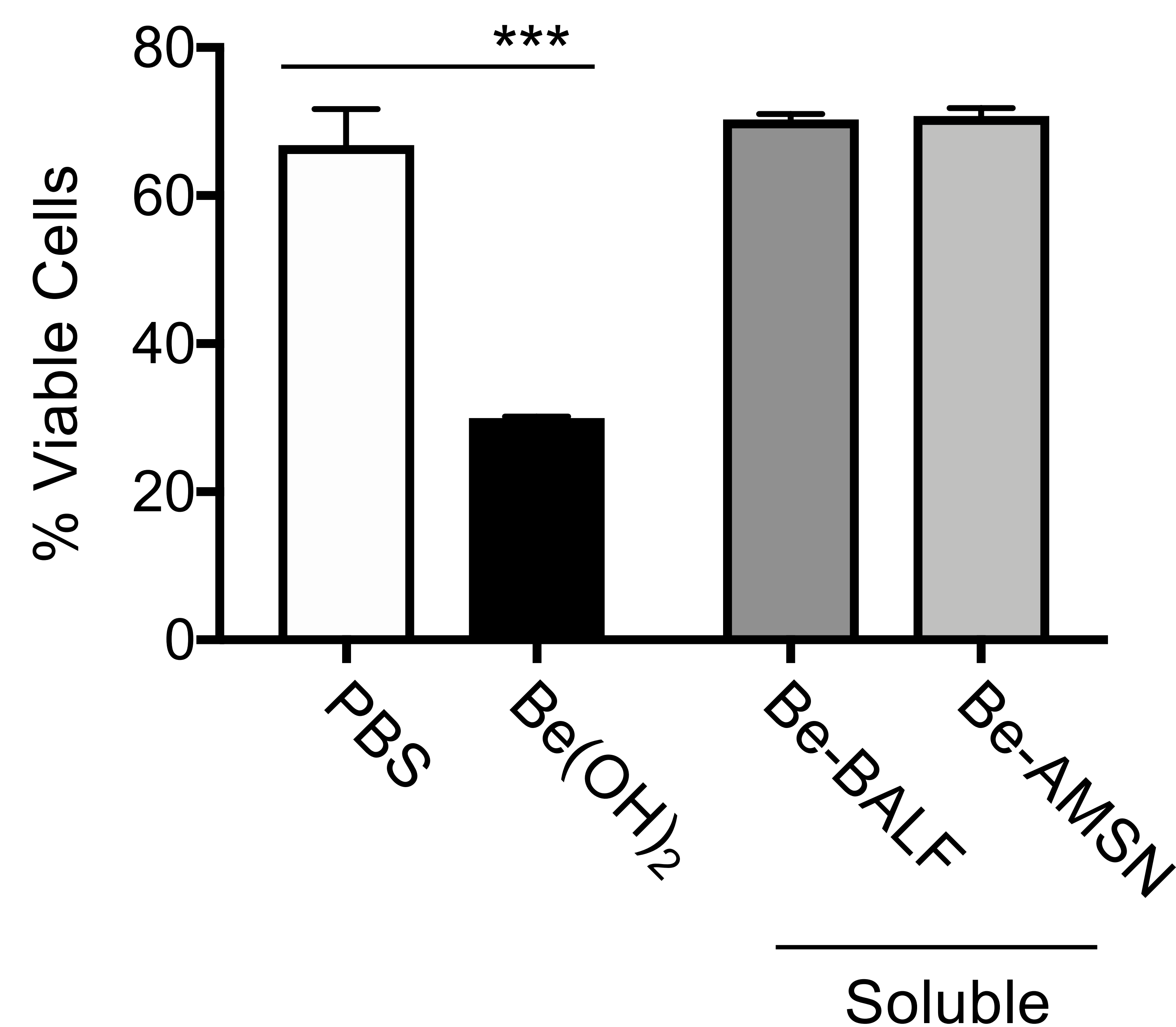


Supplemental figure 2. Activation of BMDC with CpG ODN in culture. BMDC generated from bone marrow of WT B6 mice were harvested, and cultured for 24hr in the presence of media +/- 5µg/ml CpG ODN. **A.** Unstained DCs pooled from media and CpG treated wells as a negative control and media and CpG treated DCs stained for CD11c and MHCII were gated for MHCII CD11c⁺ DCs. **B.** Gated DCs were analyzed for expression of MHCII, CD80 and CD86. Isotype control staining is shown in light gray on histograms, staining of each marker for media stimulated cells in dark grey and for CpG stimulated DCs in black. The % of cells in each sample that had high expression of each marker as defined by the gates on each histogram and median FI were determined in each treatment group. Triplicate treatments were performed in this pilot experiment for negative and positive controls, bars indicate mean values and error bars indicate SD. An unpaired t test was used to test for differences between the groups. Asterisks on graphs indicate p<0.05 (*), p<0.01 (**) and p<0.001 (***).

A.



B.



Supplemental figure 3. Assessment of BALF and alveolar macrophages supernatants (AMSN) used to detect DAMP activity. **A.** BALF isolated from cohorts of PBS and Be(OH)₂ treated mice (18hr) were centrifuged 5 min $400 \times g$ and fluid was collected, pooled from each treatment group. BALF were concentrated 10X using 3K Amicon centrifuge concentrators and centrifuged 10,000 rpm to remove additional debris and particles. The soluble fraction was collected and the pellet fraction was resuspended in an equivalent volume of PBS. DNA in each fraction was quantified and shown in A. **B.** Be(OH)₂ particles are toxic to alveolar macrophages. To confirm that these particles were sufficiently absent from the soluble fractions of BALF from Be-exposed mice or from SN from Be-exposed alveolar macrophages, 50 μ l of each were incubated with alveolar macrophages for 18hr and the viability of the alveolar macrophages was determined compared to those incubated with media +/- 50 μ g/ml Be(OH)₂.