Mycoplasma contaminants present in exosome preparations induce polyclonal B cell responses

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Abstract: Exosome fractions of dendritic cells (DC) produced in long-term cultures (LTC) were found to contain *Mycoplasma* contaminants. In this study, *Mycoplasma*-infected, -uninfected, and -re-infected cultures of DC and control cell lines have been compared for their capacity to activate lymphocytes. Using differential centrifugation, size fractionation, and inhibition assays, it has been possible to map *Mycoplasma* to the exosome or vesicle fraction purified from culture supernatant (CSN). *Mycoplasma* fractions were shown to induce proliferation of B and not T cells. The B cell response was sensitive to mitomycin C and primaquine, both known antibiotics, but resistant to protease and DNase, suggesting a role for lipoproteins. *Mycoplasma*-contaminated exosome fractions of LTC-DC were potent mitogens for naive B cells and promoted Ig secretion. In contrast to the polyclonal B cell mitogen LPS, they were unable to promote Ig isotype switching. They induced polyclonal activation of all B cell subsets, including naive B cells, the T1 and T2 subsets of transitional B cells, marginal zone (MZ), and follicular (FO) B cells. The B cell proliferative response was not antigen-specific and occurred independently of T cell help. Implications for autoimmune sequelae associated with *Mycoplasma* infection are discussed along with the possibility that primaquine could be an effective treatment for *Mycoplasma* infection in humans. This study highlights the close association between exosomes and infectious agents like *Mycoplasma* and cautions about purification procedures for preparation of exosomes for studies on immunity. *J. Leukoc. Biol.* 82: 000–000; 2007.

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INTRODUCTION

Splenic long-term cultures (LTC) generate nonadherent cells resembling immature myeloid dendritic cells (DC) with a CD11c⁺ CD11b⁺ DEC-205^{low} 33D1[−] F40/80[−] CD4[−] CD8α[−] DC phenotype; high expression of FcγII/IR, low expression of MHC-II, CD80, and CD40, and high endocytic capacity [1, 2]. LTC-DC are also refractory to LPS, TNF-α, and CD40L-induced maturation, a key step for DC in initiating T cell

responses [3]. While they can endocytose protein antigen and process this for presentation to CD4⁺ T cells, their capacity to stimulate naive T lymphocytes is limited. Many LTC-DC cannot generate a mixed leukocyte response (MLR) [1, 3], one of the first discovered functional features of DC [4, 5]. However, all LTC-DC lines tested were capable of stimulating the T_H2 CD4⁺ cell line D10G4.1 to proliferate and produce lymphokines [1, 2].

Culture supernatant (CSN) conditioned by LTC-DC has been shown to contain numerous exosome-like vesicles with similar structural properties to vesicles resembling endosomes of cultured cells [6–9]. These are thought to arise from the fusion of multivesicular endosomes with plasma membrane, resulting in expulsion of vesicles, or exosomes, into the extracellular space. Vesicles from CSN of LTC-DC display endosomal proteins like LAMP-I, but not plasma membrane proteins like CD86, suggesting that they were derived from endosomes late in the endocytic pathway [6]. Differential centrifugation was adopted for vesicle isolation according to published methods for exosome purification for their investigation as modulators of immune responses [7, 8]. Initially, exosome preparations were found to be poor stimulators of lymphocytes in vitro [6]. Here, we report that a few LTC-DC, which produce exosome fractions with potent capacity to stimulate B cell but not T cell proliferation, contain *Mycoplasma* isolated along with exosomes.

Mycoplasma are the smallest, self-replicating genus of bacteria and contain many potentially mitogenic lipoproteins [10–12]. Depending on their stage in development, *Mycoplasma* can vary in form from spherical to filamentous in shape, ranging from 125 nm to 150 μm in size [13], similar to the size distribution of APC-derived exosomes [6–9]. *Mycoplasma* have been reported to contaminate up to 80% of surveyed cell lines with limited or no effect on the growth of cultured cells [14]. Cultured DC lines found to be infected with *Mycoplasma* were therefore used to test the capacity for *Mycoplasma* to stimulate B cells.

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MATERIALS AND METHODS

Animals

Inbred mice were bred in the John Curtin School of Medical Research [Australian National University (ANU), Canberra, ACT, Australia] under specific pathogen-free conditions. B10.A[2R] and C57BL/6J male and female mice aged between 5 and 15 wk were used throughout. C57BL/6J *Ia^{-/-}* and C57BL/6J *CD40^{-/-}* mice were purchased from the Walter and Elisa Hall Institute (Melbourne, VIC, Australia). Mice were housed and handled according to the guidelines of the ANU Animal and Experimental Ethics Committee.

Cell lines

These included the monkey fibroblast cell line COS-7, the human melanoma cell line MM170, the human B cell line JY, and the murine melanoma cell line B16, kindly provided by H. Warren, J. Abbey, and C. van Broekhoven (ANU). COS-7 was maintained in sDMEM; MM170, JY, and B16 were maintained in RPMI-based medium.

Preparation and treatment of DC

LTC were generated as primary cultures of spleen from inbred mice as described previously [1, 2]. Cells were cultured in supplemented DMEM (Gibco BRL, Grand Island, NY, USA) containing 10% FCS sDMEM [3], and cultured in 5% CO₂ in air at 37°C, with the medium changed as needed. After several weeks, LTC comprised a stromal cell monolayer of fibroblastic and endothelial cells that continuously supported the proliferation and differentiation of hemopoietic cells into nonadherent dendritic-like cells. When required, LTC-DC were harvested at medium change as nonadherent cells released into CSN.

For treatment of cells before experimentation, nonadherent LTC-DC were collected and cultured without stroma in 2 ml sDMEM in the wells of a 24-well tissue culture plate for 24 h at 37°C in 5% CO₂. Primaquine treatment involved incubation of 10⁶ LTC nonadherent cells for 24 h at 37°C with 100 µg/ml of drug. Cells were then washed twice in medium before use. Cell viability was assessed by Trypan Blue exclusion. For mitomycin C treatment, 10⁶ LTC nonadherent cells were cultured with 50 µg/ml mitomycin C for 20 min at 37°C in the dark. Cells were washed three times by resuspension in 10 ml sDMEM followed by centrifugation at 300 *g* for 5 min. Cells were then cultured for 24 h prior to collection of CSN for exosome preparation.

Vesicle or “exosome” preparation

Differential centrifugation was used to isolate vesicle fractions of CSN based on previously published methods [7, 8]. Cells (5×10^3 – 1×10^7) were cultured in ≤ 10 ml sDMEM for 24 h in the absence of stroma to allow the release of vesicles into the CSN. Cells were then depleted by centrifugation at 300 *g* for 10 min at 4°C. CSN was then centrifuged at 10,000 *g* for 30 min at 4°C to sediment cell debris. In some cases, cell debris was removed from the CSN by filtration through a 800-nm cellulose cut-off filter (Millipore, Bedford, MA, USA). The vesicle fraction was then isolated by centrifugation of the remaining CSN at 200,000 *g* for 60 min at 4°C in 26 ml ultracentrifugation bottles (Nalge Company, Rochester, NY, USA) using a Ti70-fixed angle rotor (Beckman Instruments, Porterville, CA, USA). Further fractionation involved passage through 450 nm and 220 nm cut-off filters.

Enzyme treatment of exosomes

Exosome fractions were exposed to protease and DNAase treatment. Exosome fractions from 2.5 – 7×10^5 LTC-DC were incubated with 5 µg/ml pronase (protease from *S. griseus*; Calbiochem-Behring Corp. San Diego, CA, USA) or 2.5 U/75 µl DNAase (Amersham, Little Chalfont, Buckinghamshire, UK) for 30 min at 37°C. Exosomes were then diluted to 2.5 – 7×10^4 cell equivalents per milliliter in sDMEM before assessment of their lymphocyte stimulatory capacity. As a control for pronase activity in these assays, CD40L was treated in the same manner and tested for loss of capacity to stimulate lymphocytes in a MLR after pronase treatment.

Antibody staining of cells and flow cytometry

Before staining with specific antibody, cells were incubated for 15 min on ice in 25 µl sDMEM/0.1% NaN₃ containing 40 µg/ml of Fc block (antibody

2.4G2) specific for FcγII/III (CD32/CD16) (Becton Dickinson, Franklin Lakes, NJ, USA). This step was excluded if binding of antibody to FcγII/III was being assessed or if second-stage reagents bound cross-reactively to 2.4G2.

For staining cell surface markers, cells were labeled with antibody in a total volume of 50 µl sDMEM/0.1% NaN₃ for 30 min on ice. Antibodies were purchased from Becton Dickinson, as affinity purified reagents specific for CD11c (HL3), DEC-205 (NLDC-145), CD86 (GL1), CD3ε (C363-28B), CD4 (GK1.5), CD8 (53-6.7), CD21/CD35 (7G6), CD23 (B3B4), CD69 (H1.2F3), DC (33D1), macrophages (F4/80 and M1/70), B220 (RA3-6B2), Thy1.2 (53-2.1), MHC-II (IA^b) (TIB120), IgD (11-26C.2a), IgM (11/41), IgG₁ (A85-1), IgG_{2a} (R19-15), IgG_{2b} (R13-3), IgG₃ (R40-82), IgA (C10-1) and IgE (R35-72). Cells were then washed 3 times in PBS/0.1% NaN₃. As necessary, cells were incubated for 30 min on ice with a second stage fluorochrome-conjugated reagent, either FITC-conjugated sheep IgG anti-mouse Ig (Serotec, Oxford, UK), FITC-conjugated goat F(ab')₂ anti-rat Ig (Serotec) or avidin-PE (Becton Dickinson) in sDMEM/0.1% NaN₃, followed by three washes in PBS/0.1% NaN₃. For multicolor staining, the same incubation and washing procedure was repeated for the addition of other specific antibodies and fluorochrome-conjugated secondary reagents as required. Specific binding of antibody was monitored with isotype-matched control antibodies or medium in place of antibody.

For intracellular labeling, cells (2×10^5 – 10^6) were washed three times in PBS, then fixed by resuspension in ice-cold 4% paraformaldehyde/PBS while vortexing, followed by incubation for 5 min at 20°C. After washing in PBS, cells were permeabilized by resuspension in 0.5% Saponin/sDMEM/0.1% NaN₃ for 10 min at 20°C. Cells were then incubated with Fc block for 15 min followed by a 30-min incubation with specific antibody diluted in 0.5% Saponin/sDMEM/0.1% NaN₃. Cells were then stained as usual except that washes were performed in 0.5% Saponin/sDMEM/0.1% NaN₃.

Flow cytometry was performed in a FACSort flow cytometer (Becton Dickinson). Cell debris was gated out using a forward scatter threshold of 50, and 10,000–50,000 events were collected. Data analysis was performed using CellQuest software (Becton Dickinson) with postacquisition gating to obtain information on cell subsets. In some experiments, propidium iodide (PI) (2.5 µg/ml) was added to cells in PBS prior to flow cytometry for discrimination of dead cells.

CFSE labeling of cells

For assessment of lymphocyte proliferation, cells were stained with 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester [CFSE: Molecular Probes, OR, USA]. Proliferation was detected by successive dilution of stain with each cell division, measured as a decrease in fluorescence by flow cytometry. Cells ($\leq 10^7$ /ml) were resuspended in 1 ml sDMEM and mixed by vortexing with CFSE to give a final concentration of 5 mM. Cells were then incubated at room temperature for 5 min before lysis of red blood cells (RBC) with lysis buffer [0.14 M NH₄Cl, 0.017 M Tris-base (pH 7.5)], followed by three washes in medium.

Preparation and purification of effector lymphocytes

Spleens or lymph nodes were harvested from mice euthanized by cervical dislocation. Single-cell suspensions were prepared by forcing tissue through a fine wire mesh followed by repeated pipetting in 5 ml sDMEM. Cells were sedimented by centrifugation at 300 *g* for 5 min for CFSE labeling, followed by RBC lysis. Cell subsets were depleted using antibody-based protocols involving magnetic beads or FACS cell sorting. These procedures involved labeling unwanted cells with rat immunoglobulin [Ig] specific for cell surface markers for 30 min on ice in a volume of 200 µl. Cells were then washed three times in ice-cold sDMEM. For depletion by FACS sorting, cells were labeled with FITC conjugated goat IgG anti-rat Ig (Serotec, Oxford, USA) for 30 min at 4°C followed by three washes in 5 ml ice-cold sDMEM. After resuspension in PBS/1% FCS, unstained cells were sorted using a FACStar Plus flow cytometer (Becton Dickinson). This procedure was used for isolating B lymphocytes with $\leq 1\%$ contaminating cells. Antibodies used for depletion included F4/80, M1/70, 33D1, NLDC-145, 53-6.7, GK1.5, and C363-29B. For magnetic bead depletions, rat Ig-coated cells were incubated with sheep Ig anti-rat Ig Dynabeads (Dyna, Oslo, Norway) for 30 min at 4°C with rotation, using a bead-to-cell ratio of 4:1. Bead-coated cells were exposed to a magnet for 2 min, allowing aspiration of target cells. This procedure was routinely used for T cell

purification with or without subsets of APC and typically gave $\geq 90\%$ purity. DC were removed with 33D1 and NLDC-145, macrophages were removed with F4/80 and M1/70, and B cells were removed with RA3-6B2 and TIB120.

MLR and cell stimulation

RBC-depleted spleen cells were cultured in triplicate at 2×10^5 cells/well in 96-well plates together with diluting concentrations of stimulators in a total volume of 200 μ l sDMEM. Stimulators included irradiated (20Gy; ^{60}Co source) LTC-DC or vesicles prepared from LTC-DC. Control cultures comprised responders or stimulators alone. In some cases, the vesicle fraction of medium was used as a control. As a control, cells were cultured with 10 $\mu\text{g/ml}$ LPS (Sigma, St. Louis, MO, USA) or a 1/100 dilution of CD40L (prepared from paraformaldehyde-fixed CD40 ligand baculovirus-infected Sf9 cells and titrated for maximum B cell proliferation, kindly provided by Virginia McPhun, JCSMR).

DNA synthesis was assessed over the last 16 h of a 3-day culture through the addition of 1 $\mu\text{Ci/well}$ of ^3H -thymidine [$^3\text{H-T}$] in 20 μl sDMEM. Cells were then harvested on to glass-fiber filters, which were then saturated in MicroScint scintillation fluid (Packard, CT, USA). Incorporation of label was measured in a Top-Count scintillation counter (Packard). Responses were reported as mean counts per minute (CPM) $\pm$ SE of the mean of triplicate samples. Control cultures comprised responders alone or stimulators alone and in some cases the "exosome fraction" of medium was used as a control for particulates sedimented in medium. Background $^3\text{H-T}$ incorporation due to stimulators alone was <500 CPM. Alternatively, antibody staining and flow cytometry were used at 1 day to identify and gate subsets for analysis of activation by up-regulation of CD69 and CD86, and proliferation was indicated by a decrease in CFSE.

Mycoplasma detection and removal

Cells were harvested, washed, and cultured in fresh medium for 24 h for collection of DNA for PCR analysis of *Mycoplasma* infection. Since a several different *Mycoplasma* species have been reported to stimulate B cell proliferation in vitro [15], PCR analysis was performed to assess which species of

contaminants were present. DNA from LTC-DC was amplified using *Mycoplasma Plus* PCR Primer Sets, and fragmented with the restriction enzyme *Sau* 3A, which generates species-specific fragmentation of *Mycoplasma* DNA (Stratagene, La Jolla, CA, USA). *Mycoplasma* species were identified against reference products provided by the manufacturer. DNA was separated in a 1.5% agarose gel containing ethidium bromide. For treatment, cell lines infected with *Mycoplasma* were cultured for 12 days with enrofloxacin followed by several days recovery prior to use in experiments [16].

RESULTS

Lymphocyte proliferation induced by exosome fractions of LTC-DC

Some LTC-DC were found to produce exosome fractions with potent capacity for generating a dose-dependent mitotic response in both allogeneic and syngeneic spleen cells that was equivalent to that generated by the LTC-DC from which they were derived (Fig. 1A and data not shown). This activity was not observed in the vesicle fraction of culture medium (data not shown). Lymphocyte stimulation induced by the vesicle fraction was first detectable after 2 h of culture and increased to peak levels by 24h (Fig. 1B). When CSN was passed through filters of reducing pore size, including 800 nm, 450 nm, and 220 nm, the 800-nm filtrate contained the full mitogenic activity of both unfractionated CSN and the vesicle fraction isolated by differential centrifugation (Fig. 1C). Large particulates, such as cell debris removed by 800-nm filtration, were therefore not responsible for lymphocyte stimulation. Passage of CSN through a 450-nm cut-off filter significantly reduced

Fig. 1. Exosome-like vesicles induce potent lymphocyte responses. The exosome (Exo) fraction isolated from a culture supernatant (CSN) of B10.A[2R]-derived long-term cultures-dendritic cells (LTC-DC) at given times after culture of cells without stroma was assessed for lymphocyte stimulatory capacity in a semiallogeneic mixed leukocyte response (MLR). Diluting numbers of LTC-DC and exosome fractions in cell equivalents were assessed for capacity to induce $^3\text{H-T}$ incorporation in 2×10^5 C57BL/6J-derived RBC-depleted spleen cells over the last 16 h of a 72-h culture. Medium alone and the exosome fraction from the medium were used as controls. LTC-DC were irradiated (20Gy) in all assays. (A) LTC-DC and the Exo from LTC-DC were compared for immunostimulatory potential at 24 h. (B) Increased production of immunostimulatory exosomes by LTC-DC occurred over 24 h. (C) Cell-free CSN collected at 24 h and filtered through 800-nm, 450-nm, and 220-nm pore filters was compared with the exosome fraction of cell-free CSN (Exo) for capacity to stimulate a MLR. Medium alone was used as a control. Values in each graph represent mean CPM $\pm$ SE ($n=3$).

stimulatory capacity, and stimulatory factors were further depleted, although not completely after passage through a 220-nm cut-off filter (Fig. 1C). These results support the particulate nature of the lymphostimulatory factors secreted by LTC-DC and suggested a heterogeneous mix of particles of size range 200 to 800 nm consistent with exosomes [6].

Specific proliferation of B cells and not T cells

DC-derived exosomes have been reported to stimulate T cell responses, via bystander APC [17, 18]. Therefore, to determine the necessity for bystander APC in our model, T cell preparations containing either DC, macrophages, or B cells were compared with unfractionated spleen cells for capacity to respond to stimulatory LTC-DC-derived exosome fractions. LTC-DC and their vesicles stimulated lymphocyte proliferation only in the presence of B cells (Fig. 2A). Exosomes could require B cells to stimulate T cells or could stimulate B cells in their own right. In fact, semipurified B cells, but not B cell-depleted spleen cells were then shown to proliferate in response to LTC-DC and their vesicles (Fig. 2B). Antibody staining and flow cytometry of spleen cells stimulated with LTC-DC or their exosome fraction revealed increased numbers of B220⁺ B cells and decreased CD3 ϵ ⁺ T cells after 3 days of culture (data not shown). CFSE analysis of the responding cell subset confirmed that B cells and not T cells were responding (data not shown).

Exosome fractions stimulate B cells independently of T cells

The requirement for T cell help in B cell responses induced by DC is well documented. The importance of CD4⁺ T cells in the B cell response induced by LTC-DC or their vesicle fraction was therefore tested by stimulating spleen cells from mice that lack normal expression of MHC-II and have very reduced numbers of CD4⁺ T cells (Fig. 3A). LTC-DC and their vesicles fraction efficiently activated MHC-II-deficient B cells in the absence of CD4⁺ T cells inducing 50-65% of B cells from both normal and MHC-II-deficient mice to up-regulate CD69 and CD86 after 24 h of coculture and 74-82% of B cells to undergo blastogenesis and divide after 3 days of culture (Fig. 3B). This response resembled the T cell-independent LPS response. LPS contamination, however, was shown not to be

responsible for exosome-mediated B cell responses since the LPS inhibitor, polymyxin B had no effect on the capacity of LTC-DC-derived vesicles to stimulate lymphocytes but severely inhibited the ability of peak mitogenic doses of LPS to induce lymphocyte proliferation (Fig. 4A). Furthermore, CD40L, a known B cell mitogen, was also excluded since LTC-DC-derived vesicle fractions stimulated B cell proliferation equally in C57BL/6J *CD40*^{-/-} and C57BL/6J mice (Fig. 4B).

Specific inhibitors of B cell proliferation

To directly test whether the lymphostimulatory activity for B cells was due to secreted particles, the trafficking inhibitor primaquine was used to block the secretory pathway at the trans-Golgi network. This drug also prevents vesicles recycling from endocytic compartments to the plasma membrane, including transport of molecules from MHC-II-rich compartments (MIIC) to the plasma membrane in DC [19-21]. It could thus be a potential inhibitor of exosome release, which is thought to occur upon MIIC fusion with the plasma membrane. Primaquine completely inhibited the capacity of LTC-DC-derived vesicles to stimulate lymphocytes (Fig. 5A) without affecting cell viability (data not shown). Mitomycin C, reported to be an inhibitor of B cell stimulation by exosomes in CSN from mast cells [9, 22], was also found to completely inhibit the lymphostimulatory capacity of LTC-DC and their derived vesicles (Fig. 5B). Furthermore, DNase treatment of vesicles excluded a role for nucleic acid like CpG motifs (Fig. 5C). Pronase had no effect on the stimulatory capacity of the exosome fraction, despite strongly inhibiting the B cell mitogenic effect of CD40L (Fig. 5D). These data suggested a potential role for lipids in the B cell mitogenic response.

Identification of a cellular pathogen as the lymphoproliferative agent

A role for *Mycoplasma*-derived lipids was considered initially since they are frequent contaminants of cultured cells and have mitogenic activity for leukocytes [10-12]. PCR assays confirmed that a few LTC-DC maintained for over a year were infected with *Mycoplasma* and that *Mycoplasma* components were also present in LTC-DC-derived vesicle

Fig. 2. LTC-DC and their vesicles stimulate B cell but not T cell proliferation. RBC-depleted spleen cells from C57BL/6J mice were fractionated by magnetic depletion using specific antibodies. Spleen cell fractions (10⁵ cells) were compared with total spleen cells (10⁶) for response due to stimulation with 10⁴ allogeneic B10.A[2R]-derived LTC-DC, their exosome fraction (Exo) or medium alone as a control using ³H-T incorporation. (A) Responder spleen cell fractions included T cells or T cells with either DC, macrophages (Mac), or B cells. (B) Responder spleen cell fractions included B cells and spleen cells depleted of B cells. Percentages above bars indicate the proportion of contaminating cells remaining in cell preparations.

Fig. 3. B cell responses generated by LTC-DC-derived vesicles are T cell-independent. (A) RBC-depleted spleen cells (2×10^5) were derived from C57BL/6J mice or C57BL/6J $IA\alpha^{-/-}$ mice. Cells were analyzed for marker expression using an antibody specific for CD4 and Thy1.2 by flow cytometry. (B) RBC-depleted spleen cells (2×10^5) were cultured with 5×10^4 LTC-DC and their exosome fraction [Exo] or $5 \mu\text{g/ml}$ LPS and medium alone as controls. At 24 h, cells were assessed for B220 expression and up-regulation of CD69 and CD86 [% cells are shown within quadrants]. At 3 days, cells were assessed for B220 expression, proliferation through decreased CFSE intensity and increased cell size by forward scatter (FSC) (% blasting and dividing cells are shown outside gate).

Fig. 4. LPS and CD40L are not responsible for B cell proliferation. (A) The exosome fraction isolated from CSN of C57BL/6J-derived LTC-DC at 24 h after culture without stroma was assessed for capacity to stimulate syngeneic spleen cells in the presence and absence of $5 \mu\text{g/ml}$ of the LPS inhibitor polymyxin B (poly B). Control stimulators included medium alone and diluting concentrations of LPS with or without $5 \mu\text{g/ml}$ of polymyxin B. RBC-depleted spleen cells (2×10^5) were assessed for $^3\text{H-T}$ incorporation over the last 16 h of a 72-h culture. Results represent mean CPM $\pm$ SE ($n=3$). (B) CFSE-labeled RBC-depleted spleen cells (2×10^5) derived from C57BL/6J mice or C57BL/6J $CD40^{-/-}$ mice were cultured with 5×10^4 syngeneic LTC-DC and their vesicles (Exo; 5×10^4 cell equivalents) or $2.5 \mu\text{g/ml}$ LPS, CD40L in optimum stimulating amounts, and medium alone as controls. At 3 days, viable B cells (B220⁺ PI⁻) among responders were assessed for decreased CFSE intensity and increased forward scatter (FSC) using flow cytometry. The gated region in each plot represents nonblasting (low FSC) and non-dividing (high CFSE) cells. Numbers in each plot represent % cells outside gated region (i.e., blasting and dividing cells).

Fig. 5. The effect of primaquine, mitomycin C, DNAase, and pronase treatment on the capacity of LTC-DC-derived exosome-like vesicles to stimulate lymphocytes. The ability of drugs and degradative enzymes to inhibit production of the stimulatory exosome fraction from LTC-DC was assessed in a MLR. For primaquine and mitomycin C-treatment, exosome fractions were prepared from treated and untreated LTC-DC and compared (along with LTC-DC where appropriate). For pronase and DNAase treatment, exosome fractions were treated directly for 30 min at 37°C prior to dilution in medium. Values are the capacity of LTC-DC or the exosome fractions to induce ^3H -T incorporation in C57BL/6J-derived lymphocytes relative to medium alone as a control. Data represent mean CPM $\pm$ SE ($n=3$). (A) Exosome fractions (Exo) were derived from untreated LTC-DC and LTC-DC treated with 100 $\mu\text{g}/\text{ml}$ primaquine (PQN) for 24 h. (B) LTC-DC were treated with or without mitomycin C (Mito C) (50 $\mu\text{g}/\text{ml}$) for 20 min at 37°C, and then they were washed and cultured without stroma for 24 h to accumulate exosomes. (C) Stimulatory capacity of Exo untreated and treated (Exo+DNAase) with DNAase (2.5 U/75 μl). (D) Stimulatory capacity of Exo untreated and treated (Exo+pronase) with pronase (5 $\mu\text{g}/\text{ml}$). As a positive control for pronase activity, CD40L was treated in the same manner and tested for stimulatory capacity.

preparations. Both *M. arginini* and *M. orale* were detected in different cultures by PCR analysis (data not shown). Both have been reported to stimulate B cell proliferation [15]. LTC-DC-derived vesicles containing different *Mycoplasma* species were also shown to stimulate blastogenesis in B220⁺ but not CD4⁺ or CD8⁺ lymphocytes (data not shown). To investigate further the role of *Mycoplasma* in B cell proliferation, infected LTC were treated with *Mycoplasma* removal agent (enrofloxacin) and confirmed clear by PCR analysis. Treated LTC-DC and their vesicle fraction completely lost capacity to stimulate mitosis of lymphocytes (Fig. 6, A and B). Furthermore, some *Mycoplasma*-free LTC-DC never exposed to *Mycoplasma* removal agent, were incapable of generating lymphocyte responses (data not shown). Recurrence of infection in one culture also correlated with capacity to stimulate B lymphocyte responses (data not shown). Responses were due to B220⁺ lymphocytes, and not Thy1.2⁺ lymphocytes, as confirmed by antibody staining and gating of cell subsets using flow cytometry (Fig. 6C).

Mycoplasma stimulate B cells independently of DC

In line with other studies, *Mycoplasma* contaminants could not be cultured independently of LTC-DC using either liquid or solid medium, possibly a reflection of the scarcity of genes in *Mycoplasma* that are normally involved in major biochemical pathways [15]. Therefore, further experiments tested whether *Mycoplasma* in the vesicle fraction of LTC-DC-stimulated B cells independently of antigen presenting DC by assessing whether *Mycoplasma* produced by DC could transfer their B cell stimulation capacity upon infection of other cells. Several cell lines received from outside laboratories, including the monkey fibroblast cell line COS-7, the human melanoma cell line MM170, the human B cell line JY, and the murine melanoma cell line B16, were screened by PCR for *Mycoplasma* infection using primers specific for conserved gene sequences of 16S rRNA within the *Mycoplasma* genus [44]. This analysis showed that CSN from all cell lines tested except B16 contained *Mycoplasma* (Fig. 7A). CSN collected after a 24 h culture of washed cells from infected cell lines generated

Fig. 6. *Mycoplasma*-cleared LTC-DC and their exosome fraction lose capacity to stimulate lymphocytes. B6BQ LTC were treated for 7 days with 0.5 μg/ml of *Mycoplasma* Removal Agent (MRA) and were given several days to recover. Treated and untreated B6BQ LTC (A) or their Exo fractions (B) were assessed for capacity to stimulate lymphocytes in a syngeneic MLR, involving ³H-T incorporation in 2×10⁵ RBC-depleted spleen cells over the last 16 h of a 72-h culture. Controls included medium alone. Results are reported as mean CPM±SE (n=3). (C) LTC-DC regain capacity to stimulate B lymphocytes upon *Mycoplasma* reinfection. B6BQ LTC-DC were successfully treated to remove *Mycoplasma*. After reinfection over several weeks of culture, they were tested again for infection and capacity to stimulate lymphocytes in a MLR. Control stimulators included LPS and allogeneic spleen cells. Responder B [B220⁺] cells and T [Thy1.2⁺ or CD3ε⁺] cells were assessed after 3 days of culture through antibody staining and flow cytometry for proliferation assessed by decrease in CFSE intensity.

lymphocyte proliferative responses comparable to that generated by optimal concentrations of CD40L (Fig. 7B). Both whole cells and cell depleted-CSN from infected cell lines activated splenic B cells but not T cells to up-regulate CD69 after an 18-h culture, similar to responses induced by CD40L (Fig. 7C). Neither B16 cells nor B16 CSN activated any lymphocyte response (Fig. 7C).

The *Mycoplasma* infection status of cell lines was found to correlate with their capacity to stimulate B lymphocytes. Following treatment with enrofloxacin, COS-7, MM170, and JY were cleared of *Mycoplasma*, resulting in loss of B cell stimulatory capacity (Fig. 8B–D). *Mycoplasma* contaminated LTC-DC could also transfer B cell stimulatory capacity to uninfected B16 cells following exposure to CSN from infected cells (Fig. 8A). Taken together, these data strongly suggest that *Mycoplasma* can stimulate B cell proliferation, independently of DC-derived factors or exosomes. The possibility that *Mycoplasma*-infected cells also contribute to B cell stimulation cannot be ruled out. However this would have to be a phenomenon common to many different cell types.

Characteristics of the B cell response to *Mycoplasma*

B cell activation for Ig production

The opportunity existed to investigate more specifically the B cell response due to *Mycoplasma*. Infected LTC-DC and their “*Mycoplasma* fraction” were compared with LPS for capacity to stimulate proliferation of subsets of B cells. A comparison of peak responses of viable CFSE-labeled B cells after 3 days of culture showed that LTC-DC and their *Mycoplasma* fraction induced a similar number of B220⁺PI[−] B cells to undergo blasting and cell division with 74–82% of B cells responding above background (Fig. 9A). In comparison with LPS, the *Mycoplasma* fraction gave less cells undergoing 4–6 divisions (Fig. 9A). Further experiments tested whether the *Mycoplasma*

fraction isolated from LTC-DC could initiate B cell differentiation into plasma cells with isotype switching. Spleen cells were stimulated with infected LTC-DC, their *Mycoplasma* fraction, LPS, and medium as a control, and the presence of soluble Ig was detected in CSN using an ELISA assay. Both infected LTC-DC and their *Mycoplasma* fraction induced Ig secretion by 3 days of culture, peaking at 6 days at levels comparable with that induced by 5 μg/ml of LPS (data not shown). Ig levels dropped after 12 days, although levels continued to increase in cultures with added LPS (data not shown). Spleen cells stimulated with infected LTC-DC or their *Mycoplasma* fraction were also examined for both surface and intracellular expression of different Ig isotypes over time. LPS induced Ig class switching in CFSE-labeled B cells from IgM to IgG₃ by four cell divisions (Fig. 9B) [24]. However, neither infected LTC-DC nor their *Mycoplasma* fraction induced detectable surface or intracellular expression of Ig isotypes other than IgM (Fig. 9C) even after 5 or 6 divisions of B cells (all data not shown).

Polyclonal activation of all B cell subsets

The LTC-DC-derived *Mycoplasma* fraction induces a polyclonal B cell response despite the use of specific pathogen-free animals. It is not clear whether B cell responses involved only naive B cells. To assess this, cells were gated on the basis of size (forward scatter) and expression of IgD to identify small, resting naive B cells. These were then assessed for activation after culture with *Mycoplasma*-containing fractions by up-regulation of CD69 expression. After 0.5 days culture of spleen cells with either infected LTC-DC, their *Mycoplasma* fraction, or LPS, both large and small IgD⁺ B cells increased surface expression of CD69, although larger cells gave better responses (Fig. 10). IgD⁺ B cells also underwent several rounds of division by 4 days of culture with infected LTC-DC and their *Mycoplasma* fraction (data not shown). Like LPS, infected LTC-DC and their *Mycoplasma* fraction stimulated both large B

Fig. 7. *Mycoplasma*-contaminated cell lines and their CSN stimulate B cell responses. PCR was used to assess *Mycoplasma* infection of the monkey fibroblast cell line COS-7, the human melanoma cell line MM170, the human B cell line JY, and the murine melanoma cell line B16. (A) DNA prepared from CSN of confluent cell lines was amplified using primers specific for conserved *Mycoplasma* 16S rRNA. Products were separated by electrophoresis in 1.5% agarose gel containing 100 $\mu\text{g}/\text{ml}$ ethidium bromide. Positive controls were DNA from *Mycoplasma* contaminated LTC-DC samples. These were amplified both in parallel and within each test sample. Negative controls contained no DNA template. A 1-kb GeneMarker DNA ladder is shown. (B and C) Each cell line and its CSN were tested for capacity to stimulate lymphocytes. Cells were harvested from confluent cultures and cultured in fresh medium in a new flask for 20 h. CSN from cultures was then depleted of

cells by centrifugation at 300 g for 10 min. (B) CSN was assessed for capacity to induce $^3\text{H-T}$ incorporation [$\text{CPM} \pm \text{SE}$ ($n=3$)] in C57BL/6J-derived RBC-depleted spleen cells (2×10^5) over the last 16 h of a 2-day culture. (C) Cells lines and CSN collected from cell lines at 20 h of culture were assessed by flow cytometry for capacity to induce upregulation of CD69 among B220⁺ B cells and Thy1.2⁺ T cells in C57BL/6J-derived RBC-depleted spleen cells (2×10^5) at 18 h after coculture. Controls included CD40L and medium alone. Peak responses only are shown.

cells and small, resting naive B cell populations, consistent with polyclonal activation.

Mature B cells comprise two distinct types, marginal zone (MZ) B cells, which are CD23 low and CD21 high, and follicular (FO) B cells, which are CD23 high and CD21 intermediate [25]. These are thought to respond differently upon activation, with MZ B cells, inducing a more rapid blast response than FO B cells after LPS treatment [25]. Precursor B cells also exist in spleen. These are divided into the transitional B cell subsets, T1 and T2, representing stages in selection of the mature B cell repertoire [26]. Like MZ B cells, transitional B cells express high levels of IgM not expressed on FO B cells. T1 B cells can be further discriminated from T2 B cells by low or undetectable levels of CD23, as for MZ B cells. T1 can be distinguished from MZ B cells by the absence of CD21 [26]. Spleen cells were cultured for 3 days with the *Mycoplasma* fraction of LTC-DC and divided into T1, T2, MZ, and FO B cells on the basis of CD21, CD23, and IgM marker expression. The response of splenic B cell subsets to the vesicle fraction of infected LTC-DC was then compared with LPS as a control mitogen.

Both LTC-DC-derived vesicles and LPS were mitogenic for all B cell subsets. Overall, 0.4-2.1% of lymphocytes displayed T1 markers, 16-19% displayed T2 markers, 4.8-11% displayed MZ markers and 41-50% displayed FO B cell markers after 3 days of culture under all conditions of stimulation (**Fig. 11A**). However, there was an approximate 2- to 5-fold increase in population size after stimulation with the *Mycoplasma* containing fraction or LPS, indicated by a specific increase in the total cell number within each B cell subset (Fig. 11B). The majority [57-90%] of B cells in each subpopulation responding to vesicles or LPS were blasts. There was no specific induction of blastogenesis within a particular B cell subset after stimulation with vesicles, as opposed to LPS.

DISCUSSION

This study shows that *Mycoplasma* contaminants in LTC-DC and their exosome preparations are responsible for inducing B cell mitogenesis either directly or in conjunction with cell

Fig. 8. Mycoplasma generates B cell responses independent of host cell type. To associate *Mycoplasma* contamination with mitogenic activity for B cells, comparison was made between various infected and uninfected cell lines. (A) B16 cells were infected by contaminated CSN from the LTC-X1 to allow *Mycoplasma* infection. (B–D) COS-7, MM170, and JY were treated with enrofloxacin at 70 mM to clear existing *Mycoplasma* infections. Infected/treated cell lines and their uninfected/untreated counterparts were cultured in fresh medium in new flasks for 20 h. CSN was collected as a source of *Mycoplasma* and depleted of cells by centrifugation at 300 g for 10 min. CSN was then tested for lymphocyte stimulatory capacity by culture with 2×10^5 C57BL/6J-derived RBC-depleted spleen cells. Medium alone was used as a control. After 20 h, spleen cells were assessed for CD69 up-regulation on B220⁺ or Thy1.2⁺ cells by antibody staining and flow cytometry. Spleen cells were also assessed for ³H-T incorporation over the last 16 h of a 96 h culture. Results represent mean CPM $\pm$ SE ($n=3$).

factors. *Mycoplasma* isolated as a vesicle fraction of infected cells can stimulate B cell responses independently of LTC-DC, and the responsible components are resistant to proteases and DNases, suggesting a role for lipoproteins probably *Mycoplasma*-derived. This response was inhibited by mitomycin C [9, 22] and primaquine, an inhibitor of membrane transport. Nonviable contaminants were excluded as causative since the production of stimulatory particles correlated with in vitro culture of LTC-DC. Furthermore, polymyxin B inhibition assays preclude a role for the lipid A component of LPS. These results have implications for our understanding of *Mycoplasma*-induced immunomodulation and on how the study of exosomes and cellular immune responses can be affected by microbial contaminants.

Previous studies have shown that LTC-DC, but not their exosome fraction, can initiate an MHC-II-restricted response in TCR-Tg CD4⁺ T cells [3]. This confirms that LTC-DC can generate T cell responses independently of *Mycoplasma* infection. These findings may also explain results demonstrating that mitomycin C has an inhibitory effect on the ability of LTC-DC and their exosome fraction to induce an MLR but has no effect on the capacity of LTC-DC to stimulate the T cell clone, D10.G4.1 [27]. Mitomycin C is known to crosslink DNA with absolute specificity for the sequence CpG [28]. Because of

the abundance of CpG in prokaryote cells, mitomycin C is a potent antibiotic and a potent *Mycoplasma*-inhibiting drug [29]. Whether *Mycoplasma* infection has other effects on LTC-DC is not clear. *Mycoplasma* can induce human monocyte-derived DC to undergo maturation and to secrete cytokines, like IL-6 and IL-12 [30]. Preliminary investigations, however, indicate that the partially mature properties displayed by LTC-DC are not altered by *Mycoplasma* infection. These include expression levels of immunomodulatory molecules, like MHC-II and CD86, endocytotic capability, inability to stimulate a normal repertoire of naive T cells, and refractoriness to LPS activation (data not shown).

The size of the B cell stimulatory agent in LTC-DC CSN compares with the size of exosome-like vesicles observed previously in CSN [6]. *Mycoplasma* also fit this size distribution [13]. Published reports showing whole mount electron micrographs of *Mycoplasma* demonstrate them to be highly pleomorphic in structure [31]. They do not resemble the uniform spherical structures observed by whole mount electron microscopy of exosome fractions derived from LTC-DC [6]. Furthermore, antibody staining of exosome fractions has indicated expression of LAMP-1, a protein reported to be expressed by APC-derived exosomes [7], and also expressed intracellularly by LTC-DC. The finding of *Mycoplasma* as a contaminant of

Fig. 9. *Mycoplasma* have high capacity to induce B cell proliferation and immunoglobulin secretion without isotype switching. *Mycoplasma*-infected C57BL/6J-derived LTC-DC (5×10^4) and their exosome fraction were assessed for capacity to stimulate RBC-depleted syngeneic spleen cells (2×10^5) relative to LPS (5 µg/ml) or medium alone as controls. (A) Viable CFSE-labeled splenic B cells ($B220^+ PI^-$) were analyzed after 3 days of culture using antibody staining and flow cytometry to measure decreased CFSE intensity and increased forward scatter (FSC). Gated regions represent nonblasting (low FSC) and nondividing (high CFSE) cells. Number of cell divisions and % cells in each division was measured on the basis of CFSE intensity. Data shown represent % cells outside gated regions (i.e., blasting and dividing cells). (B) $B220^+$ responder cells after 4 days of culture were assessed for Ig isotype expression by surface labeling using antibody staining and flow cytometry. Numbers represent % cells expressing isotype. (C) $B220^+$ responder cells after 4 days of culture were assessed for Ig isotype expression by surface and intracellular labeling using antibody staining and flow cytometry on fixed, permeabilized cells.

some LTC does not detract from the finding that LTC-DC do, in fact, produce exosome-like vesicles. Overall, it appears that exosome isolation procedures can also result in coisolation of *Mycoplasma*. To date, *Mycoplasma* have not been observed under electron microscopy of either cells or vesicle fractions. *Mycoplasma* express cytoadherence and accessory proteins, which allow adherence to host cell surfaces [32]. Other studies have also reported *Mycoplasma* within cells, or capable of fusing their outer membrane with the plasma membrane of host cells, resulting in cell fusion [32].

These findings have important implications for studies on exosomes. Previous studies ruled out the possibility that exosomes were derived from plasma membrane components or apoptotic bodies [6–8, 33]. However, there has been no investigation of exosome fractions for *Mycoplasma* or other microorganisms. It is important to note that much of the work done on leukocyte-derived vesicles, including functional studies, has involved cell lines, like the B cell lines JY and RN [7], the DC line D1 [8, 17], and the mast cell lines P815 and MC/9 [9, 34]. Because of the ease with which *Mycoplasma* infection can

occur in in vitro cultured cell lines [14] and the difficulty of isolating exosomes away from *Mycoplasma*, it will be important to assess the impact of these results on the broader field of exosome biology. Size restriction filters and sucrose gradients are current methods used to fractionate vesicles from other cellular components or cell debris [17, 35]. These cannot be guaranteed to isolate vesicles free of *Mycoplasma* contaminants.

Previous studies have identified exosomes to be mediators of both T and B lymphocyte activation, cytokine production by leukocytes, and DC maturation [8, 9, 34]. These are also known functions of *Mycoplasma* [15]. However, most studies have failed to directly relate the lymphocyte modulation induced by the exosome fraction with vesicles present in this fraction. For instance, CSN factors derived from mast cells can replace cells in generating nonspecific responses in T cells and also resting B cells, leading to blastogenesis, mitosis, and IgM secretion [22]. This response has been attributed to exosome-like vesicles present in the CSN of mast cells [9]. Vesicles are also thought to be responsible for the capacity of CSN factors from

Fig. 10. *Mycoplasma* activate naive B cells. RBC-depleted spleen cells (2×10^5) derived from C57BL/6J mice were cultured with syngeneic LTC-DC (2×10^4) and their *Mycoplasma*-containing exosome fraction or 5 μ g/ml LPS and medium alone as controls. After 15 h, responder cells were stained with antibodies specific for B220 and CD69 and analyzed by flow cytometry. Small [region 1 (R1)] and large (R2) B220⁺ cells were gated on the basis of forward scatter and assessed for IgD and CD69 expression post acquisition. Numbers in quadrants represent % cells.

mast cells to induce maturation of DC in vitro and to have adjuvant effects on cytokine secretion in vivo similar to LPS [34]. The mast cell-derived agent was isolated by differential centrifugation of mast cell CSN, from which exosome-like vesicles could also be isolated [9]. However, *Mycoplasma* contaminants can also be isolated using these procedures. Importantly, mast cell-derived factors mediating the responses observed were found to be protease-resistant but extremely sensitive to mitomycin C treatment [22]. These properties resemble those of the *Mycoplasma*-contaminated exosome fraction derived from LTC-DC.

Most work characterizing B cell responses induced by *Mycoplasma*-infected cells or their isolated vesicle fraction were performed using cells derived from one LTC culture, B6BQ. Cells were contaminated by *M. arginini*, which can act as a T cell-independent polyclonal B cell mitogen, resulting in growth and Ig secretion of resting B cells with no effect on T cells [12, 36]. This study extends those findings and shows that polyclonal T cell-independent B cell responses induced by *M. arginini* are triggered through a lipid A (polymyxin B)-independent mechanism that is DNAase- and pronase-resistant but sensitive to mitomycin C and primaquine treatment. Furthermore, *M. arginini*-infected cells induce up-regulation of CD69 and several rounds of division in small naive [IgD⁺] B cells, as well as the outgrowth of every known subpopulation of B cells in the spleen, including the T1, T2, MZ, and FO subsets.

B cell mitogenic effects of *Mycoplasma* appear to be due to lipoproteins. *Mycoplasma* express high levels of lipoproteins in

diverse compositions anchored to the outer leaflet of their plasma membrane bilayer [10]. All membrane-anchored lipoproteins contain a lipolated amino-terminal cysteine residue [10], with immunomodulatory activity independent of attached protein [37, 38]. *M. fermentans* contains lipid-associated membrane proteins that are potent inducers of murine T cell-independent B cell proliferation and Ig secretion without induction of isotype switching [11]. Studies have identified a lipopeptide, MALP-2, in *M. fermentans*, with similar properties to the endotoxin LPS but acting through a pathway independent of Toll-like receptor (TLR)-4, which is required for LPS signaling [39, 40]. MALP-2 is identical to the N-terminal lipid moiety present on two other *M. fermentans* immunogenic lipoproteins, M161Ag and P48 [37]. MALP-2 and M161Ag signal through TLR-2, which binds the N-terminal lipid moiety of a number of different lipoproteins [37, 39].

The finding that *Mycoplasma*-infected cells or vesicles produced by those cells can stimulate polyclonal activation of all B cell subsets in spleen, including the T1 and T2 transitional populations of B cells, could have important implications for autoimmune sequelae associated with *Mycoplasma* infection [41, 42]. Immature B cells from bone marrow develop into transitional T1 B cells, which migrate selectively to the spleen [26]. Here, they differentiate into all major B cell populations, including T2 transitional B cells, mature IgD⁺ FO B cells, and mature MZ B cells. Transitional B cells are highly sensitive to negative selection dependent on B cell receptor interaction with antigen in a process that has not yet been fully charac-

Fig. 11. *Mycoplasma* promote the outgrowth of all B cell subsets in the spleen. RBC-depleted spleen cells (2×10^5) derived from C57BL/6J mice were cultured with the *Mycoplasma*-containing exosome fraction derived from 2×10^4 syngeneic LTC-DC or 5 $\mu\text{g/ml}$ LPS and medium alone as controls. After 3 days, cells were analyzed by antibody staining and flow cytometry. (A) Lymphocytes were analyzed for IgM, CD23, and CD21 expression to identify transitional T1 and T2 B cells, mature follicular type B cells (FO) and marginal zone B cells (MZ). The proportion of B cell subsets among all lymphocytes is shown as % cells (numbers next to gated regions in each dot plot). (B) The total number of cells and blasting cells (large cells by FSC) in each population (using the displayed counting beads for reference) is displayed in the bar graph.

terized [26, 43, 44]. If *Mycoplasma* can promote the outgrowth of T1 and T2 transitional B cells, then it could induce proliferation of potentially autoreactive B cells. *Mycoplasma pneumoniae* in humans and *Mycoplasma pulmonis* in mice are frequently described in association with production of polyclonal IgM autoreactive to red blood cells and autoimmune hemolytic anemia [41, 42]. This finding may provide insight into a potential mechanism involved in *Mycoplasma*-induced autoimmune disease.

The strong inhibitory effect of primaquine on *Mycoplasma*, with no concomitant effect on the viability of host LTC-DC, indicates that it may be an effective treatment for *Mycoplasma*-induced disease. Primaquine is already used for treatment of *Pneumocystis* and *Plasmodium* infections in humans [45, 46], and *Mycoplasma* have increasing importance, as a number of diseases implicate *Mycoplasma* as a causative agent, including pneumonia [47]. Increased resistance to macrolide drugs, often used to treat *Mycoplasma* respiratory diseases, makes the search for new drugs very important [48].

This study has characterized B lymphocytes responding to *Mycoplasma*-infected cells, and particularly to cells infected with *M. arginini*. Although the B cell responses generated by *M. arginini*-infected cells are similar to those induced by the most recognized bacterial modulin, LPS, they are qualitatively different acting through moieties independent of lipid A. This

study emphasizes the potential of *Mycoplasma*-infected cells for generating T cell-independent polyclonal outgrowth of all major B cell subsets in spleen.

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