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Mycobacterium tuberculosis Membrane Vesicles Inhibit T Cell Activation

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Mycobacterium tuberculosis **utilizes multiple mechanisms to evade host immune responses, and inhibition of effector CD4$^+$ T cell responses by *M. tuberculosis* may contribute to immune evasion. TCR signaling is inhibited by *M. tuberculosis* cell envelope lipoglycans, such as lipoarabinomannan and lipomannan, but a mechanism for lipoglycans to traffic from *M. tuberculosis* within infected macrophages to reach T cells is unknown. In these studies, we found that membrane vesicles produced by *M. tuberculosis* and released from infected macrophages inhibited the activation of CD4$^+$ T cells, as indicated by reduced production of IL-2 and reduced T cell proliferation. Flow cytometry and Western blot demonstrated that lipoglycans from *M. tuberculosis*–derived bacterial vesicles (BVs) are transferred to T cells, where they inhibit T cell responses. Stimulation of CD4$^+$ T cells in the presence of BVs induced expression of GRAIL, a marker of T cell anergy; upon restimulation, these T cells showed reduced ability to proliferate, confirming a state of T cell anergy. Furthermore, lipoarabinomannan was associated with T cells after their incubation with infected macrophages in vitro and when T cells were isolated from lungs of *M. tuberculosis*–infected mice, confirming the occurrence of lipoarabinomannan trafficking to T cells in vivo. These studies demonstrate a novel mechanism for the direct regulation of CD4$^+$ T cells by *M. tuberculosis* lipoglycans conveyed by BVs that are produced by *M. tuberculosis* and released from infected macrophages. These lipoglycans are transferred to T cells to inhibit T cell responses, providing a mechanism that may promote immune evasion.** ***The Journal of Immunology*****, 2017, 198: 2028–2037.**

Mycobacterium tuberculosis infection results in the release of extracellular vesicles (EVs) containing bacterial content from infected macrophages (1–4). EVs produced during infection with mycobacterial species are able to regulate uninfected macrophages (2–9). We have shown that EVs from *M. tuberculosis*–infected macrophages consist of two distinct and separable fractions: bacteria-derived membrane vesicles (bacterial vesicles [BVs]) and host exosomes (3). Whereas BVs released from infected macrophages contained *M. tuberculosis* components and had activity to regulate uninfected macrophages, exosomes from infected macrophages (when separated from BVs) lacked these components and activities, demonstrating the importance of BVs in determining the export of *M. tuberculosis* components from infected macrophages (3).

M. tuberculosis produces BVs both during macrophage infection and in axenic culture; the BVs produced under these two conditions carry overlapping content (1–3, 10–12) and similar immune-modulatory properties (3, 12–14). The content and immune-modulatory properties of exosome preparations from infected macrophages (1, 5, 10) are also overlapping with BVs (11, 12, 15), although our interpretation is that this is due to the presence of BVs in the exosome preparations (3). BVs from mycobacteria in axenic cultures and from infected macrophages have been assessed for mycobacterial components by proteomic and biochemical studies. They contain numerous bacterial proteins, including lipoproteins (e.g., LpqH, LprG), lipoglycans and glycolipids (e.g., lipoarabinomannan [LAM], lipomannan [LM], and phosphatidylinositol mannoside [PIM] species), and Ags (e.g., Ag85B) (1–3, 10–12). These components may contribute to both host immune responses and immune evasion mechanisms, for example, provision of Ag to drive T cell responses, lipoproteins to activate TLR2 signaling and inhibit macrophage Ag presentation, and LAM to inhibit phagosome maturation (16–26). Thus, BV release provides a mechanism to broadcast *M. tuberculosis* components beyond infected macrophages; this mechanism has the potential to either expand host defense or to promote immune evasion.

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Abbreviations used in this article: BV, bacterial vesicle; Ctr EV, EV from uninfected macrophage; EV, extracellular vesicle; Inf EV, EV from *M. tuberculosis*–infected macrophages; LAM, lipoarabinomannan; LM, lipomannan; PIM, phosphatidylinositol mannoside.

Prior studies of BVs and EV preparations from infected macrophages have investigated the effects of these vesicles on macrophages (3–6, 8, 12, 14), but these studies have not addressed direct effects of these vesicles on T cells. Of significant interest are the *M. tuberculosis* lipoglycans LAM and LM. These major components of the *M. tuberculosis* cell wall are found in BVs isolated from axenic *M. tuberculosis* culture and from infected macrophages. LAM has been shown to inhibit activation of $CD4^+$ T cells, leading to decreased proliferation and cytokine production upon TCR stimulation (27–30). In this context, LAM inhibits TCR signaling, as manifested by decreases in Lck, LAT, and ZAP70 phosphorylation (27, 28). Importantly, exposure of $CD4^+$ T cells to LAM during T cell activation induces anergy, manifested by decreased T cell responses upon subsequent stimulation and increased expression of anergy markers such as the E3 ubiquitin ligase GRAIL (29). However, exposure of T cells to BVs and LAM may primarily occur in the lung, and LAM may primarily impact effector T cells as opposed to priming naive T cells. Also, it is still unclear whether LAM can be transferred to T cells from macrophage phagosomes, where *M. tuberculosis* is sequestered, and a mechanism for LAM trafficking from infected macrophages to T cells has not been demonstrated.

We hypothesized that LAM is trafficked by BVs that are produced by *M. tuberculosis* in phagosomes and released by macrophages to reach $CD4^+$ T cells in the lung and inhibit their responses, supporting bacterial immune evasion. In these studies, we demonstrate that EVs from infected macrophages, but not EVs from uninfected macrophages, inhibit T cell activation, an inhibition attributable to the presence of BVs. This inhibition may be due in part to the trafficked LAM, but additional bacterial components of the BVs may also contribute. BVs inhibited the in vitro activation of Th1 effector $CD4^+$ T cells as well as naive T cells. The ability to inhibit Th1 effector responses is of particular potential significance, as this mechanism could limit protective Th1 responses to *M. tuberculosis* at the site of infection (where BVs are most likely to encounter T cells). Moreover, we demonstrate that pulmonary $CD4^+$ T cells acquire LAM in the course of aerosol infection of mice with virulent *M. tuberculosis*, demonstrating that exposure of T cells to LAM occurs in vivo. Taken together, our results support the hypothesis that BV trafficking of LAM and other mycobacterial components inhibits responses by $CD4^+$ T cells to *M. tuberculosis* infection, potentially contributing to bacterial immune evasion.

Materials and Methods

Reagents and Abs

BSA, chemicals, and detergents were from Sigma-Aldrich (St. Louis, MO). Abs (monoclonal unless otherwise stated) included polyclonal rabbit anti–*M. tuberculosis* (Gen-Way Biotech, San Diego, CA), CS-35 anti-LAM (hybridoma from BEI Resources, Manassas, VA), polyclonal rabbit anti-GRAIL (Thermo Fisher, Grand Island, NY), EM-04 anti-mouse CD9 (Pierce, Rockford, IL), NVG-2 anti-mouse CD63 (BioLegend, San Diego, CA), 145-2C11 anti-mouse CD3ε and its PE conjugate (BD Biosciences, San Jose, CA), 37.51 anti-mouse CD28 (BD Biosciences), allophycocyanin-conjugated RM4-5 anti-mouse CD4 (BD Biosciences), PE/Cy7-conjugated M1/70 anti-mouse CD11b (BioLegend), PE/Cy7-conjugated rat IgG2b (isotype control, clone eB149/10H5; eBioscience, San Diego, CA), PE-conjugated Armenian hamster IgG control clone eBio299Arm (eBioscience), Alexa Fluor 647–anti-mouse CD3ε (BioLegend), Alexa Fluor 647–Armenian hamster IgG (isotype control; BioLegend), Alexa Fluor 488–anti-mouse CD11b (BioLegend), polyclonal rabbit IgG (isotype control; Thermo Fisher), and DyLight 488–conjugated polyclonal donkey anti-rabbit IgG (BioLegend). CS-35 mAb was purified from hybridoma culture supernatants using protein G affinity chromatography. The polyclonal anti–*M. tuberculosis* Ab detects LAM and LM, although its specificity is not limited to these lipoglycans. *M. tuberculosis* strains H37Ra and H37Rv were obtained from American Type Culture Collection (Manassas, VA). Bacteria were cultured at 37°C in Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 10% Middlebrook ADC enrichment (BD Biosciences), 0.05% Tween 80, and 0.2% glycerol. Bacteria were grown to midlog phase (OD_{600} = 0.5–0.6) and frozen in 25% glycerol for infection stocks. To prepare H37Ra stocks, bacteria were declumped by seven passages through a 25-gauge needle and centrifuged at 100 × *g* for 5 min to remove clumps. The supernatant was frozen in 1-ml aliquots for later use, and a portion was assessed by serial dilution and culture on Middlebrook 7H11 agar plates to determine the stock titer (~4×10^6 CFU/ml). For experimental use, H37Ra stocks were thawed and similarly declumped. To prepare H37Rv stocks, bacteria were declumped by vortexing with 3-mm glass beads for 2 min, centrifuged at 100 × *g* for 5 min to remove clumps, and frozen in 1-ml aliquots at low titer (2×10^6 CFU/ml) or high titer (4×10^7 CFU/ml); titers were determined by serial dilution and culture on Middlebrook 7H11 agar plates. For infection of mice, H37Rv stocks were thawed, declumped by vortexing with glass beads as above, and then further declumped by needle aspiration as described below for aerosol infection.

Mammalian cell culture

The Institutional Animal Care and Use Committee of Case Western Reserve University approved all animal studies protocols (protocol no. 2015-0023). Mice were housed under specific pathogen-free conditions. C57BL/6J mice (8- to 12-wk-old females) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mycobacterial Ag85B-specific TCR-transgenic (P25) mice were provided by Kiyoshi Takatsu (University of Tokyo) (31). P25 T cells recognize a peptide ($Ag85B_{240–254}$; NH2-FQDAYNAAGGHNAVF-COOH) derived from *M. tuberculosis* Ag85B in the context of MHC class II $I\text{-}A^b$ (31). Unless otherwise indicated, all cell culture incubations were at 37°C in a humidified, 5% CO_2 atmosphere. Macrophages were cultured from suspensions of bone marrow cells as described previously (25). Bone marrow was flushed from femurs and tibias, and cell suspensions were homogenized and filtered through a 70-μm screen. Bone marrow cells were cultured in DMEM (HyClone, Logan, UT) supplemented with 10% heat-inactivated FBS (Life Technologies, Carlsbad, CA), 50 mM 2-ME (Bio-Rad Laboratories, Hercules, CA), 1 mM sodium pyruvate (HyClone), 10 mM HEPES (HyClone), 100 U/ml penicillin, and 100 mg/ml streptomycin (HyClone) (complete medium, referred to as D10F), with 25% LADMAC cell-conditioned medium as a source of M-CSF. The medium was changed on days 5 and 7 of culture, and macrophages were used on day 8. $CD4^+$ T cells were isolated from spleens as described previously (27, 29). Spleens were dissociated through a 40-μm screen in 5 ml of DMEM, and RBCs were lysed in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA [pH 7.4]). Splenocytes were plated in 100-mm tissue culture plates and incubated for 1 h at 37°C. Nonadherent cells were collected; $CD4^+$ T cells were purified by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA) and incubated overnight in D10F supplemented with 10 ng/ml IL-7 (Miltenyi Biotec). For all inhibition assays, $CD4^+$ T cells were washed and resuspended in HL-1 medium (BioWhittaker, East Rutherford, NJ) supplemented with nonessential amino acids, penicillin/streptomycin, L-glutamine, HEPES, and 2-ME. For some experiments, untouched naive $CD4^+$ T cells were isolated from splenocytes using a naive $CD4^+$ T cell isolation kit (Miltenyi Biotec), which removes $CD44^{hi}$ memory T cells and lineages other than $CD4^+$ T cells. Th1 effector cells were generated from naive $CD4^+$ T cells by incubation for 3 d in D10F on plates previously coated with 1 μg/ml anti-CD3ε, with the addition of 1 μg/ml anti-CD28, 5 μg/ml anti–IL-4, and 10 ng/ml IL-12. Th1 cells were then removed from stimulation and rested for 5 d in D10F with 10 ng/ml IL-7 prior to use in inhibition assays.

Infection of macrophages and EV purification

Macrophage infection and EV purification were performed as reported previously (3, 32). Briefly, macrophages were plated, allowed to recover for 1 d, incubated for 4 h with *M. tuberculosis* strain H37Ra at a multiplicity of infection of 10 in antibiotic-free D10F, and washed to remove extracellular bacteria. The medium was replaced with antibiotic-free D10F that was centrifuged prior to use at 100,000 × *g* for 18 h to remove exosomes and other particles that are present in FBS (vesicle production medium), and the cells were cultured for 20 h (24 h total infection time). To remove cells and larger particulates, the culture medium was centrifuged at 4°C sequentially at 500 × *g* for 15 min, 2000 × *g* for 15 min, and 10,000 × *g* for 30 min. The supernatant was then centrifuged at 100,000 × *g* for 70 min using the Beckman SW28 rotor to pellet EVs. The vesicle pellet was washed in 5 ml of PBS and recovered by centrifugation for 2 h at 100,000 × *g* in a Beckman SW50.1 rotor. The final EV pellet was suspended in PBS (to a concentration of vesicles produced from 10^6 cell

equivalents per milliliter, ~1 mg/ml protein concentration) and frozen at −80°C.

BV purification from axenic culture

H37Ra from log-phase cultures (2 ml) was seeded into 2 l of 7H9 medium (BD Biosciences) supplemented with ADC (BD Biosciences) and glycerol (Thermo Fisher). Cultures were grown for 10 d in shaker flasks at 37°C. Bacteria were removed by centrifugation for 15 min at 1500 × *g* and 3000 × *g*. The supernatant was filtered through a 0.45-μm polyvinylidene difluoride filter (Millipore, Billerica, MA) and centrifuged at 10,000 × *g* for 30 min to remove larger particles. BVs were pelleted at 100,000 × *g* for 2 h, suspended in 1 ml of PBS, and further purified using a qEV size exclusion column (Izon Science, Christchurch, New Zealand). Fractions containing BVs were centrifuged at 100,000 × *g* for 2 h. BVs were resuspended in PBS and stored at −80°C.

Western blotting

Proteins were separated by 12% SDS-PAGE under reducing conditions and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were incubated in blocking buffer (5% milk in PBS with 0.1% Tween 20), followed by incubation with primary Abs overnight at 4°C in blocking buffer. Membranes were washed and then incubated with HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Membranes were washed three times in PBS with 0.1% Tween 20, and detection was performed with ECL Western blotting substrate (Pierce).

T cell functional analysis

$CD4^+$ T cells (10^5 cells per well) were treated in triplicate with purified vesicles or LAM for 1 h. BVs were added at a LAM equivalent concentration of 10 μg/ml unless otherwise indicated. EVs from uninfected or infected macrophages were added at 100 μg/ml EV protein content. Cells were activated for 24 h with plate-bound anti-CD3ε (1 μg/ml) and soluble anti-CD28 (1 μg/ml) in the continued presence of vesicles or LAM; supernatants were harvested, and IL-2 was quantitated by ELISA using the murine IL-2 DuoSet (R&D Systems, Minneapolis, MN). Alternatively, T cells were activated as above for 48 h, and proliferation was measured as described (29) by addition of 1 μCi of [^{3}H]thymidine per well (Amersham Pharmacia Biotech, Piscataway, NJ) for an additional 16 h. [^{3}H]thymidine incorporation was measured by liquid scintillation counting. Results were expressed as mean counts per minute of triplicate values ± SD. Alternatively, proliferation was measured by dye dilution using a CellTrace Violet cell proliferation kit (Invitrogen, Carlsbad, CA). Cells were incubated with CellTrace Violet prior to BV or LAM treatment and activation. Cells were harvested at 72 h and stained with Live/Dead Fixable Yellow (Invitrogen) and PE–anti-CD3ε Ab prior to detection of cell proliferation by flow cytometry. In some experiments, cells were removed from stimulation after 24 h and rested for 7 d in D10F with 10 ng/ml IL-7. Rested cells were then activated an additional 24 h with anti-CD3ε and anti-CD28, and cytokine production and proliferation were measured as before. Where indicated, $CD4^+$ T cells were activated with PMA (16 ng/ml) and ionomycin (0.1 μg/ml), or ionomycin alone, and analyzed as described. In some experiments, P25 $CD4^+$ T cells were treated as indicated for 1 h at 37°C and then primed by coculture for 48 h at a 1:2 ratio with paraformaldehyde-fixed macrophages with 1 μg/ml *M. tuberculosis* Ag 85B peptide. IL-2 was measured as described previously. After 48 h, cells were collected by vigorous pipetting, washed three times with DMEM, and rested for 5 d in D10F with 10 ng/μl IL-7. Rested cells were restimulated with fixed macrophages as described earlier for 24 h before harvesting supernatants for IL-2 ELISA or for 48 h before incubation with [^{3}H]thymidine as above to measure T cell proliferation. In some experiments, CellTrace Violet–labeled $CD4^+$ T cells were activated in the presence of unlabeled $CD4^+$ T cells that had previously been incubated with or without BVs and washed; proliferation was measured by CellTrace dye dilution, and IL-2 production was determined by ELISA.

Quantitative PCR analysis of gene expression

Total RNA was isolated from $CD4^+$ T cells using the RNeasy Plus mini kit (Qiagen, Valencia, CA). RNA quantity was determined using a NanoDrop ND-2000c spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized using the QuantiTect reverse transcription kit (Qiagen) following the manufacturer's protocol. Equal quantities of cDNA for each experimental condition were amplified by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories). Primers were for *Gapdh* (forward, 5′-AAC GAC CCC TTC ATT GC-3′, reverse, 5′-TCC ACG ACA TAC TCA GCA C-3′) (33) and *Rnf128* (encoding GRAIL) (forward, 5′-GCG CAG TCA GCA AAT GAA-3′, reverse, 5′-TGT CAA CAT GGG GAA CAA CA-3′) (34). Samples were amplified using a hot start at 95°C for 3 min, followed by 50 cycles of 10 s at 95°C, 10 s at 59°C, 30 s at 72°C, and a postamplification melting curve ramping from 65 to 95°C in increments of 0.5°C per 5 s. The abundance of each transcript was calculated relative to GAPDH gene expression, using the equation $2^{[CT(\text{target gene}) - CT(\text{GAPDH gene})]}$ (35), normalizing the cycle threshold (*CT*) for each sample using the *CT* for the GAPDH gene amplified in parallel.

Flow cytometry for BV content in T cells

$CD4^+$ T cells from C57BL/6 mice were incubated with BVs at 10 μg/ml LAM equivalent concentration for 24 h and analyzed by flow cytometry for incorporation of bacterial content. After exposure to BVs, cells were washed three times with PBS, stained with Live/Dead Fixable Yellow (Invitrogen), incubated with CS-35 or polyclonal anti–*M. tuberculosis* Abs for 30 min on ice, washed with PBS, and incubated with Alexa Fluor 488–anti-mouse or Alexa Fluor 488–anti-rabbit (BioLegend) for 30 min on ice. Cells were washed with PBS and incubated for 30 min with Alexa Fluor 647–anti-CD3ε, washed in PBS, and fixed in 2% paraformaldehyde with 1% BSA. Flow cytometry was performed on an LSR II (BD Biosciences). Data were analyzed using FlowJo version 10 (Tree Star, Ashland, OR).

BV transfer in coculture

Macrophages were prepared and infected as described above for 4 h, and then washed three times to remove extracellular bacteria. P25 $CD4^+$ T cells were isolated by untouched $CD4^+$ selection (as above) and incubated 1:1 with infected macrophages for 24 h with addition of 1 μg/ml Ag85B peptide. Nonadherent cells were collected, washed three times in PBS, stained with Live/Dead Fixable Yellow, Alexa Fluor 647–anti-CD3ε, and PE/Cy7–anti-CDllb, and then fixed. Live/$CD11b^-$/$CD3\varepsilon^+$ cells were sorted on a FACSAria (BD Biosciences). Sorted cells were analyzed for content by Western blot.

Quantifying LAM transfer to T cells by BVs

$CD4^+$ T cells from C57BL/6 mice were incubated overnight with LAM or BVs at equivalent LAM concentrations (10 μg/ml). Cells were washed in PBS, suspended in MojoSort buffer (BioLegend), incubated with biotinylated anti-CD4 Ab (RM4-5; BioLegend) for 15 min at 4°C, washed in MojoSort buffer, incubated with MojoSort streptavidin magnetic beads (BioLegend) for 15 min at 4°C, washed twice in MojoSort buffer, and resuspended. The suspension was exposed to the magnet for 5 min, and supernatant was removed. Magnetic sorting was repeated twice with fresh buffer. Sorted cells were resuspended in sample buffer and analyzed by Western blot.

Aerosol infection of mice and preparation of lung cells

For low- or high-dose aerosol infections, respectively, low- or high-titer H37Rv frozen stocks (1 ml) were thawed, declumped by vortexing with glass beads and centrifugation as above, and diluted by addition of 5 ml of LPS-free sterile water. Bacterial clumps were further disrupted by three sequential aspirations through a sterile 26-gauge hypodermic needle, and 5 ml of the suspension was placed in the glass nebulizer chamber of an inhalation exposure system (Glas-Col, Terre Haute, IN). Female C57BL/6J mice (8–16 wk old, The Jackson Laboratory) were exposed to bacterial aerosol for 50 min with input and output airflow rates set at 7 and 24 l/min, respectively. The initial *M. tuberculosis* inoculum was determined from lung homogenates prepared 1 d postinfection and ranged from 50 to 100 lung CFU/mouse for low-dose infection and 1500 to 2000 lung CFU/mouse for high-dose infection, as developed in prior infection studies (36, 37). At day 28 postinfection, mice were anesthetized with 750 μl of 1.25% 2,2,2-tribromoethanol (Sigma-Aldrich) in 40% 2-methyl-2-butanol (*tert*-amyl alcohol; Sigma-Aldrich) and exsanguinated by cutting the renal arteries. The diaphragm and the lower ribcage were removed to expose the lungs. Lungs were removed and collected directly into gentleMACS C tubes (Miltenyi Biotec) containing 5 ml of PBS, and 125 μl of PBS was added containing collagenase (125 U/ml final concentration; Sigma-Aldrich) and DNase (30 U/ml final concentration; Invitrogen). Lungs were homogenized using a gentleMACS dissociator (Miltenyi Biotec) following the manufacturer's standardized protocol. Lung homogenates were passed through 70-μm nylon filters into 50-ml tubes; 5 ml of ACK buffer was added to each tube to lyse RBCs. Lung cells were pelleted at 1500 rpm for 15 min at 4°C. Supernatants were decanted and pellets were washed once with 5 ml of cold PBS. Cells were counted using a LUNA automated cell counter (Logos BioSystems, Anyang, South Korea).

Purification of CD4 T cells from infected lungs

$CD4^+$ T cells were immunoaffinity purified from lung homogenates by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec). To check for bacterial contamination, an aliquot of these cells was diluted in Middlebrook 7H9 medium containing 0.05% SDS for 10 min at room temperature, serially diluted in PBS, plated on Middlebrook 7H11 agar, incubated at 37°C, and monitored for growth of bacterial colonies. To further purify T cells, the immunoaffinity-purified cells were washed three times in PBS, stained with Alexa Fluor 647–anti-CD3ε mAb and Alexa Fluor 488–anti-CD11b, and then fixed. From this population, $CD11b^-/CD3\varepsilon^+$ cells were isolated by FACS with a FACSAria (BD Biosciences). Sorted cells were checked for purity by flow cytometry and analyzed for LAM content by solubilization in reducing sample buffer (5×10^6 cells/ml; Bio-Rad Laboratories) followed by SDS-PAGE and Western blot analysis as described above (20 μl with 10^5 cell equivalents was loaded in each lane).

Statistical analyses

Data were analyzed using Prism 5 (GraphPad Software, La Jolla, CA). An unpaired Student *t* test with a Welch correction was used to assess significance for individual comparisons where indicated ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

Results

EVs released by M. tuberculosis*–infected macrophages inhibit $CD4^+$ T cell activation*

M. tuberculosis–infected macrophages release EVs (EVs from *M. tuberculosis*–infected macrophages [Inf EVs]). Our recent work (3) demonstrated that Inf EVs consist of a mixture of two distinct vesicle populations: host-derived EVs, identified by host markers CD9 and CD63, and BVs, identified by *M. tuberculosis* lipoglycans and lipoproteins, including LAM (Fig. 1A). Because LAM inhibits TCR signaling and T cell activation (27), we considered the hypothesis that Inf EVs could deliver LAM to inhibit T cell activation. $CD4^+$ T cells were incubated for 1 h with Inf EVs, EVs from uninfected macrophages (Ctr EVs), or medium without EVs; the cells were then stimulated with anti-CD3ε and anti-CD28 Abs in the continued absence or presence of EVs for 24 h. T cell activation was assessed by IL-2 secretion as measured by ELISA. IL-2 production was significantly inhibited by Inf EVs relative to Ctr EVs or medium without EVs (Fig. 1B). We conclude that Inf EVs inhibit T cell activation, whereas Ctr EVs do not. Because the difference between Inf EVs and Ctr EVs is the presence of *M. tuberculosis*–derived BVs in the Inf EV preparation (whereas both contain host-derived exosomes), we inferred that the BVs inhibit T cell activation and proceeded to test that hypothesis directly.

BVs inhibit T cell activation following TCR stimulation

BVs are released by *M. tuberculosis* both during infection of macrophages and in axenic cultures (3, 12). We purified BVs from axenic *M. tuberculosis* cultures by differential centrifugation and size exclusion chromatography. As predicted, BVs contained LAM (as well as LM) (Fig. 2A), revealing a lipoglycan content that is similar to Inf EVs (Fig. 1A). Fractions containing BVs were pooled, and the LAM content of the BV preparation was assessed by Western blot using LAM standards (Fig. 2B); this approach was subsequently used to define concentrations of BVs to achieve a given LAM equivalent concentration. To assess the effect of BVs on $CD4^+$ T cell activation, $CD4^+$ T cells were treated with BVs or LAM at varying concentrations for 1 h and then activated by anti-CD3ε/anti-CD28 TCR stimulation for 24 h in the continuing presence of BVs or purified LAM. Both LAM and BVs produced a dose-dependent inhibition of IL-2 production by T cells (Fig. 2C) (27, 29). Notably, BVs were highly potent and efficacious in inhibiting IL-2 production; a given concentration of purified LAM produced less inhibition than did a concentration of BVs containing

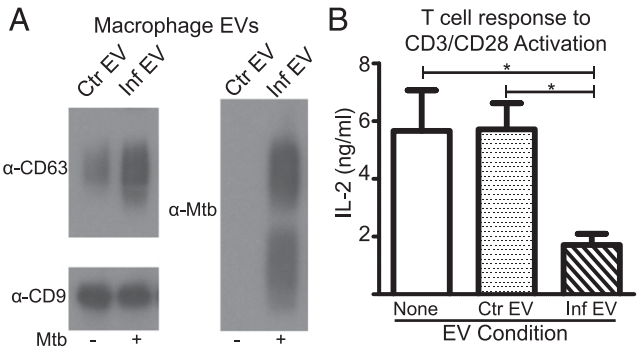

FIGURE 1. EVs from *M. tuberculosis*–infected macrophages inhibit TCR activation of $CD4^+$ T cells. (**A**) EVs were purified from uninfected or *M. tuberculosis*–infected macrophages to produce Ctr EVs or Inf EVs, respectively. EVs (5-μg protein content) were assessed by Western blot for host CD9 and CD63 (left) and *M. tuberculosis* lipoglycans (right, top band represents LAM and bottom band represents LM). (**B**) $CD4^+$ T cells from spleens of C57BL/6 mice were treated for 1 h with Inf EVs or Ctr EVs (100 μg/ml EV protein concentration) and activated for 24 h with 1 μg/ml plate bound anti-CD3ε and 1 μg/ml soluble anti-CD28 in the continued presence of the EVs. IL-2 was measured by ELISA. Data are shown as mean ± SD of triplicate samples from one representative experiment ($n >$ 3 experiments). A Student *t* test with a Welch correction was used to assess statistical significance. $*p \leq 0.05$.

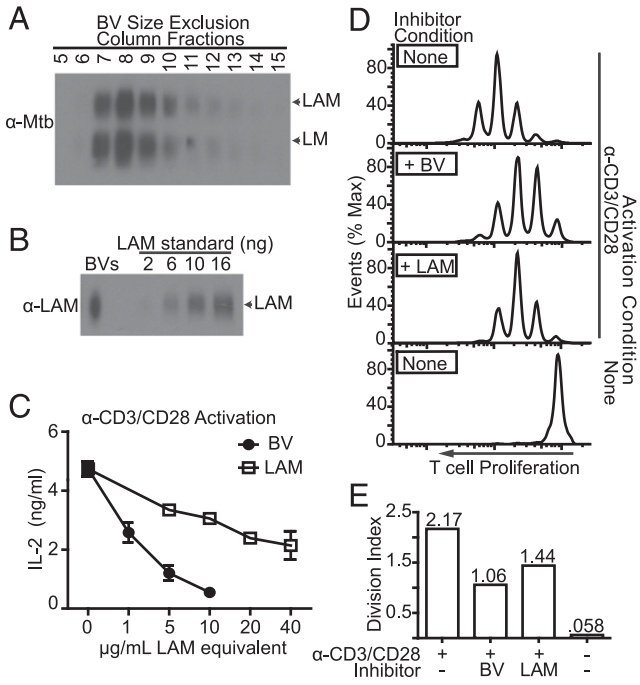

FIGURE 2. BVs from axenic *M. tuberculosis* inhibit TCR-induced activation of $CD4^+$ T cells. (**A**) BVs from 2 l of axenic *M. tuberculosis* culture were purified by differential centrifugation and size exclusion chromatography. Western blot with polyclonal anti–*M. tuberculosis* Ab shows LAM and LM in size exclusion chromatography fractions. (**B**) BV-containing fractions (nos. 6–11) from (A) were pooled, concentrated, and analyzed by Western blot with CS-35 Ab (anti-LAM) in comparison with a purified LAM standard used for quantification. (**C**) $CD4^+$ T cells derived from spleens of C57BL/6 mice were treated for 1 h with BVs or purified LAM and then stimulated for 24 h with 1 μg/ml plate-bound anti-CD3ε and 1 μg/ml soluble anti-CD28 in the continued presence or absence of BVs or LAM. IL-2 was measured by ELISA. (**D** and **E**) Proliferation was measured by dye dilution in 72 h cultures by flow cytometry. Data are shown as mean ± SD of triplicate samples from one representative experiment ($n = 3$ experiments).

the same amount of LAM, and the maximum level of inhibition was greater with BVs than LAM (Fig. 2C). LAM and BV-mediated inhibition of IL-2 production were both specific for IL-2 induced by TCR activation, as IL-2 production induced by PMA/ionomycin was not inhibited by BVs or LAM (data not shown). $CD4^+$ T cells incubated with BVs for 24 h were ~90% viable as assessed by Live/Dead staining (data not shown). Both BVs and LAM also inhibited proliferation of $CD4^+$ T cells in response to anti-CD3ε/anti-CD28 TCR stimulation (Fig. 2D). The number of divisions was reduced by ~50% in the presence of BVs or ~30% in the presence of LAM (Fig. 2E). $CD4^+$ T cells demonstrated minimal IL-2 production or proliferation when activated with anti-CD28 alone, whereas activation with anti-CD3ε alone was robust and was also inhibited by prior BV treatment (Supplemental Fig. 1), indicating that BVs inhibit TCR/CD3-mediated T cell activation independent of CD28 costimulatory signaling mechanisms. Inhibition of $CD4^+$ T cell proliferation was directly mediated by cell-intrinsic effects of BVs, as opposed to induction of inhibitory molecules acting intercellularly, as BV-treated T cells did not inhibit proliferation of cocultured $CD4^+$ T cells that were not directly exposed to BVs (Supplemental Fig. 2). We conclude that BVs, similar to LAM, inhibit T cell activation induced through TCR signaling, as manifested by reduced T cell proliferation and production of IL-2. Moreover, BVs are particularly potent at inhibiting T cell responses.

The greater potency of BVs relative to LAM for inhibiting T cell responses may be due to additional inhibitory components contained within BVs, or other possible mechanisms such as more efficient T cell uptake of LAM from BVs relative to purified LAM. We first examined whether BVs mediate transfer of LAM to T cells with greater efficiency than purified LAM. T cells were incubated with BVs or purified LAM, and the T cells were then separated from free BVs by immunolabeling with MojoSort magnetic streptavidin-conjugated beads coated with biotinylated anti-CD4 Ab. The T cells were then washed, solubilized, and assessed for LAM content by Western blot. LAM was associated with T cells after incubation with either LAM (10 μg/ml) or BVs (10 μg/ml LAM equivalent concentration). At the same LAM equivalent concentration, BVs did not result in more LAM delivery to T cells than purified LAM (Fig. 3A), indicating that LAM delivery efficiency is not the explanation for the potency of BVs in inhibiting T cell activation. Accordingly, we focused on the possibility that BVs contain other inhibitors, in addition to LAM, which increase their ability to inhibit T cell activation. *M. tuberculosis* produces other lipoglycans related to LAM, for example, LM, and smaller glycolipids, for example, PIMs (38–40). BVs contain LM as well as LAM (Figs. 1B, 2A), and possibly other lipoglycan/glycolipid species. Hence, we hypothesized that LM and possibly other lipoglycans/glycolipids contribute to the ability of BVs to inhibit $CD4^+$ T cell activation. We incubated $CD4^+$ T cells with purified LAM, LM, PIM1/2, or PIM6 for 1 h; the cells were then incubated for 24 h with anti-CD3ε/anti-CD28 in the continued presence of the lipoglycans/glycolipids. T cell IL-2 production was inhibited by all of these species (PIM1/2 to a lesser extent; Fig. 3B). These data demonstrate the potential for additional lipoglycan and glycolipid components of BVs to inhibit T cell activation; species other than the ones we have tested may also contribute.

BVs traffic LAM and other lipoglycans from infected macrophages to T cells

Our model suggests that BVs mediate the trafficking of LAM and other lipoglycans from infected macrophages to T cells. To explore this hypothesis, we extended our observation that BVs can traffic LAM to T cells (Fig. 3A) by assessing BV delivery of additional

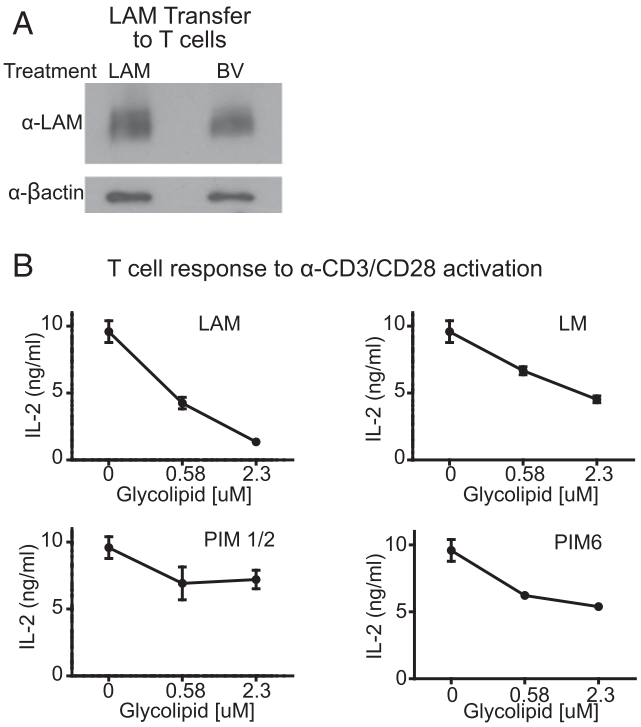

FIGURE 3. Multiple *M. tuberculosis* lipoglycans can contribute to BV inhibition of $CD4^+$ T cells. (**A**) $CD4^+$ T cells from spleens of C57BL/6 mice were incubated for 24 h with BVs (10 μg/ml LAM equivalent concentration) or LAM (10 μg/ml), repurified by magnetic sorting, and assessed for LAM content by Western blot with CS-35 Ab. (**B**) $CD4^+$ T cells were treated with lipoglycans LAM, LM, PIM1/2, or PIM6 for 1 h prior to activation with plate-bound anti-CD3ε (1 μg/ml) and soluble anti-CD28 (1 μg/ml) for 24 h. IL-2 was measured by ELISA. Data are shown as mean ± SD of triplicate samples from one representative experiment (n = 3 experiments).

molecules (e.g., LM) and by tracking the transfer of lipoglycans from infected macrophages to T cells. First, we used a polyclonal anti–*M. tuberculosis* Ab that detects LM as well as LAM. $CD4^+$ T cells were incubated with BVs at 10 μg/ml LAM equivalent concentration for 24 h and then washed and stained with this Ab. Flow cytometry revealed association of *M. tuberculosis* components with T cell membranes (Fig. 4A). To track transfer of lipoglycans from infected macrophages to T cells, we cocultured *M. tuberculosis*–infected macrophages with *M. tuberculosis* Ag85B-specific P25 $CD4^+$ T cells, separated the T cells from the macrophages by FACS of nonadherent cells, solubilized the T cells, and performed Western blots with the polyclonal anti–*M. tuberculosis* Ab. In the T cell lysates, we observed bands corresponding to the positions of LAM and LM (Fig. 4B). We conclude that BVs can traffic LAM and other *M. tuberculosis* content, such as LM, from infected macrophages to T cells.

LAM traffics to $CD4^+$ T cells in the lung during in vivo infection

We extended our in vitro observations that *M. tuberculosis* lipoglycans traffic from infected macrophages to T cells by using an in vivo mouse model of *M. tuberculosis* infection. C57BL/6J mice were infected at low aerosol dose (50–100 lung CFU) or high aerosol dose (1500–2000 lung CFU) and housed for 28 d prior to lung harvest, immunoaffinity purification of $CD4^+$ T cells from lung homogenates (yielding 90% purity of $CD4^+CD11b^-$ cells), and further purification by FACS to isolate $CD3^+CD4^+CD11b^-$ cells of ≥99% purity. These cells were solubilized, and lysates were analyzed by SDS-PAGE and Western blot for LAM. Fig. 5

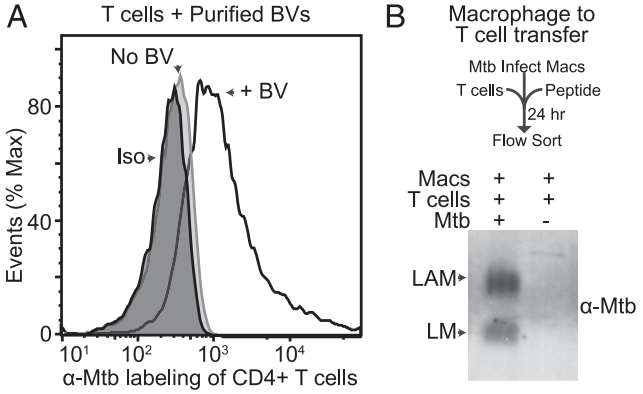

FIGURE 4. BVs facilitate the trafficking of LAM and LM from infected macrophages to $CD4^+$ T cells. (**A**) $CD4^+$ T cells from spleens of C57BL/6 mice were incubated with or without BVs (10 μg/ml LAM equivalent concentration) for 24 h, stained with anti-CD3ε and anti–*M. tuberculosis* Abs or isotype control Abs, fixed, and assessed by flow cytometry with polyclonal anti–*M. tuberculosis* Ab. Open outline indicates BV-treated T cells anti–*M. tuberculosis* Ab; light gray outline indicates untreated T cells anti–*M. tuberculosis* Ab; dark gray outline indicates BV-treated T cells with isotype control Ab. (**B**) P25 $CD4^+$ T cells were cultured with *M. tuberculosis*–infected or uninfected macrophages for 24 h with the addition of P25 peptide (1 μg/ml). $CD4^+$ T cells were then purified from macrophages by FACS with exclusion of $CD11b^+$ cells and selection of $CD3\varepsilon^+$ cells. Purified T cells were assessed for *M. tuberculosis* lipoglycan content by Western blotting with polyclonal anti–*M. tuberculosis* Ab.

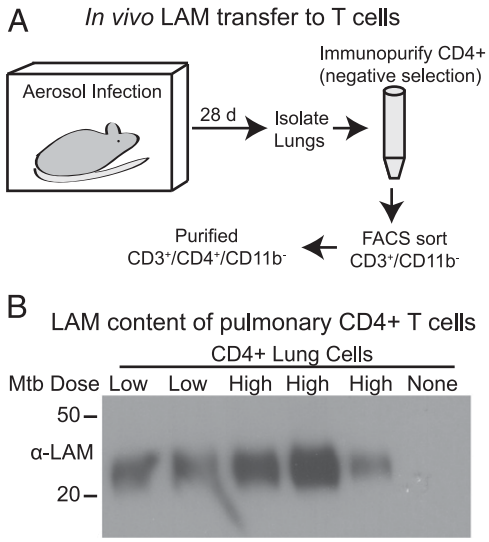

FIGURE 5. LAM trafficking to $CD4^+$ T cells in vivo. (**A**) Schematic of experimental design. Mice were aerosol infected with *M. tuberculosis* H37Rv at low or high dose. After 28 d of infection, lungs were harvested and $CD4^+$ T cells were isolated by immunoaffinity purification. $CD4^+$ T cells were further purified by FACS to select $CD3^+CD11b^-$ cells (≥99% purity), providing the preparation of $CD3^+CD4^+CD11b^-$ cells for LAM analysis. (**B**) Western blot analysis of LAM content in lysates of purified $CD3^+CD4^+CD11b^-$ lung cells after low-dose or high-dose aerosol infection of mice with *M. tuberculosis* H37Rv ("None" indicates lysate of immunoaffinity-purified $CD4^+$ T cells from uninfected mouse lung). Each lane represents lysate from 10^5 cells derived from a single animal (the two low-dose infections are from a single experiment, and the three high-dose infections are from another experiment). LAM was detected in $CD3^+CD4^+$ $CD11b^-$ cells from lungs of mice after both low- and high-dose infections, although with some variability in level.

shows that LAM is associated with $CD4^+$ T cells from lungs of mice after low- or high-dose aerosol infection with *M. tuberculosis*. LAM was not detected in samples containing lysates of a similar number of immunoaffinity-purified $CD4^+$ lung cells from uninfected mice, demonstrating specificity of the Western blot assay (Fig. 5). The FACS-purified lung T cell preparation was free of *M. tuberculosis* CFU, excluding the possibility that the LAM signal was due to intact extracellular bacteria present in the preparation. These results support the conclusion that LAM trafficking to $CD4^+$ T cells occurs in vivo during infection with *M. tuberculosis*.

BVs inhibit activation of naive and Th1 effector $CD4^+$ T cells

The results reported above indicate that BVs can inhibit T cell activation but do not explore the relative ability of BVs to inhibit naive versus effector T cells. The potential ability to inhibit effector T cell responses is important, because Th1 responses are induced by *M. tuberculosis* infection and are critical for immunologic control of the infection. Moreover, *M. tuberculosis*–infected macrophages are present primarily in the lung (and BVs are likely also primarily in the lung), where they are more likely to encounter effector T cells. In contrast, most naive T cells reside in secondary lymphoid organs, where they are less likely to encounter infected macrophages (and presumably BVs). These considerations suggest that BV-mediated inhibition of T cell responses may have most impact on effector T cells in the lung as opposed to priming of naive T cell responses. Our prior studies showed that LAM inhibits responses of both naive and effector T cells (28, 29). Therefore, we hypothesized that *M. tuberculosis* BVs inhibit activation of Th1 effector as well as naive $CD4^+$ T cells. To test this hypothesis, we generated naive $CD4^+$ T cells and in vitro–primed Th1 effector cells and tested the ability of BVs to inhibit these cell populations. *M. tuberculosis* BVs inhibited anti-CD3ε/anti-CD28 activation of naive $CD4^+$ T cells from C57BL/6 mice as measured by both IL-2 production and cell proliferation (Fig. 6A, 6B). Similarly, BVs inhibited activation of naive P25 $CD4^+$ T cells by antigenic peptide presented by fixed macrophages (Fig. 6C). BVs also inhibited activation of Th1 effector T cells by anti-CD3ε/anti-CD28 as assessed by induction of IL-2 and IFN-γ (Fig. 6D, 6E). We conclude that BVs are capable of inhibiting activation of both naive and effector cells.

BVs induce anergy in $CD4^+$ T cells

In addition to its ability to inhibit naive T cell activation, LAM induces anergy when present during TCR stimulation of naive T cells, and this is associated with increased expression of GRAIL (29). GRAIL may also be important in regulating effector T cell responses, as GRAIL-knockout Th1 cells have been reported to overproduce IFN-γ (41). We tested whether BVs lead to the same anergy phenotype as the single component LAM. $CD4^+$ T cells from spleens of C57BL/6 mice were treated with BVs (10 μg/ml LAM equivalent concentration) or LAM (10 μg/ml) prior to activation; this treatment was found to upregulate expression of GRAIL as detected at both the mRNA and protein levels (Fig. 7A, 7B). To test for induction of functional anergy, naive $CD4^+$ T cells were incubated with BVs during primary stimulation with anti-CD3ε/anti-CD28, rested for 5 d, and then restimulated (without BVs) with anti-CD3ε/anti-CD28. The presence of BVs during primary stimulation caused decreased proliferation upon restimulation (Fig. 7C), although IL-2 production upon restimulation was not inhibited by the presence of BVs during primary stimulation (Fig. 7D). Similar results were obtained when T cells were stimulated with peptide presented by fixed macrophages (upon both initial activation and restimulation, Fig. 7E, 7F). In contrast to naive $CD4^+$ T cells, Th1 cells first exposed to BVs during stimulation of the effector T cells (first effector stimulation) were

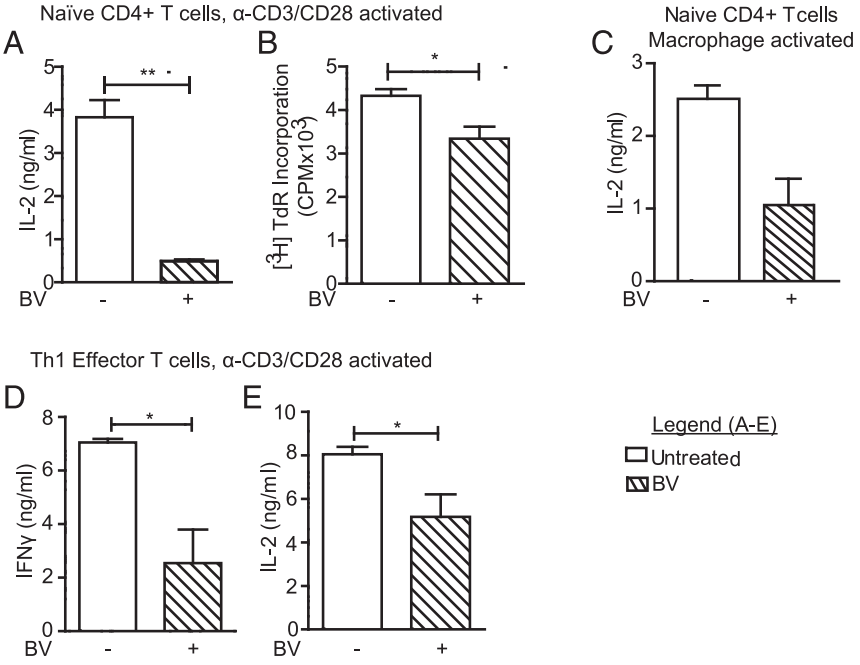

FIGURE 6. BVs inhibit the activation of both naive and Th1 effector CD4$^+$ T cells. (**A** and **B**) Naive CD4$^+$ T cells from C57BL/6 mice were incubated for 1 h with BVs at 10 μg/ml LAM equivalent concentration and stimulated with anti-CD3ε/anti-CD28 in the continued presence of BVs. After 24 h, IL-2 was measured in the supernatant by ELISA, and proliferation was measured after 48 h by [^{3}H]thymidine incorporation. (**C**) Naive P25 CD4$^+$ T cells were activated with and without BVs as in (A), except T cells were stimulated with fixed macrophages presenting Ag85B peptide. (**D** and **E**) Th1 effector CD4$^+$ T cells were prepared from splenocytes of C57BL/6 mice and activated as in (A); after 24 h supernatants were harvested, and IFN-γ (D) and IL-2 (E) were measured by ELISA. Data are shown as mean ± SD of triplicate samples from one representative experiment [n = 3 experiments, except n = 2 for (C)]. A Student t test with a Welch correction was used to assess statistical significance. *$p \leq 0.05$, **$p \leq 0.01$.

not impaired in their ability to respond to a subsequent stimulation (data not shown), although their response to the first effector stimulation was impaired (Fig. 6D, 6E). We conclude that BVs are able to induce a state of partial anergy when present during primary stimulation of naive T cells; this state is characterized by decreased proliferative response to secondary stimulation but persistent IL-2 responses. Alternatively, BV inhibition of effector T cells appears to be limited to the period when BVs interact with effector T cells and does not involve lasting anergy beyond the period of BV exposure. This could result in local inhibition of Th1 responses at sites of infection without systemic inhibition of T cell responses.

Discussion

M. tuberculosis employs many tactics to facilitate suppression and evasion of host immune responses. In this study, we show evidence for a mechanism by which *M. tuberculosis* can directly inhibit CD4$^+$ T cell responses via release of LAM and other lipoglycans in BVs, allowing their delivery to T cells. The role of LAM in inhibiting T cell responses has been previously established (27–30, 42, 43), but evidence has been lacking for a mechanism by which LAM could traffic from the bacterium, typically sequestered intracellularly within macrophage phagosomes, to reach T cells. Studies of EVs from *M. tuberculosis*–infected macrophages and BVs from axenic *M. tuberculosis* culture have shown that lipoglycans such as LAM are released from *M. tuberculosis* in vesicles (3, 4, 12), but the ability of these vesicles to deliver LAM to T cells has been unaddressed. In this study, we have demonstrated the ability of BVs to deliver *M. tuberculosis*–derived materials, including LAM, to CD4$^+$ T cells, resulting in inhibition of T cell responses. Moreover, we have demonstrated that LAM is transferred to pulmonary CD4$^+$ T cells in vivo during infection with virulent *M. tuberculosis*, supporting the in vivo relevance of this mechanism.

Anti-CD3ε/anti-CD28 TCR stimulation was used as the experimental approach for most in vitro experiments with T cells; this approach has an important advantage over stimulating T cells with APCs in that one can exclude the possibility that BVs directly affect the APCs and only affect the T cells indirectly. Our results with this reductionist system demonstrate that BVs do directly impact T cells and induce T cell–intrinsic inhibitory mechanisms. Additionally, we confirmed these results in studies with fixed APCs (macrophages) presenting an *M. tuberculosis* antigenic peptide from Ag85B to TCR transgenic T cells specific for this peptide. The data shown in Figs. 6C, 7E, and 7F show that BVs also inhibit responses of CD4$^+$ T cells to macrophages presenting specific peptide Ag. Thus, the BV inhibitory effect is observed in this more physiologically relevant system and is not limited to the anti-CD3/anti-CD28 stimulation system.

Our results also address the potentially conflicting influences of LAM and *M. tuberculosis* lipoproteins in determining the effects of BVs on T cell responses. Studies with purified *M. tuberculosis* lipoproteins have shown that their TLR2 agonist activity provides a costimulatory effect to enhance CD4$^+$ T cell activation (18, 44). Studies of EVs from *M. tuberculosis*–infected macrophages and BVs from axenic *M. tuberculosis* cultures have shown that these vesicles contain *M. tuberculosis* lipoproteins (e.g., LpqH and LprG) as well as lipoglycans (e.g., LAM) (1, 3, 4, 6, 12). Because BVs contain a mixture of these lipoproteins (with potential activating influence) and lipoglycans (with potential inhibitory influence), the balance of these influences in affecting the outcome of T cell responses was previously unclear. In our present studies, the ability of BVs to inhibit T cell responses indicates that the inhibitory mechanisms dominate in the context of BV interactions with T cells.

We observed that BVs inhibited CD4$^+$ T cell responses to a greater degree than purified LAM at the level contained within the BVs (Fig. 2C, 2D). We hypothesized that this could be due to more efficient transfer of LAM into T cell membranes when delivered by BVs as opposed to purified LAM preparations, or due to inhibitory contributions of other components of the BVs. We determined that the LAM transfer to T cells is not greater with BVs relative to purified LAM (Fig. 3A). Alternatively, we found that *M. tuberculosis* lipoglycans that can inhibit CD4$^+$ T cell activation include LM as well as LAM (Fig. 3B); both LM and LAM were detected in BVs by Western blot (Figs. 1A, 2A) and were transferred to T cells cocultured with infected macrophages (Fig. 4B). Additionally, the smaller glycolipid species PIM1/2 and PIM6 also inhibited CD4$^+$ T cell responses (Fig. 3B); although our experiments did not directly address the level of PIM species in BVs,

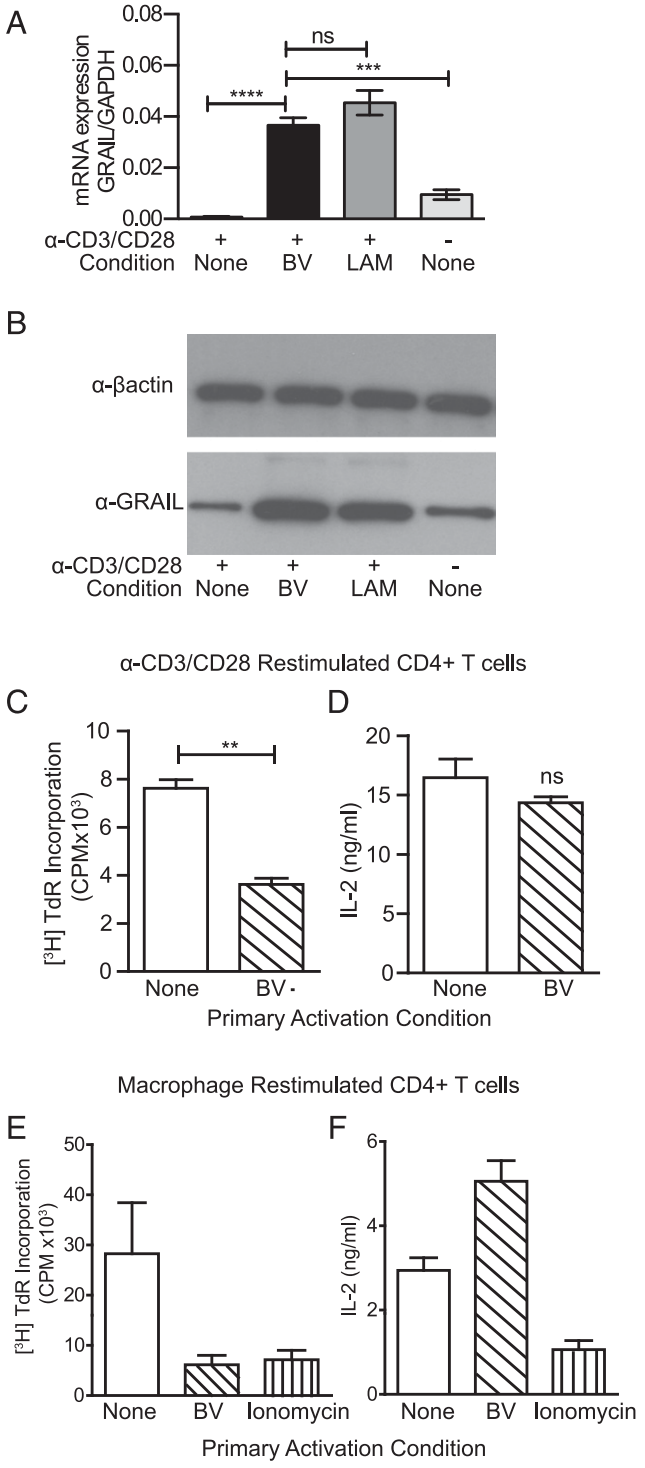

FIGURE 7. BVs induce GRAIL expression and functional anergy in naive CD4$^+$ T cells. (**A**) CD4$^+$ T cells were isolated from spleens of C57BL/6 mice and activated with anti-CD3ε/anti-CD28 in the presence or absence of BVs (10 μg/ml LAM equivalent concentration) or LAM (10 μg/ml). GRAIL expression was measured by quantitative PCR after 24 h. (**B**) Western blot analysis of GRAIL protein expression in CD4$^+$ T cells treated as in (A). (**C** and **D**) Naive CD4$^+$ T cells were activated as in Fig. 6A in the presence or absence of BVs, rested for 5 d with 10 ng/ml IL-7, and then restimulated with anti-CD3ε/anti-CD28. After 48 h of restimulation, proliferation was measured by addition of [^{3}H]thymidine for an additional 16 h (C). After 24 h of restimulation, supernatants were harvested and IL-2 was measured by ELISA (D). (**E** and **F**) Naive P25 CD4$^+$ T cells were activated for 48 h with fixed macrophages presenting Ag85B peptide in the presence or absence of BVs or ionomycin, rested for 5 d with 10 ng/ml IL-7, and restimulated with fixed macrophages and peptide. Proliferation and IL-2 were measured as in (C) and (D). Data are shown as mean ± SD of triplicate samples from one representative experiment [$n > 3$; except (E) and (F), $n = 2$]. A Student t test with a Welch correction was used to assess statistical significance. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

they have been detected in such vesicle preparations in other studies (12, 44–46). We conclude that multiple *M. tuberculosis* lipoglycans and glycolipids can contribute to the inhibitory capacity of BVs. Whether these lipoglycans and glycolipids induce the same inhibitory mechanisms as LAM remains to be determined.

The mechanisms for association of LAM and other lipoglycans/glycolipids with T cells remain unclear. Previous work has demonstrated close association of LAM with T cell membranes; LAM resists removal from T cells by washing, and it colocalizes with lipid raft proteins, likely representing integration into the T cell plasma membrane (27, 29). We demonstrate in these studies that BV components can be detected by flow cytometry as associated with the T cell surface, and are not removed by washing (Figs. 3A, 4A); this may reflect transfer of BV contents to the T cell plasma membrane, although this has not been strictly determined. BVs from nonmycobacterial species are known to use membrane lipid rafts to facilitate transfer of their content to target cells (47–50). Further studies will be necessary to determine the mechanisms for transfer of content from *M. tuberculosis* BVs to T cells.

Direct regulation of T cell responses by *M. tuberculosis* BVs presents a new mechanism of immune regulation by *M. tuberculosis* that is of potential significance given the important roles of T cells in limiting *M. tuberculosis* infection (51–55). Our results demonstrate that activation of CD4$^+$ T cells by a TCR stimulus is inhibited by BVs, resulting in decreased T cell responses (Figs. 1, 2). BVs did not reduce responses of T cells to activation with PMA/ionomycin, indicating that the BV effect was not due to generalized toxicity and suggesting specific relevance to activation of T cells via TCR signaling. This finding parallels our earlier observation that LAM inhibits early TCR signaling steps (27, 28). This parallel is consistent with the hypothesis that LAM contained within the BVs is an active mediator of the inhibitory effect, although there may be contributions of other lipoglycans with similar effects, or perhaps other *M. tuberculosis*–derived molecules.

We investigated the ability of BVs to inhibit responses by different CD4$^+$ T cell subsets. Some of our experiments (Figs. 1–4, 7A, 7B) used T cells purified from splenocytes by immunomagnetic isolation of untouched CD4$^+$ cells; these cells may be comprised primarily of naive CD4$^+$ T cells, but they were not selected for expression of naive T cell markers. To address this issue, we performed experiments with untouched naive CD4$^+$ T cells purified from splenocytes by immunomagnetic depletion of CD44^{hi} memory T cells and of lineages other than CD4$^+$ T cells (Figs. 6A–C, 7C–F), and we investigated the ability of BVs to inhibit responses by effector T cells by using in vitro–primed Th1 cells (Fig. 6D, 6E). These studies demonstrated the ability of BVs to inhibit both naive and effector CD4$^+$ T cell responses during the period of exposure of T cells to BVs (lasting anergy after the exposure period was seen only with naive T cells). Because exposure of T cells to BVs may occur primarily at the site of infection in the lung, where T cells are primarily of the effector subset, we propose that the most significant potential role for BV regulation of T cell responses may lie in the inhibition of Th1 responses. The inability of effector T cells to adequately respond

to clear infection has been established (56); contributing mechanisms may include inhibition of Ag presentation (22, 57–62) as well as other mechanisms to reduce T cell responses such as direct inhibition of Th1 responses by BVs.

Our results establish a new mechanism for delivery of *M. tuberculosis* components to T cells, enabling *M. tuberculosis* to directly influence and inhibit T cell responses in a manner distinct from other known immune evasion mechanisms, for example, those involving macrophages. BVs are produced by *M. tuberculosis* within macrophage phagosomes and traffic to the extracellular space by exocytosis. This enables them to reach T cells, where they deliver *M. tuberculosis* molecules, including lipoglycans such as LAM, which result in inhibition of T cell responses. This model advances understanding of immune evasion mechanisms and pathogenesis of *M. tuberculosis* infection. It also raises the possibility that further research to understand these mechanisms may enable the design of host-directed therapeutic approaches to rescue responsiveness of T cells in *M. tuberculosis* infection.

Disclosures

The authors have no financial conflicts of interest.

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