Dendritic Cells Pulsed with Intact Streptococcus pneumoniae Elicit both Protein- and Polysaccharide-specific Immunoglobulin Isotype Responses In Vivo through Distinct Mechanisms

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Abstract

Immature bone marrow–derived myeloid dendritic cells (BMDCs) are induced to undergo phenotypic maturation and secretion of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-12, and IL-10 when pulsed in vitro with intact Streptococcus pneumoniae. After transfer to naive mice, pulsed BMDCs induce immunoglobulin (Ig) isotype responses specific for both protein and polysaccharide pneumococcal antigens, having in common the requirement for viable BMDCs, T cells, and B7-dependent costimulation in the recipient mice. Whereas primary Ig isotype responses to bacterial proteins uniformly require BMDC expression of major histocompatibility complex class II, CD40, and B7, and the secretion of IL-6, but not IL-12, similar requirements for antipolysaccharide Ig responses were only observed for the IgG1 isotype.

Key words: immunity • APCs • cellular • antibody formation • Gram-positive bacteria

Introduction

Two major categories of antigen have been recognized, thymus-dependent (TD)* and thymus-independent (TI) antigens, based on their relative ability to induce Ig antibody responses in athymic nude mice, which are markedly deficient in T cells (1). TI antigens, such as bacterial polysaccharides, are unable to bind to MHC molecules (2) and hence cannot directly trigger cognate interactions between APCs and T cells. These observations suggested a minimal, if any, role for APCs and T cells in humoral immune responses to polysaccharide antigens. The presence of repeating, identical antigenic epitopes expressed by polysaccharide molecules suggested instead, that they directly mediated B cell activation through potent membrane Ig-mediated activation which in concert with some form of non-T cell help induced Ig secretion (3). However, considerable evidence has accumulated indicating that T cells, acting in a noncognate manner, can play a significant role in regulating specific antibody responses to bacterial polysaccharides (1, 4–7). Nevertheless, little is known regarding the potential role of APCs in these responses. Only in vitro studies describing a direct role of macrophages in B cell activation in response to TNP-haptenated antigens such as TNP-Ficoll, TNP-LP, and TNP-Brucella abortus, have been reported (8, 9).

Dendritic cells (DCs) are APCs with the unique property of inducing primary immune responses, thus allowing establishment of immunological memory (10). Immature DCs in peripheral tissues, internalize (11, 12) and process bacteria and other particulate antigens for MHC class I and MHC class II antigen presentation to T cells (13), migrating to secondary lymphoid organs, where DCs prime naïve T cells (14, 15), naïve and memory B cells (16, 17) and activate NK cells (18) to induce specific immune responses. A role for DCs in conferring antimicrobial immunity is suggested by studies in which in vitro antigen-pulsed DCs transferred into naïve hosts conferred protection against a further challenge with the pathogen (19, 20). These observations indicate that DCs play a key role in linking innate and adaptive immunity, critical for host protection against microbial pathogens.

Protection against extracellular bacteria is largely conferred by Ig specific for bacterial polysaccharide and protein

*Abbreviations used in this paper: AP, alkaline phosphatase; BMDC, bone marrow–derived myeloid dendritic cell; Cps14, capsular polysaccharide type 14; CT LA, cytotoxic T lymphocyte antigen; MFI, mean fluorescence intensity; PC, phosphorylcholine; PsA, pneumococcal surface adhesion A; PspA, pneumococcal surface protein A; TD, thymus dependent; TI, thymus independent.
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antigens (21). We demonstrated previously the ability of the Gram-positive extracellular bacteria, *Streptococcus pneumoniae*, to induce polysaccharide- and protein-specific Ig isotype responses in vivo, in which both responses were dependent on T cell help and B7-dependent costimulation (6, 22). On the basis of these data we wished to determine DCs to initiate in vivo humoral responses to “TI” antigens, expressed by an intact extracellular bacteria, and underscore striking differential DC requirements for induction of protein- versus polysaccharide-specific responses.

### Materials and Methods

**Mice.** C57BL/6N mice were obtained from the National Cancer Institute. IL-6−/−, IL-10−/−, IL-12−/−, CD40−/−, MHC class II−/− (locus Ma) mice and their corresponding C57BL/6j controls were obtained from The Jackson Laboratory. B6129SF2/J mice were used as controls for the TNF-α−/− mice both obtained also from The Jackson Laboratory. Double B7−1−/− × B7−2−/− (CD80/CD86) mice and the corresponding BALB/c controls were provided by Arlene Sharpe (Brigham and Women’s Hospital, Boston, MA). Mice were used at 8–10 wk of age and were maintained in a pathogen-free environment at Uniformed Services University of the Health Sciences (USUHS).

**Cell Culture Media.** The cell culture media consisted of RPMI 1640 supplemented with 5% FCS (BioWittaker), 50 μM 2-ME, 20 μg/ml gentamycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 25 mM Hepes.

**S. pneumoniae Strains and Bacterial Antigens.** The *S. pneumoniae* capsular type 14 was provided by Mr. Sam Wilson (USUHS). The capsular type 14 variant R36A of type 2 were all gifts of David E. Briles (University of Alabama, Birmingham, AL). Recombinant pneumococcal surface protein A (PspA), a gift of Luba Grinberg (BioSynexus, Inc., Rockville, MD), was expressed in *Saccharomyces cerevisiae* BJ3505 as a His6-tagged fusion protein, and purified by Ni-NTA affinity chromatography (24). Recombinant pneumococcal surface adhesion A (PsaA), provided by James C. Paton (Adelaide University, Adelaide, Australia), was expressed in *Escherichia coli* as a His6-tagged fusion protein, and purified by Ni-NTA affinity chromatography (25). Phosphorylcholine (PC)-KLH, a gift of Andrew Lees (BioSynexus, Inc.), was synthesized as described previously (6). The resulting conjugate had a substitution degree of 19 PC/KLH molecule. Purified capsular polysaccharide type 14 (Cps14) was purchased from American Type Culture Collection.

**Bacterial Culture and Preparation of the Bacterial Inoculum.** Isolated colonies in Columbia blood agar were grown in Todd Hewitt broth to mid-log phase, collected, and heated killed by incubation at 60°C for 1 h. Sterility was confirmed by subculture on blood agar plates. After extensive washings, the bacterial suspension was adjusted with PBS to give an absorbance reading at 650 nm of 0.6 which corresponded to 10⁶ CFU/ml. Bacteria were then aliquoted at 10⁴ CFU/ml and frozen at −80°C until their use as antigen for mouse immunizations or to pulse bone marrow–derived myeloid dendritic cells (BMDCs) in culture. Bacteria treated with 0.1% paraformaldehyde for 16 h at 20°C, were preserved and aliquoted similar to heat-killed bacteria, and were used in a preliminary set of experiments.

**Fluorescent Labeling of S. pneumoniae with CM-DiI.** Bacteria were labeled with the fluorescent lipophilic cell tracer chloromethylbenzamido derivative of DiI (CM-DiI; Molecular Probes). Specifically, suspensions of 10⁹ bacteria per milliliter were incubated at 37°C for 10 min, in a solution containing 5 mM CM-DiI in PBS. The suspension was then incubated for an additional 30 min at 4°C. After the incubation, the excess of fluorescent tracer was removed by extensive washings in cell culture media.

**BM Cell Culture.** DCs were cultured from bone marrow, essentially as described previously (26), with slight modifications. Briefly, bone marrow was flushed from femurs and tibia, homogenized and the red blood cells removed by lysing with ACK lysis buffer (GIBCO BRL). T cells, B cells, and other MHC class II-expressing cells were removed from the bone marrow cell suspension by incubation for 30 min at 4°C with a mAb cocktail composed by AF6–120.1 (mouse IgG2ak anti-I-A²), RA3–6B2 (rat IgG2ak anti-CD45R/B220), and 53–58 (rat IgG1k anti-CD8b.2), and negatively selected by magnetic bead cell sorting using a mixture of affinity purified polyclonal goat anti-rat IgG and goat anti-mouse IgG-conjugated beads (BioSpheres). All the mAbs were purchased from BD Pharmingen. The BM cells obtained were cultured at 1.25 × 10⁶ cells per milliliter in cell culture media supplemented with 10 ng/ml of murine recombinant GM-CSF, provided by Luba Grinberg. The cells were cultured at 37°C in an atmosphere of 7% CO₂ and 95% humidity. On day 3 the supernatant was removed and replaced with fresh media containing GM-CSF. On day 5, ~2/3 of the supernatant was removed, replaced with 2 volumes of fresh media containing GM-CSF, and the cultures were split into two wells. Typical experiments were performed with the nonadherent and loosely adherent cell population from cultures at days 6–8. At the end of the culture period, ≥95% of the cells displayed the morphology and cell surface markers characteristic of myeloid DCs.

**Flow Cytometric Analysis of BMDC Surface Antigen Expression.** All steps were performed on ice. For staining BMDCs for flow cytometry, the Fc receptors were specifically blocked with 2.5 μg/ml/10⁶ BMDCs of anti-CD16/CD32 mAb (clone 2.4G2; BD Pharmingen) in PBS containing 1% FCS (staining buffer), 45 min before the staining, and during the staining. Cells were stained by incubation for 30 min with biotinylated or PE-conjugated mAbs (BD Pharmingen) specific for CD11c (clone 11C1), CD8a (clone 53–6.7), CD11b (M1/70), I-A⁴ (clone AF6–120.1), I-A² (clone AMS-32.1), H2-K⁰ (AF6–88.5), H2-K¹ (SF1–1.1), CD40 (clone 3/23), CD80 (16–10A1), CD86 (clone GL1), CD25 (clone PC61), and CD54 (clone 3E2). The incubation with biotinylated mAbs was followed by staining with PE-streptavidin conjugate for 15 min. For detection of CD16/CD32 expression with the specific antibody (clone 2.4G2), the Fc blocking step was omitted. Irrelevant isotype and species-matched mAbs were used as staining controls. Cells were analyzed on an EPICS XL–MCL (Beckman Coulter). Dead cells and debris were excluded from analysis by gating on the appropriate forward and side scatter profile.

For detection of PC on the surface of pulsed BMDCs, the Fc receptor-blocked cells were directly stained with FITC-conjugated murine IgM (clone HPCM2) or IgG2a (clone G2a2.A1) anti-PC mAbs, or indirectly with unconjugated IgG2b (clone 1B8E5) followed by staining with FITC-conjugated rat anti-mouse IgG2b mAb (clone R12–3; BD Pharmingen). For detection of intracellular PC, pulsed BMDCs were fixed and permeabilized with Cytofix/Cytoperm Plus (BD Pharmingen).
according to manufacturer’s instructions, and stained with FITC-conjugated murine IgG2a anti-PC (clone G2a2.A1). The PC-specific mAbs were gifts of Anna Lustig and James J. Kenny (National Institutes of Health). The specificity of the mAbs were confirmed by ELISA. A similar indirect approach using mouse polyclonal antibodies to Cps14 followed by staining with FITC-conjugated rat anti–mouse IgG2b mAb or PE-conjugated polyclonal goat anti–mouse IgM (Southern Biotechnology Associates, Inc.) were used to detect surface Cps14. Controls included pulsed BMDCs stained with FITC-conjugated murine IgM anti–TNP mAb (G155–228) or FITC-conjugated murine IgG2a anti–TNP mAb (G155–178), and for indirect stainings purified IgG2b anti–mouse IgD* (AMS9.1). Staining of free S. pneumoniae analyzed in the same was used as a positive control.

**Bacterial Pulsing of BMDCs for In Vivo Transfer Experiments.** BMDCs collected after 6–8 d of culture were washed two times in cell culture media. Residual macrophages were removed through plastic adherence during 1 h of culture at 37°C. BMDCs were plated at 10^6 BMDCs per milliliter per well in 24-well cell culture plates (Costar). After 30 min, to allow cells to settle, 100 µl of a bacterial suspension in cell culture media containing the desired amount of inactivated S. pneumoniae was added to the cultures. In experiments involving transfer of in vitro–pulsed BMDCs into naive mice, the BMDCs were pulsed at ratios of 800 bacteria per individual BMDCs during a time pulse of 4–5 h in media containing GM-CSF. After the pulse, the excess of bacteria was extensively washed, at least six times each for 10 min at 350x g at 4°C. A control tube containing a mixture of thymocytes and CM-DiI fluorescent labeled S. pneumoniae at the same bacterial density as at the onset of BMDC pulsing was used to monitor the progress of the washings. If >100 free bacteria were found in the resulting examination of the pelleted cells in an inverted fluorescent microscope, the washings were continued. The pulsed BMDC pellet obtained was resuspended in fresh cell culture media at a final density of 2 x 10^7 BMDCs per milliliter and doses of 200 µl (10^5 BMDCs) were intravenously injected into the mice. To test the immunogenicity of the phagocytized bacteria by themselves, half of the pulsed BMDCs preparation ready for immunization, was subjected to three sequential freeze-thaw cycles performed by immersion in liquid nitrogen and thaw at 37°C. No further washings were performed before immunization (in order to retain the released bacterial products during the freeze-thaw cycles).

For viability experiments, immediately after the pulse and after extensive washing performed at 4°C, pulsed BMDCs were fixed for 30 min in 1% paraformaldehyde in PBS at RT or for 1 min in 70% ethanol in PBS. Cells were washed again three times and the viability and cell count determined before injection.

**Phagocytic Capacity of BMDCs.** BMDCs were pulsed with varying ratios of CM-DiI–labeled S. pneumoniae/DC as indicated above for the transfer experiments. At different times (0, 0.5, 5, and 24 h), the BMDCs were collected, washed in PBS, and viable BMDCs were analyzed by flow cytometry gating to exclude free bacteria by size. BMDCs pulsed with unlabeled bacteria at the same ratios were used to estimate the background of nontpecific fluorescence. To estimate the average maximal number of bacteria phagocytosed per individual BMDC, the mean fluorescence intensity (MFI) of the gated BMDCs pulsed with CM-DiI–labeled bacteria, after subtracting background fluorescence, was divided by the MFI of the CM-DiI–labeled free bacteria used for the pulse. Because CM-DiI is lipophilic, an increased fluorescence emission due to the aggregation of the labeled particles after DC internalization, and due to label insertion in DC membranes as a result of bacterial processing by the DCs could be expected. Thus, the obtained value must be considered as maximal average uptake.

**Cytokine ELISA.** The concentrations of specific cytokines released into the media of BMDC cultures were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin–alkaline phosphatase (AP) were purchased from BD Pharmingen. Streptavidin–AP was used in combination with PNPP (p-nitrophenyl phosphate disodium; Sigma–Aldrich) as substrate to detect the specific binding. Standards were included in every plate and the samples were tested in duplicate. The detection limits of the respective ELISAs were 20 pg/ml IL–2, 35 pg/ml IL–3, 5 pg/ml IL–4, 4 pg/ml IL–6, 150 pg/ml IL–10, 6 pg/ml IL–12 (p40/p70), 125 pg/ml IL–12 (p70), 12 pg/ml TNF–α, and 80 pg/ml IFN–γ.

**“Real-Time” RT-PCR for Measurement of Cytokine-specific mRNA.** Total RNA was extracted from BMDCs, collected at different times during the pulse with S. pneumoniae using RNAzol B (TEL–TEST, Inc.). Control samples contained mRNA obtained from BMDCs cultured for the same time period in medium alone. Total RNA was then reversed–transcribed using the Superscript II Preamplification System for first strand cDNA synthesis (Invitrogen) according to manufacturer’s instructions. 180 ng RNA was subsequently used as template for each “Real-Time” PCR reaction. All PCR reactions for cytokine–specific mRNA were performed on an ABI PRISM 7700 Sequence Detector System (PE Applied Biosystems) using proprietary cytokine–specific primers and probes from ABI Applied Biosystems. Relative cytokine mRNA levels were determined by normalization of signal with that for ribosomal RNA. In initial studies, twofold dilutions of cDNA generated a linear signal curve over at least a 30-fold range of cDNA concentrations. mRNA induction in pulsed BMDCs was reported as –fold increases over BMDCs cultured in medium alone.

**Protocols for Immunization and Cell Transfer.** Mice were immunized intraperitoneally with 5 x 10^6 free, heat-killed S. pneumoniae in PBS, or injected intravenously with 10^5 viable BMDCs. For in vivo Ig induction studies using pulsed BMDCs obtained from wild-type versus knockout mice, serum samples were obtained 14 d after BMDCs transfer into naive wild-type mice.

**In Vivo T Cell Depletion and B7 Blockade.** For acute depletion of T cells, mice were injected intravenously with 1 mg each of purified rat IgG2b anti–mouse CD4 (clone GK1.5) and rat IgG2b anti–mouse CD8 (clone 24.2.3) mAbs 2 d before the injection with BMDCs. Control mice received 2 mg of purified rat IgG2b mAb (J1.2) of irrelevant specificity. In vivo blockade of B7 interactions was accomplished through intravenous injection, 1 d before immunization, of 0.2 mg of a murine fusion protein of cytototic T lymphocyte antigen (CTLA)-4 and an Ig domain (CTLA4Ig) provided by William C. Gause (USUHS). Control mice received 0.2 mg of L6, a control fusion protein. The effects of these treatments has been reported previously in detail (6, 27).

**Measurement of Serum Titers of Ig Isotypes Specific to Cps14, PC, and PspA by ELISA.** Immulon–4 HBX (Dynex Technologies) microtiter plates were coated with 0.5 µg/well of Cps14 in 0.1 M bicarbonate buffer, pH 9.6, overnight at 4°C. After the plates were washed four times in 0.1 M Tris, pH 8.3, containing 0.05% Tween 20 and 5% ovalbumin (EBTA), threefold dilutions of the serum samples diluted in EBTA were then added starting at 1/10 or 1/25, and the plates incubated overnight at 4°C. The plates were washed three times with EBTA and incubated during 1 h at 37°C with polyclonal goat anti–mouse IgM, IgG1, IgG2a,
IgG2b, or IgG3 conjugated to AP. Plates were washed five times with PBS containing 0.05% Tween 20, and the enzymatic reaction developed for 2 h at RT using as substrate, 1 mg/ml solution of PNPP in 1 M Tris-Cl buffer, pH 9.8, and 0.3 mM MgCl₂. In every plate was included one high titer antiserum pool as standard to normalize the interassay results. Titers were expressed as the dilution of sera giving an absorbance at 405 nm equal to 1.0. If the absorbances obtained were under 1 the titer was extrapolated from the standard curve.

The ELISAs for the measurement of PC and PspA-specific Ig isotype titers have been reported previously (6). Briefly, Immunolone 4 HBX immunoplates were coated overnight with 0.5 μg/well of PC-KLH or recombinant PspA in PBS at 4°C. The plates were washed in PBS containing 0.05% Tween 20 and unspecific binding blocked with PBS containing 2% BSA. The protocols were similar to those described for detection of Cps14-specific Ig.

Statistics. Data were expressed as arithmetic mean ± SEM of the individual titer. Levels of significance of the differences between groups were determined by the Student’s t test. P values <0.05 were considered statistically significant.

Results

Internalization of Heat-killed S. pneumoniae by Cultured BMDCs Is Essentially Nonsaturable. To determine a potential role of DCs in mediating protein- and polysaccharide-specific Ig isotype responses to S. pneumoniae we prepared a relatively pure population of DCs by culturing preselected BM cells in GM-CSF. After 6–8 d of culture we obtained a ≥95% population of cells with a morphology (data not shown) and cell surface phenotype (Fig. 1 C) consistent with immature myeloid (CD11c⁺, CD11b⁻, CD8α⁻) DCs (28). We first wished to determine the average rate of DC uptake of CM-DiI–labeled S. pneumoniae as a function of bacterial density. As is shown in Fig. 1 A, uptake of nonopsonized S. pneumoniae (nonencapsulated variant of capsular type 3, JD6/11) by cultured BMDCs was essentially nonsaturatable. Similar kinetics of uptake as a function of bacterial density were also obtained with encapsulated (type 3 or type 14) or nonencapsulated (type 2) strains (data not shown). At the higher bacterial densities a plateau in uptake was observed after a prolonged culture period (>24 h) (Fig. 1 A), most likely due to the decrease in uptake of bacteria that is typically associated with DC maturation (Fig. 1 C).

S. pneumoniae Induces BMDCs to Secrete TNF-α, IL-6, IL-12, and IL-10 with Different Kinetics. BMDCs constitutively secreted relative low levels of IL-12 (p40/p70) (210 ± 25 pg/ml) during the culture period, but released no detectable TNF-α (<12 pg/ml), IL-6 (<4 pg/ml), IL-10 (<150 pg/ml), IL-2 (<20 pg/ml), IL-3 (<70 pg/ml), IL-4 (<5 pg/ml), or IFN-γ (<80 pg/ml). Stimulation with S. pneumoniae induced BMDCs to secrete TNF-α, IL-6, IL-12, and IL-10 with distinct kinetics (Fig. 1 B), but did not stimulate detectable IL-2, IL-3, IL-4, or IFN-γ. TNF-α was the first to be detected (30 min) after exposure to S. pneumoniae followed closely by IL-6 and IL-12 (1–2 h) then later by IL-10 (5 h) (Fig. 1 B). Detection of IL-12 as p40/p70 or p70 resulted in similar kinetics (data not shown). In pulse-chase experiments (Fig. 1 B) we observed a rapid downregulation of TNF-α secretion. After 5 h no further secretion of TNF-α could be detected. Secretion of IL-12 and IL-10 dropped significantly after 5 h but was still detectable for an extended period (up to 24 h). In contrast, IL-6 secretion was sustained for >48 h at levels 1,000× over the background (Fig. 1 B, data not shown). The ki-
netics of cytokine secretion correlated well with the kinetics of induction of cytokine-specific mRNA (Fig. 1 B). These data strongly suggest that *S. pneumoniae* induces, with distinct kinetics, transcriptional upregulation and secretion of both proinflammatory (IL-6, IL-12, and TNF-α) and antiinflammatory (IL-10) cytokines by BMDCs.

*S. pneumoniae* Induces Maturation of BMDCs in Culture. As shown in Fig. 1 C, *S. pneumoniae* also induced BMDC maturation in vitro, as evidenced by upregulation of MHC class II, CD40, CD80 (B7–1), CD86 (B7–2), and CD86 (B7–2) in downregulation of CD16/CD32 (Fcγ II/III receptor). The expression levels of CD11c, CD11b, and H2-K were unchanged after *S. pneumoniae* exposure, whereas CD8α was undetectable at all time points. The induction of BMDC maturation was dose-dependent with little phenotypic change occurring at 40 bacteria/DC, thus establishing a stimulation threshold for this process. In pulse-chase experiments, optimal induction of BMDC maturation required no more than 2–5 h exposure to *S. pneumoniae* (data not shown).

BMDCs Pulsed In Vitro with *S. pneumoniae* Induced both Protein- and Polysaccharide-specific Ig Isotype Responses In Vivo. In preliminary experiments we established a set of conditions for inducing optimal in vivo protein- and polysaccharide-specific Ig responses upon transfer of *S. pneumoniae*-pulsed DCs (“pulsed BMDCs”) into naive mice (data not shown). On the basis of these studies, we chose to inject each mouse intravenously with 10⁶ viable BMDCs that were pulsed with a ratio of 800 bacteria per individual DC for a 4–5 h period. This resulted in each DC internalizing an average of ≤10 bacteria (see Fig. 1 A). As illustrated above, these conditions for BMDCs pulsing further allowed for in vitro autocrine/paracrine exposure of BMDCs to stimulatory cytokines, but without significant exposure to immunosuppressive IL-10, and for continued cytokine secretion, with the exception of TNF-α, by BMDCs upon transfer in vivo. As shown in Fig. 2, BMDCs pulsed with heat-killed *S. pneumoniae* and transferred into naive mice stimulated a primary Ig response specific for PspA, Cps14, and PC (hapten component of the cell wall C-polysaccharide). BMDCs pulsed with formalinized bacteria induced comparable primary antigen-specific Ig responses (data not shown). A primary Ig response specific for PsaA, a membrane-bound lipoprotein, was also induced (data not shown). In contrast, unpulsed BMDCs failed to induce any specific primary Ig response.

The kinetic and isotype distribution of the primary humoral responses induced by pulsed BMDCs were generally similar to that observed after immunization with free *S. pneumoniae* (Fig. 2). The Ig isotype profiles were dependent on the eliciting antigen. PspA-specific Igs were dominated by IgG2b and IgG2a, with lower amounts of IgG1. No PspA-specific IgA, IgM, or IgG3 were detected. In contrast, polysaccharide-specific responses (anti-PC and anti-Cps14) included all Ig isotypes, but IgM and IgG3 were prevalent. The primary anti-PC response displayed kinetics characteristic of a classical T1 response with Ig levels peaking around day 7. However, the primary anti-Cps14 response showed more delayed kinetics similar to those exhibited by the PspA-specific Ig response (Fig. 2).

Mice Primed with *S. pneumoniae*-pulsed BMDCs Developed Immunological Memory. The anti-PspA response elicited by pulsed BMDCs was highly boosted for all Ig isotypes after secondary immunization with a suboptimal dose of free PspA-specific antigens.

![Figure 2](image_url)

**Figure 2.** Kinetics of the in vivo antiprotein and anti-polysaccharide Ig isotype induced by in vivo transfer of BMDCs pulsed with *S. pneumoniae* type 14. Groups of C57BL/6 mice (n = 6) received an intravenous injection of 10⁶ BMDCs pulsed with *S. pneumoniae* type 14 (black symbols) or 10⁶ unpulsed BMDCs as control (white symbols) at day 0. At day 21, both groups of mice received an intraperitoneal immunization with 5 × 10⁶ free *S. pneumoniae* type 14 (indicated by arrows). The data shows the serum titers of Ig isotypes specific for PspA (top), PC (middle), and Cps14 (bottom) on different days after immunization. The data shown are representative of two independent experiments.
bacteria (17–19-fold enhancements) (Fig. 2). In contrast, mice receiving unpulsed BMDCs followed by immunization with free bacteria elicited a barely detectable anti-PspA response. These data indicated that pulsed BMDCs induced PspA-specific memory. In contrast, the IgM, IgG3, and IgG2b anti-PC responses in mice receiving pulsed or unpulsed BMDCs were equivalent after boosting with free bacteria. An exception was IgG1 anti-PC which was ~10-fold higher in boosted mice receiving pulsed versus unpulsed BMDCs (Fig. 2). Mice receiving two immunizations (21 d apart) with free bacteria alone, showed no boosted anti-PC responses for any isotype, arguing against significant carry-over of free bacteria during primary immunization with pulsed BMDCs. The ability to similarly boost the anti-Cps14 response in mice receiving pulsed BMDCs was also Ig isotype-dependent (Fig. 2). Thus, significant boosting was again seen for the IgG1 isotype, but also for IgG3, whereas little if any boosting was observed for IgM or IgG2b anti-Cps14 relative to mice receiving unpulsed BMDCs. Collectively, these data indicate the ability of BMDCs pulsed with intact *S. pneumoniae* to uniformly elicit strong immunologic memory for a bacterial protein. Memory generation for an antipolysaccharide response was Ig isotype dependent.

**Viable Pulsed BMDCs Are Specifically Required for Induction of Ig Responses In Vivo.** We next wished to determine whether pulsed DCs played an active role in initiating in vivo Ig responses, or perhaps were serving as nonspecific and passive carriers of antigen. Thus, BMDCs were first pulsed with *S. pneumoniae* type 14, then the excess of bacteria was washed and immediately fixed in formaldehyde before transfer into naive mice. As shown in Fig. 3, BMDCs viability was critical for both anti-PspA and antipolysaccharide Ig responses. Similar results were obtained when BMDCs were pulsed with the nonencapsulated R36A strain, then fixed in ethanol before transfer (data not shown). The Ig induction was specific for BMDCs since viable thymocytes incubated with bacteria also failed to induce detectable antigen-specific Ig responses (Fig. 3), ruling out the possibility that carry-over of free bacteria was responsible for the observed Ig responses in vivo. Moreover, immunization with BMDCs which were first pulsed with *S. pneumoniae*, then freeze-thawed immediately before injection, did not induce detectable antiprotein- or antipolysaccharide-specific Ig responses (Fig. 4). These data strongly suggest that internalized bacteria, in the absence of viable BMDCs, were not immunogenic by themselves. Collectively, these data demonstrate an active role for pulsed BMDCs in mediating both protein- and polysaccharide-specific Ig responses in vivo.

**Both Antiprotein and Antipolysaccharide Ig Responses Induced by Pulsed BMDCs Are T Cell-dependent and Require B7-dependent Costimulation.** To determine the requirement for T cells in mediating Ig responses to pulsed-BMDCs we acutely depleted T cells in the recipient mice with anti-CD4 and anti-CD8 mAbs before injecting pulsed BMDCs. As shown in Fig. 5, endogenous T cells were critical for development of IgG specific for both anti-PspA and polysaccharide antigens, whereas IgM antipolysaccharide responses showed only a partial dependence on T cells. We further wished to determine whether B7-dependent costimulation was required for these T cell–dependent effects. Mice were therefore injected with CTLA4Ig before transfer of *S. pneumoniae*-pulsed BMDCs. As shown in Fig. 5, functional interaction of T cells with B7 expressed on either recipient cells and/or on the injected BMDCs pulsed with *S. pneumoniae*, were critical for both the IgG anti-PspA and IgG anti-Cps14 responses. Significant suppres-

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**Figure 3.** BMDCs viability is critical for the induction of antigen-specific antibody responses. Groups of five B6.129 mice were injected intravenously with either 10^6 viable BMDCs pulsed with *S. pneumoniae* type 14 (live BMDCs), 10^6 pulsed BMDCs that were fixed with formaldehyde immediately before transfer (fixed BMDCs), 10^6 viable thymocytes incubated with the same ratio of bacteria (thymocytes) or, 10^6 BMDCs not exposed to bacteria (live unpulsed BMDCs). Sera samples were collected at day 14 and serum titers of antigen specific Ig isotypes were determined by ELISA. The data show the arithmetic mean ± SEM of the individual titers.
The induction of anti-PC and anti-Cps14 Ig responses by S. pneumoniae–pulsed BMDCs did not require BMDC expression of MHC Class II molecules and were not associated with BMDC surface presentation of polysaccharide epitopes. To further understand the mechanism(s) underlying the T cell–mediated induction of antiprotein and antipolysaccharide responses to pulsed BMDCs we used BMDCs obtained from mice genetically deficient in expression of MHC class II molecules. As shown in Fig. 6, MHC class II−/− BMDCs pulsed with S. pneumoniae failed to induce anti-PspA responses of any isotype upon transfer to naive mice. In contrast, with the exception of IgG1, both anti-PC and anti-Cps14 Ig responses to MHC class II−/− and wild-type pulsed BMDCs were similar (Fig. 6). The impairment of the IgG1 response using MHC class II−/−...
BMDCs was more marked for the Cps14-specific relative to the PC-specific response. Flow cytometric analysis using three anti-PC mAbs and one polyclonal anti-Cps14 mAb, in BMDCs pulsed for 24 h with *S. pneumoniae*, failed to detect either PC or Cps14 on the cell surface of pulsed BMDCs, but did easily detect the presence of intracellular PC (data not shown). These data argue against a direct BMDCs surface presentation of B cell epitopes for the polysaccharides studied.

**BMDC Expression of CD40 or B7 Is Required for an Optimal Anti-PspA Response, but Only for an Optimal IgG1 Antipolysaccharide Response.** To further determine the costimulation requirements for Ig induction in response to pulsed BMDCs, we directly evaluated the importance of BMDC expression of CD40 and B7−1/B7−2 by using BMDCs obtained from mice genetically deficient in CD40, or mice doubly deficient for B7−1 and B7−2. As shown in Fig. 7, BMDCs lacking CD40, or B7 expression were markedly defective in inducing anti-PspA responses of all isotypes relative to wild-type BMDCs. In contrast, BMDCs lacking these cell surface molecules were defective only in induction of polysaccharide-specific IgG1. The differences in the IgG anti-PC and anti-Cps 14 responses observed when using CTLA4Ig (Fig. 5) versus B7-deficient BMDCs (Fig. 7), may reflect either compensatory costimulatory ability of BMDCs lacking B7, or more likely B7-dependent costimulation by B cells and/or macrophages in the recipient. In this regard, BMDCs derived from CD40−/−, B7−1/B7−2−/−, and MHC class II−/− mice, did not differ significantly in vitro, in phagocytic capacity, cytokine secretion, or cell surface phenotype relative to wild-type BMDCs (data not shown).

**BMDC Production of IL-6 Is Required for Optimal Anti-PspA and Antipolysaccharide Ig Responses.** Finally, to determine whether BMDC-derived cytokines play a role in Ig
responses to *S. pneumoniae*, we used BMDCs obtained from mice genetically deficient in TNF-α, IL-6, IL-12, and IL-10. IL-6−/− BMDCs showed a striking defect in the induction of IgG anti-PspA of all isotypes, and IgG1 and IgG2b anti-Cps14, with a more modest reduction in IgG1 anti-PC (Fig. 8). BMDCs lacking in IL-10 or TNF-α demonstrated much more limited defects; IL-10−/− BMDCs elicited a diminished IgG1 anti-PspA response relative to wild-type BMDCs whereas TNF-α−/− BMDCs elicited reduced IgG2b anti-Cps14. A lack of IL-12 expression by BMDCs had no significant effects on either the anti-PspA or antipolysaccharide responses. BMDCs obtained from TNF-α−/−, IL-6−/−, or IL-12−/− mice did not differ significantly from wild-type BMDCs in their in vitro phagocytic capacity, secretion of cytokines, and cell surface phenotype before or after exposure to *S. pneumoniae* (data not shown). However, whereas IL-10−/− and wild-type BMDCs showed a similar cell surface phenotype before pulsing with *S. pneumoniae*, exposure to bacteria resulted in a significantly greater upregulation of MHC class II and CD86 in IL-10−/− BMDCs relative to wild-type BMDCs (2–3-fold increase in MFI). Enhanced expression of MHC class II and CD86 was also observed in cultures of wild-type, pulsed BMDCs treated with a neutralizing anti–IL-10 mAb. Finally, addition of IL-10 to IL-10−/− pulsed BMDC cultures reversed the increased enhancement of MHC class II and CD86 relative to wild-type BMDCs (data not shown). These data indicated that IL-10 downregulates BMDC maturation in an autocrine manner.

**Discussion**

Little is known regarding the cellular mechanism(s) by which intact extracellular bacteria elicit in vivo antipolysaccharide, as well as antiprotein Ig isotype responses. The inability of polysaccharide antigens to associate with MHC molecules (2) and hence their failure to recruit cognate T cell help for antipolysaccharide responses, has long called into question the role, if any, of APCs, as well as T cells in this process. Although polysaccharides may deliver potent membrane Ig-mediated signals to the B cell, by virtue of their multivalency, in vitro studies strongly suggest that additional B cell signals are required for induction of Ig class switching and secretion (1, 29).

It is well established that DCs can internalize both Gram-positive and Gram-negative bacteria and can be stimulated by these pathogens to undergo maturation and cytokine secretion (11, 12, 30–32). However, the potential role of DCs in mediating polysaccharide-, as well as protein-specific humoral responses in vivo, upon exposure to an intact bacterial pathogen is unknown. In this regard, we demonstrate that immature BMDCs not only mature and secrete cytokines in response to the Gram-positive extracellular bacteria, *S. pneumoniae*, but that such DCs internalize nonopsonized *S. pneumoniae* and subsequently mediate primary Ig responses specific for a bacterial-derived protein (PspA), lipoprotein (PsaA), capsular polysaccharide (Cps14), and a haptenic component (PC) of the cell wall C-polysaccharide (teichoic acid). Pulsed BMDCs also induce immunologic memory which is most pronounced for the antiprotein response. The mechanisms underlying BMDC induction of protein- versus polysaccharide-specific Ig are distinct as will be discussed below.

The requirement for active participation, and hence potential physiologic relevance, for BMDC induction of Ig in response to intact *S. pneumoniae*, are underscored by the demonstrated requirement for BMDC viability after bacterial uptake and processing, cellular selectivity as evidenced
by the inability of viable thymocytes to similarly induce Ig, the absence of BMDC cell surface retention of S. pneumoniae after a 24-h pulse, the poor immunogenicity of pulsed BMDCs subjected to several freeze-thaw cycles before injection, a strong requirement for BMDC secretion of IL-6, and cell surface expression of MHC class II, CD40, and B7–1/B7–2. Thus, passively carried intact bacteria, cell membrane fragments containing MHC molecules loaded with processed antigen, or cell debris containing partially processed bacteria cannot account for BMDC induction of the antigen-specific Ig responses. In this regard, DCs pulsed with S. pneumoniae in vitro, then formalin-fixed, localized to the spleen 1 h after intravenous injection at levels comparable to that seen using pulsed viable DCs (ratio 1:1.3 fixed: viable; data not shown). These data further support the notion that passive DC-mediated transport of bacteria to the spleen cannot account for the Ig-inducing activity of viable pulsed DCs. However, the transfer of antigen by injected BMDCs to endogenous APCs for their subsequent reprocessing and presentation could also be important in the induction of the Ig response. This process could be mediated either through synapse formation with endogenous APCs (33, 34) or exosome secretion by the injected BMDCs (35) as has been demonstrated in vitro.

In addition to a requirement for viable BMDCs, the protein- and polysaccharide-specific IgG responses induced by pulsed BMDCs also share a requirement for T cells and B7-dependent costimulation, whereas the IgM antipolysaccharide response is relatively less dependent on these forms of help. Cognate interactions between APCs and T cells result in bidirectional signaling that is mediated by a number of receptor–ligand pairs (36). Specifically, T cells become fully activated through engagement of TCR with MHC–peptide complexes on APCs, in concert with a costimulatory signal typically, but not exclusively, delivered by the interaction of CD28 on the T cell with B7–1/B7–2 on the APC. TCR cross-linking in turn, induces CD40-ligand on the T cell which then can activate the APC through CD40. This serves, in part, to upregulate B7 and MHC molecules on the APC as well as costimulate release of inductive cytokines making them more effective APCs for T cells. In this regard, the absolute requirement for BMDC expression of MHC class II, CD40, and B7–1/B7–2 for induction of PspA-specific Ig of all IgG isotypes, collectively support the notion that cognate DC–T cell interactions play a critical role in mediating this response. Interestingly, a similar pattern to that observed for induction of PspA-specific Ig, including the induction of immunologic memory, was observed for the IgG1 anti-PC and anti-Cps14 responses. This strongly suggests that the induction of IgG1 antipolysaccharide responses by DCs, partly required cognate DC–T cell help. That help may be mediated first through the priming of T cells specific for bacterial protein epitopes presented by the DCs. Polysaccharide-specific B cells may then internalize bacteria or bacterial fragments containing microbial protein and present MHC-peptide to the primed T cells in order to receive cognate help. Of interest, conjugation of polysaccharides to protein carriers (e.g., “conjugate vaccines”) alters the antipolysaccharide response from TI to TD and this is associated with a shift to the IgG1 isotype and memory development (1, 37, 38).

In striking contrast, the IgG3 and IgG2b, as well as the IgM, anti-PC, and anti-Cps14 responses are induced normally by pulsed BMDCs lacking either MHC class II, CD40, or B7–1/B7–2. These data suggest the possibility that the T cell help for the IgG3 and IgG2b antipolysaccharide response is noncognate. In support of this, recent observations in our laboratory indicate a relatively rapid, B7-dependent but TCR-nonspecific T cell helper activity for the IgG anti-PC response to free S. pneumoniae in vivo (22, 39). Further, the lack of a requirement for BMDC expression of B7–1/B7–2 for the IgG3 and IgG2b antipolysaccharide response, despite its inhibition by CTLA4Ig, suggests that expression of B7 on other cell types, mostly likely B cells and/or macrophages, can mediate the required T cell costimulation.

BMDCs secreted both proinflammatory (IL-6, TNF-α, and IL-12) and antiinflammatory (IL-10) cytokines with distinctively different kinetics and duration after in vitro exposure to S. pneumoniae, and this was mirrored at the transcriptional level. Of note, TNF-α was secreted most rapidly, then quickly downregulated relative to the other cytokines, suggesting that a major role in vivo of DC-derived TNF-α, as with TNF-α secreted by other myeloid cells, is to activate the proinflammatory process in a paracrine fashion. In light of the time during which BMDCs were exposed in vitro to S. pneumoniae before being transferred into recipient mice, we anticipate that BMDCs released relatively little TNF-α during its elicitation of the Ig response in vivo. Consistent with this, the Ig responses elicited by TNF-α−/− and wild-type BMDCs were essentially similar. TNF-α has been reported to induce maturation of BMDCs (40) and immature DC lines in vitro (31, 32), and perhaps serves to costimulate maturation of DCs induced by bacteria. However, we observe that BMDCs from TNF-α−/−, as well as from IL-6−/− and IL-12−/− mice matured and secreted other cytokines normally in vivo, relative to wild-type BMDCs.

In contrast, IL-10 in an autocrine fashion, limited BMDC maturation in response to S. pneumoniae. Our observation that IL-10 released by BMDCs was delayed relative to IL-6, TNF-α, and IL-12, suggests that this IL-10 serves to limit DC activation after its initiation by other stimuli. IL-10 has also been shown by others to impair DC induction of T cell responses and to induce antigen-specific tolerance (41, 42). In this regard, the length of time during which BMDCs were pulsed with S. pneumoniae in vitro before transfer into recipient mice, greatly limited their exposure to artifically high levels of IL-10 in culture. Of interest, IL-10−/− and wild-type BMDCs elicited similar Ig responses in vivo, with the unexplained exception of the IgG1 anti-PspA response, which was markedly reduced in response to pulsed IL-10−/− BMDCs.

Pulsed BMDCs secreted IL-12 with kinetics similar to IL-6. Of interest, BMDCs used in this study were of the myeloid type (CD11c+CD8α−), whereas lymphoid DCs

10 Dendritic Cells Induce Polysaccharide-specific Ig Responses
(CD11cCD8α+) appear to be the major source of IL-12 release in vivo in the systems studied (43). IL-12 plays a key role in mediating a type 1 cytokine response, characterized by IFN-γ, but not IL-4 production. Type 1 responses typically show a skewing of Ig toward the IgG2a isotype whereas IL-4-dominated responses show a shift toward the production of IgG1 (44). DCs have been implicated as a key initial source of IL-12 for directing a subsequent type 1 production of IgG1 (44). DCs have been implicated as a key initial source of IL-12 for directing a subsequent type 1 response. Nevertheless, S. pneumoniae-pulsed BMDCs elicited a mixed Ig isotype response suggesting a nonpolarized pattern of cytokine production, and IL-12−/− and wild-type BMDCs elicited similar Ig isotype responses in vivo.

The most dramatic effect of BMDC-derived cytokines on the humoral response was seen with IL-6, as evidenced by drastic impairments of both the antiprotein and antipolysaccharide responses using IL-6−/−-pulsed BMDCs. Specifically, marked reductions were observed in anti-PspA of all IgG isotypes, IgG1 and IgG2b anti-Cps14, and IgG1 anti-PC. IL-6 could act directly on B cells to induce maturation (45). In this regard, DCs have been reported to secrete soluble IL-6Rα which, when complexed to IL-6 enhance the differentiation of CD40 activated B cells to IgG-secreting cells (36). Alternatively, IL-6 may stimulate humoral responses, through induction of C3 synthesis by local macrophages (46). Antigen-bound C3 is important for CD21-dependent B cell antigen receptor signaling, as well as retention of antigen by follicular dendritic cells (47). Of interest, a previous study has implicated DCs as a primary source of IL-6 during a TD Ig response in vivo (46).

The parameters governing the anti-Cps14 versus the anti-PC responses were also somewhat different. Specifically, the anti-Cps14 response showed a greater dependence on T cell help and B7-dependent costimulation, as well as BMDC secretion of IL-6. Potentially relevant to these differences is the fact PC is an environmental antigen expressed by a broad range of commensal and pathogenic microorganisms, as well as by host glycoconjugates (48), whereas Cps14 is unlikely to be widespread in the environment. Thus, B cells specific for PC, but not Cps14, may be constitutively activated through their surface Ig receptors, and thus have less stringent activation requirements for induction of IgG isotype switching and maturation into IgG-secreting cells.

In summary, our data are the first to demonstrate an active role for DCs in the uptake and presentation of both polysaccharide and protein antigens expressed by an intact extracellular bacteria, for induction of a humoral response in vivo. The distinctly different parameters involved through which DCs mediate antiprotein and antipolysaccharide responses, and even environmental versus nonenvironmental polysaccharides suggest novel pathways of immune regulation and a powerful model system for their further investigation. Of particular interest will be an elucidation of how DCs process bacterial pathogens for the eventual presentation of polysaccharide antigens to the immune system.

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