Opposing Signals from Pathogen-Associated Molecular Patterns and IL-10 Are Critical for Optimal Dendritic Cell Induction of In Vivo Humoral Immunity to Streptococcus pneumoniae

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Interleukin-10 is widely regarded as an inhibitor of immunity in part through its ability to inhibit dendritic cell (DC) function. The present study suggests a modification of this view by demonstrating instead that a critical balance exists between signals mediated by pathogen-associated molecular patterns and IL-10 for optimization of DC induction of an in vivo humoral immune response. Bone marrow-derived, CD8α- DC pulsed with Streptococcus pneumoniae in vitro induce in vivo protein- and polysaccharide-specific Ig isotype responses upon adoptive transfer into naive mice. Following bacterial activation, DC have a limited time during which they can function as effective APCs in vivo due to the onset of maturation-associated apoptosis. Autocrine IL-10, by limiting the time during which DC are responsive to widely varying levels of bacterial stimulation, delays the onset of DC apoptosis and thus prolongs the time during which DC are able to elicit in vivo humoral immunity. These data demonstrate a requirement for properly balanced positive and negative signaling in DC to optimize an in vivo immune response to a pathogen. The Journal of Immunology, 2003, 171: 3508–3519.

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3 Abbreviations used in this paper: DC, dendritic cell; Act D, actinomycin D; AP, alkaline phosphatase; BMDC, bone marrow-derived DC; CM, chloromethylbenzamido; Cps14, capsular polysaccharide type 14; DII, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; MFI, mean fluorescence intensity; PAMPs, bacterial pathogen-associated molecular patterns; PC-KLH, phosphorylcholine-keyhole limpet hemocyanin; PL, propidium iodide; PopA, pneumococcal surface protein A; 1/2 (1.5–2.9 days) (10).

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of apoptotic or necrotic fragments of Ag-carrying DC that had migrated to the lymphoid organ (15). Although cross-priming for CTL induction has been well described, via DC uptake of virally infected apoptotic cells (16), little is known in this regard concerning the mechanism of DC presentation of bacterial Ags. In vitro, contradictory results have been obtained using different apoptotic cell types. Thus, whereas DC that internalize *Salmonella*-infected macrophage-apoptotic bodies efficiently present bacteria-encoded Ag on MHC class I and II molecules (17), uninfected apoptotic neutrophils inhibit the ability of LPS-activated DC to prime naive T cells (18).

In the present study, we investigated the parameters that regulate DC longevity in response to bacterial challenge and the associated impact on the ability of DC to induce in vivo humoral immunity. We demonstrate a requirement for properly balanced positive and negative signaling in DC following exposure to bacteria that appears to be critical for optimizing DC induction of an in vivo antibacterial humoral immune response.

Materials and Methods

**Mice**

C57BL/6N and BALB/c mice were obtained from the National Cancer Institute (Gaithersburg, MD). IL-10−/− mice and their corresponding C57BL/6J controls were obtained from The Jackson Laboratory (Bar Harbor, ME). 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, Bethesda, MD). The experiments in this study were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare (National Institutes of Health) 78-23.

**Streptococcus pneumoniae strains and bacterial Ags**

The *S. pneumoniae* capsular type 14 strain (Pn14) was generously provided by S. Wilson (USUHS). Recombinant pneumococcal surface protein A (PspA) was expressed in *Saccharomyces cerevisiae* BJ3505 as a His6-tagged fusion protein, and purified by Ni-NTA affinity chromatography (19). Phosphorylcholine-keyhole limpet hemocyanin (PC-KLH), a gift of Dr. M. Lees (Biosynexus, Rockville, MD), was synthesized, as described previously (19). The resulting conjugate had a substitution degree of 19 PC-Ag on MHC class I and II molecules (17), uninfected apoptotic neutrophils inhibited the ability of LPS-activated DC to prime naive T cells (18).

**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, heat killed by incubation at 60°C for 1 h, extensively washed in PBS, and adjusted with PBS to 106 CFU/ml by absorbance reading of 0.6 at 650 nm. Sterility was confirmed by subculture on blood agar plates. Bacteria were then aliquoted at 1010 CFU/ml and frozen at −80°C until thawed for use.

**Cell culture medium**

The cell culture medium consisted of RPMI 1640 supplemented with 5% FCS (BioWhittaker, Walkersville, MD), 50 μM 2-ME, 20 μg/ml gentamicin, 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 25 mM HEPES.

**Bone marrow cell culture**

DC were cultured from bone marrow, essentially as previously described (21), with slight modifications (8). Briefly, bone marrow cell suspensions were depleted of T cells, B cells, and other MHC class II-expressing cells by incubation for 30 min at 4°C with a mAb cocktail composed of AF6-120.1 (mouse IgG2a anti-I-Ak), RA3-6B2 (rat IgG2a anti-CD45RB/CD220), 53-8.8 (rat IgG1 anti-CD8α), and RB6-8C5 (rat IgG2b anti-rat-ly–6G), 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; Biosource, Camarillo, CA). All the mAbs were purchased from BD PharMingen (San Diego, CA). The bone marrow cells obtained were cultured at a 1.25 × 106 cells/ml in cell culture medium supplemented with 10 ng/ml murine rGM-CSF, kindly provided by L. Grinberg (Biosynexus). On days 3 and 5, the supernatant was removed and replaced with fresh medium containing GM-CSF.

**Trypan blue exclusion assay**

Cell membrane permeability and viability were tested by the trypan blue exclusion assay by adding a mixture of a 0.4% solution of trypan blue (Sigma-Aldrich, St. Louis, MO) in cell-containing medium at a 1/5 v/v dilution. Percentage of viable cells was counted by light microscopy using a hemocytometer.

**DNA labeling and flow cytometric analysis**

The percentage of apoptotic nuclei was measured by flow cytometry, after propidium iodide (PI) staining in hypotonic buffer, essentially as described by Nicoletti et al. (23). Briefly, BMDC were collected at different time points during the pulse with bacteria or drug treatment and washed extensively, and the cell pellet of 1 × 106 BMDC was gently resuspended in 1 ml of hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate plus 0.1% Triton X-100). The tubes were placed at 4°C in the dark overnight, and the PI fluorescence of individual nuclei was measured by flow cytometric analysis (23). BMDC treated in culture for 12–24 h with 1 μg/ml actinomycin D (Act D; Sigma-Aldrich) were used as positive controls of apoptosis. BMDC treated with 0.2% saponin (Sigma-Aldrich) were used as positive control of necrosis.

**Inhibition of caspase-10 activity**

Caspase-10 activity was irreversibly inhibited by treatment of BMDC with 50 μM of the cell-permeable Z-DEVAD caspase inhibitor (R&D Systems, Minneapolis, MN). The inhibitor was added to the cultures 30 min before the pulse with bacteria and maintained in the culture medium during the pulse. This treatment efficiently blocked the BMDC apoptosis induced by heat-killed *S. pneumoniae* (22).

**Flow cytometric analysis of BMDC surface Ag expression**

All steps were performed on ice. For staining BMDC for flow cytometry, the Fc receptors were specifically blocked with 2.5 μg/ml/106 BMDC of anti-CD16/CD32 mAb (clone 2.4G2, BD PharmMingen) in PBS containing 1% FCS, 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 HLE), I-Ak (clone AF6-120.1), I-Ak (clone AMS-32.1), H2-Kk (AF6-88.5), H2-Kk (SF1-1.1), CD40 (clone 5/23), CD80 (16-10A1), CD86 (clone GL1), and CD25 (clone PC70). 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 Ab (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, Miami, FL). Dead cells and debris were excluded from analysis by gating on the appropriate forward and side scatter profile.

**Phagocytic capacity of BMDC**

The time course of bacterial internalization was tracked by pulsing the BMDC with *S. pneumoniae* labeled with the fluorescent lipophilic cell tracker chloromethylbenzamido derivative of 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (CM-Di; Molecular Probes, Eugene, OR), as previously reported (8). Briefly, at different time points of the pulse, BMDC were collected and washed in PBS, and viable BMDC were analyzed by flow cytometry with gating by size, to exclude...
free bacteria. BMDC pulsed with unlabeled bacteria at the same ratios were used to estimate the background of nonspecific fluorescence.

**Cytokine ELISA**

The concentrations of specific cytokines released into the medium 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 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 IL-6, 4 pg/ml; IL-10, 70 pg/ml; IL-12 (p40/p70), 6 pg/ml; IL-12 (p70), 125 pg/ml; TNF-α, 12 pg/ml.

**Bacterial pulsing of BMDC for in vivo transfer experiments**

In experiments involving transfer of in vitro pulsed BMDC into naive mice, the BMDC were pulsed at ratios of 800 bacteria per individual BMDC during a time pulse of 5, 24, or 44 h. After the pulse, the excess of bacteria was extensively washed, at least six times each for 10 min at 350 × 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 medium at 0.5 × 10^7 BMDC/ml and doses of 200 µl (10^7 BMDC) were injected i.v. into the mice. Alternatively, BMDC were replated and cultured for an additional 18–20 h in medium alone (i.e., chase) before transfer to the recipient mice. For the experiments comparing capacity of BMDC subjected to different times of pulse and chase with bacteria to induce Ag-specific Ig isotype responses in vivo, the addition of bacteria was timed to allow the injection of the BMDC at the same time in all the experimental groups of mice.

**Immunization and cell transfer**

For in vivo Ig induction studies, naive mice were injected i.v. with 1 × 10^6 viable trypan blue-excluded, pulsed wild-type or IL-10−/− BMDC following various in vitro treatments. Serum samples were obtained 14 days after BMDC transfer.

**Measurement of serum titers of Ig isotypes specific to Cps14, PC, and PspA by ELISA**

Immulon-4 HBX (Dynex Technologies, Chantilly, VA) microtiter plates were coated with 0.5 µg/well of the Cps14 of S. pneumoniae 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% OVA (EBTA), 3-fold dilutions of the sera samples diluted in EBTA were added starting at 1/10 or 1/25, and the plates were incubated overnight at 4°C. The plates were washed three times with EBTA and incubated 1 h at 37°C with biotinylated mAbs used for detection, and streptavidin-alkaline phosphatase, were purchased from BD PharMingen. Streptavidin-AP was used in combination with 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 IL-6, 4 pg/ml; IL-10, 70 pg/ml; IL-12 (p40/p70), 6 pg/ml; IL-12 (p70), 125 pg/ml; TNF-α, 12 pg/ml.

**Results**

**Protein- and polysaccharide-specific Ig isotype responses elicited in vivo by BMDC pulsed in vitro with S. pneumoniae decreased with the time of pulse**

As we previously reported (8), BMDC pulsed in vitro for 5 h with heat-killed S. pneumoniae, then transferred into naive recipients, elicited Ig isotype responses specific for bacterial protein (i.e., PspA) and polysaccharide (i.e., PC, determinant of C-polysaccharide or Cps14) Ags. However, as shown in Fig. 1, we observed that the time of pulse with bacteria strongly impacted on the capacity of BMDC to induce in vivo Ag-specific Ig isotype responses, despite the fact that in all cases equal numbers of pulsed BMDC, excluding trypan blue, were injected. Thus, BMDC exposed to heat-killed S. pneumoniae for 24 h in vitro before transfer into naive mice were strikingly less able to stimulate IgG anti-PspA, anti-PC, and anti-Cps14 responses in vivo, relative to BMDC pulsed in vitro for only 5 h before transfer. In contrast, the time of pulse had no significant effect on the IgM anti-PC or IgM anti-Cps14 response. As further illustrated in Fig. 1, this difference was not due to a longer in vivo exposure of BMDC to free bacteria in the culture medium, because a 5-h pulse with S. pneumoniae, followed by resuspension of washed BMDC in medium alone for an additional 18 h (i.e., an 18-h chase) also led to strongly reduced Ig responses in vivo after BMDC transfer to naive mice, similar to that observed for BMDC receiving the continual 24-h pulse. Further extending the time of in vitro pulse to 44 h or to a 24-h pulse, followed by a 20-h chase before BMDC transfer in vivo, led to a

![FIGURE 1](image-url)
We recently demonstrated that intact \textit{S. pneumoniae} not to direct toxicity correlated with DC activation and phenotypic maturation and BMDC, beginning 24 h after bacterial exposure, which was equally impaired with pulse times of 24 or 44 h, relative to 5 h. Once again, the IgM anti-PC and IgM anti-Cps14 responses at 44 h were similar to those in which pulse times were either 5 or 24 h. These data indicated that a 5-h exposure to heat-killed \textit{S. pneumoniae} induces, in vitro, a time-dependent process within BMDC that eventually diminishes their capacity to induce an in vivo IgG antiprotein and antipolysaccharide response when transferred into naive mice.

\textbf{Heat-killed \textit{S. pneumoniae} induced significant apoptosis of BMDC, beginning 24 h after bacterial exposure, that was correlated with DC activation and phenotypic maturation and not to direct toxicity}

We recently demonstrated that intact \textit{S. pneumoniae} induce DC apoptosis (22). Thus, loss of BMDC function upon adoptive transfer after prolonged in vitro culture following activation with \textit{S. pneumoniae} could be secondary to the subsequent onset of apoptosis. To quantify BMDC apoptosis, we measured the percentages of hypodiploid nuclei, using propidium iodide staining and flow cytometry (23), at various times following continual exposure of BMDC to heat-killed \textit{S. pneumoniae} in vitro. At a bacteria:BMDC ratio of 800:1, used for the experiments illustrated in Fig. 1, pulsed BMDC at 24 h exhibited only a modest degree (<20%) of apoptosis compared with pulsed BMDC at 5 h or unpulsed BMDC at 24, 44, or 72 h, which exhibited a basal level of hypodiploid cells (Fig. 2, A and B). In contrast, at 44 and 72 h after bacterial exposure, up to 60 and >80%, respectively, of BMDC exhibited apoptosis. Similar kinetics, although somewhat lower percentages of apoptotic cells, were observed following a 5-h pulse of BMDC at 800:1 bacteria:BMDC ratio, followed by reculture of washed BMDC in medium alone (chase) (Fig. 2B). In contrast to the delayed kinetics of induction of BMDC apoptosis observed in response to heat-killed \textit{S. pneumoniae}, a primary inducer of apoptosis, Act D, rapidly induced apoptosis, with ~80% hypodiploid nuclei observed at ~10 h after exposure.

The degree of apoptosis induction was dependent upon the bacteria:BMDC ratio with progressive reduction in the percentage of apoptotic cells observed over time as the ratio was lowered (Fig. 2B). Of interest, the bacteria dose response for induction of apoptosis of BMDC closely followed that observed for the degree of induction of cytokine secretion (i.e., TNF-\(\alpha\) and IL-6) (Fig. 2C) and phenotypic maturation, as evidenced by increases in cell surface expression of CD86, CD40, MHC class II, and CD25, and decreased expression of CD16/CD32 (Fig. 2D). These data strongly suggest that the BMDC pulsed with heat-killed \textit{S. pneumoniae} for 24 or 44 h, as opposed to 5 h, are ineffective at inducing Ig when transferred into naive mice, because they are poised to undergo apoptosis at these latter time points. Furthermore, these data suggest that the delayed induction of apoptosis by heat-killed \textit{S. pneumoniae}, as opposed to Act D, is associated to BMDC activation/maturation program and not a direct toxic effect of the bacteria itself.

\textbf{Autocrine IL-10 diminishes BMDC apoptosis in response to heat-killed \textit{S. pneumoniae}}

Cytokines released by DC in response to bacterial challenge could play a role in modulating the subsequent induction of apoptosis either directly via the apoptotic signaling pathway and/or indirectly through effects on cellular maturation. In turn, this could influence the ability of such DC to induce Ag-specific Ig responses. We previously demonstrated that BMDC secrete IL-6, IL-12, TNF-\(\alpha\), and IL-10 in response to \textit{S. pneumoniae} (8). IL-10 concentrations detected in culture supernatant were of 200 ± 66 pg/ml, 5 h, and 1615 ± 220, 20 h after a pulse with 800 bacteria per BMDC. Thus, we added 25 \(\mu\)g/ml of either neutralizing anti-

\textbf{FIGURE 2.} Bacterial dose dependence and kinetics of induction of BMDC nuclear fragmentation, cytokine secretion, and phenotypic maturation. A, Flow cytometric analysis of PI-stained DC cultured in medium alone or pulsed (bacteria:BMDC 800:1) for the time periods indicated. The percentages of hypodiploid nuclei are shown in every graph. B, Kinetics of induction of hypodiploid nuclei at different bacteria:BMDC ratios. Hatched lines represent 1\% of the hypodiploid nuclei are shown in every graph. C, Concentrations of secreted TNF-\(\alpha\) and IL-6 in culture supernatant over time using different bacteria:BMDC ratios were determined by ELISA. D, BMDC were cultured for 20 h with the indicated ratios of bacteria:BMDC, and the level of expression of several cell surface proteins typically modulated during maturation was determined by flow cytometry. The expression of these markers is indicated as fold change of the mean fluorescence intensity (MFI) relative to BMDC cultured in medium alone. For the DNA fragmentation studies, the arithmetic mean ± SEM of three independent experiments in triplicate is shown.
IL-6, anti-IL-12, anti-TNF-α, or anti-IL-10 mAb to BMDC cultures at the time of addition of heat-killed *S. pneumoniae*. After 72 h of culture, BMDC were analyzed by flow cytometry for percentage of hypodiploid nuclei. These mAbs efficiently neutralized the released cytokines to undetectable levels, as measured by ELISA (data not shown). As shown in Fig. 3, neutralization of IL-10 resulted in a substantial augmentation in apoptosis of pulsed BMDC. In contrast, neutralization of TNF-α led to a modest, although significant, decrease in apoptosis. IL-10 modulated apoptosis only in response to bacterial challenge, in that addition of IL-10, as well as IL-6, IL-12, or TNF-α or IL-2 or IL-4 to cultures of unpulsed BMDC at concentrations from 10 pg/ml to 10 ng/ml, had no effect on apoptosis over the 72-h culture period (data not shown).

**Autocrine IL-10-mediated reduction in BMDC apoptosis is associated with diminished BMDC maturation**

We next wished to determine whether the reduction of BMDC apoptosis secondary to autocrine IL-10 was associated with changes in BMDC maturation. At 24 h after pulsing with *S. pneumoniae*, BMDC from IL-10−/− mice showed significantly greater expression of cell surface MHC class II, CD40, CD25, CD80, and CD86, and lower levels of CD16/CD32 relative to IL-10+/+ BMDC (Fig. 4A). A similar effect on BMDC phenotype was observed when neutralizing anti-IL-10 mAb was added to cultures of pulsed IL-10+/+ BMDC when compared with pulsed BMDC treated with a control mAb (Fig. 4B). Consistent with these data, the reverse effect on phenotype was seen when IL-10−/− BMDC were treated with IL-10 (Fig. 4B). IL-10−/− and IL-10+/+ BMDC showed similar degrees of bacterial uptake 5 h after *S. pneumoniae* addition, but by 24 h, IL-10−/− BMDC showed significantly less overall uptake than IL-10+/+ cells (Fig. 4C) consistent with BMDC in a more advanced stage of maturation. These data indicate that the reduction in apoptosis due to autocrine IL-10 is associated with a decrease in BMDC maturation, with a consequent decrease in bacterial uptake.

**Late, but not early, release of cytokines in response to *S. pneumoniae* is down-modulated by autocrine IL-10**

Unlike BMDC maturation, which is clearly manifest ~24 h after exposure to *S. pneumoniae*, cytokine production is initially decreased within several hours after BMDC activation. To determine the effect of autocrine IL-10 on BMDC activation, we measured the concentration of secreted IL-12 (Fig. 4D) and IL-6 (data not shown) by IL-10−/− vs IL-10+/+ BMDC at varying times after addition of *S. pneumoniae*. The secretion of IL-12 by IL-10−/− and IL-10+/+ BMDC, 2 h after addition of *S. pneumoniae*, was similar (Fig. 4D). However, starting at ~4 h, the IL-10−/− BMDC exhibited a progressive enhancement in IL-12 secretion relative to IL-10+/+ cells. Similar data were obtained for IL-6 secretion (data not shown). This same proportional effect occurred over a wide range of bacteria:BMDC ratios (the lower ratio induced 50–100 times less IL-12 and IL-6 than the higher ratio). Consistent with these data (Fig. 4E), pulsed IL-10−/− BMDC treated with IL-10 also showed no difference in IL-12 secretion at 2 h, relative to untreated cells, over a wide bacteria:BMDC ratio, but exhibited a progressively attenuated response starting at 4 h. Similar effects were observed for IL-6 secretion (data not shown). These data indicate that autocrine IL-10 down-modulates BMDC function, beginning only after at least 4 h of initial contact with bacteria, to the same proportional degree regardless of the stimulating dose of bacteria and the associated level of induced cytokine.

**BMDC are receptive to IL-10-mediated functional modulation only within the first ~4 h following exposure to *S. pneumoniae***

In the next set of experiments, we wished to determine the time during which IL-10 mediated its functional effects on BMDC following *S. pneumoniae* activation. Thus, neutralizing anti-IL-10 mAb was added to BMDC cultures at varying times after addition of *S. pneumoniae* and both phenotypic maturation (Fig. 5A) and cytokine secretion (Fig. 5B) were determined 20 h after initiation of culture. Delay of addition of anti-IL-10 mAb up to 4 h after initiation of culture led to a progressively greater autocrine IL-10-mediated reduction of CD86 expression and secretion of IL-6, IL-12, and TNF-α and a concomitant increase in expression of CD16/CD32. By 6 h, no significant effect of anti-IL-10 mAb was observed. In a complementary experiment (Fig. 5C), IL-10 was added to cultures of IL-10−/− BMDC at varying times after addition of *S. pneumoniae*, and cytokine secretion was measured 20 h after initiation of culture. Delay of addition of IL-10 up to 4 h led to a progressive loss of IL-10-mediated inhibition of secretion of IL-6, IL-12, and TNF-α, with no significant effect of IL-10 observed when added at 6 h. Collectively, these data indicate that IL-10 signaling only within the first 4–6 h following contact with *S. pneumoniae* results in the subsequent modulatory effects of IL-10 on DC activation and maturation.

**Increasing bacterial activation necessitates higher IL-10 concentrations for optimal inhibition of IL-12 secretion**

To determine the relationship between the level of bacterial stimulation and the IL-10 dose requirements for modulation of BMDC activation, we established cultures with different bacteria:BMDC ratios (200:1, 50:1, and 10:1), which resulted in progressively lower IL-12 responses. All BMDC were obtained from IL-10−/− mice. To each of these groups we added varying amounts of IL-10 (0.1, 1.0, and 10 ng/ml) either at the initiation of culture, or 3 or 6 h later, and measured the concentrations of secreted IL-12 at 20 h after start of culture (Fig. 5D). When added at initiation of culture, all concentrations of IL-10 were effective at suppressing IL-12 secretion, regardless of the bacteria:BMDC ratio. However, when addition of IL-10 was delayed by 3 h, the lowest dose of IL-10 (0.1 ng/ml) was relatively ineffective at suppressing IL-12 secretion at the highest bacteria:BMDC ratio, but was partially suppressive for the two lower ratios. In contrast, the higher doses of IL-10 (1.0 and 10 ng/ml) were partially effective at inhibiting IL-12 secretion at

![FIGURE 3](https://example.com/figure3.png)
the two higher bacteria:BMDC ratios (200:1 and 50:1) and were able to completely suppress IL-12 secretion at the lowest ratio (10:1). Partial, although far less inhibition of IL-12 secretion was still attainable in most groups when IL-10 was added at 6 h. In light of the positive correlation between bacterial dose and release of IL-10 by IL-10+/- BMDC, these data suggest that autocrine IL-10 counterbalances DC activation induced by varying levels of bacterial stimuli.

**IL-10 controls the time during which BMDC are responsive to the bacterial stimuli**

IL-10 is widely regarded as an inhibitor of DC activation and maturation. However, our data that IL-10 works within the 4-h time period following bacterial activation, but that its effects are not observed until after the time period of its action is over (>4 h), led us to hypothesize that IL-10 does not directly inhibit DC activation, but perhaps limits the time during which DC are able to respond to bacteria-mediated signaling. To resolve this question, IL-10+/- BMDC and IL-10-/- BMDC in the presence or absence of exogenous IL-10 were cocultured with *S. pneumoniae* for varying periods of time (pulse). After the period of pulse, BMDC were extensively washed and recultured in medium alone for an additional 20 h (chase), upon which the concentrations of secreted IL-12 were measured. As indicated in Fig. 6, increasing the *S. pneumoniae* pulse time up to 4 h led to modestly progressive increases of IL-12 secretion over the subsequent 20-h chase period for all BMDC cultures. However, pulse times longer than 4 h led to a progressive loss of IL-12 secretion by IL-10+/- BMDC and by IL-10-/- BMDC treated with IL-10. In distinct contrast, IL-10-/- BMDC continued to secrete ever higher amounts of IL-12 as the exposure time to *S. pneumoniae* was increased. These data
untreated BMDC. and data are expressed as the fractioned fold change of treated (IL-10) vs untreated BMDC. and 10:1), and varying doses of IL-10 (0.1, 1.0, and 10 ng/ml) were added at various times after coculture of IL-10*/+ S. pneumoniae with untreated BMDC, even in the presence of exogenous IL-10, demonstrated minimal apoptosis at 72 h. Addition of S. pneumoniae induced ~20% of the IL-10*/+ BMDC to undergo apoptosis, whereas in contrast 85% of IL-10−/− BMDC were apoptotic at this time point. Addition of 10 ng/ml IL-10 1 h before pulse of IL-10−/− BMDC with S. pneumoniae resulted in a level of apoptosis (~20%) comparable to that observed for pulsed IL-10*/+ BMDC alone. Lower concentrations of IL-10 resulted in progressively higher levels of apoptosis. Delaying the addition of IL-10 by adding it at either 0 or 1 h led to a progressive increase in apoptotic cells among the IL-10−/− BMDC population, an effect that was also dose dependent. Addition of any dose of IL-10 at 3 h after initiation of culture of IL-10−/− BMDC with S. pneumoniae had no effect at all on the percentage of apoptotic cells. Thus, IL-10 must act on BMDC no later than 1–3 h after initial contact with bacteria to have a significant impact on the subsequent onset of bacteria-induced apoptosis.

In a complementary study, we wished to determine the duration of time that was required in order for autocrine IL-10 to down-regulate BMDC apoptosis in response to S. pneumoniae. Thus, neutralizing anti-IL-10 mAb was added on different times after coculture of IL-10*/+ BMDC with S. pneumoniae and apoptotic cells were enumerated 72 h after initiation of culture (Fig. 7B). Once again, unpulsed BMDC underwent minimal apoptosis in the presence of either anti-IL-10 mAb or isotype-matched control mAb. Likewise, pulsed BMDC treated with an isotype-matched control mAb exhibited ~20% apoptotic cells similar to what was illustrated in Fig. 6A. In contrast, addition of anti-IL-10 mAb from 1 h before to 1 h after initiation of culture led to the generation of 50–65% apoptotic BMDC. Delay of addition of anti-IL-10 mAb by 3 h allowed for sufficient IL-10 action during this first 3 h of

strONGLY suggest that IL-10 signaling during the first 4 h after bacterial activation results in a progressive loss of DC responsiveness to continued bacterial signaling, and not to a direct inhibition of DC activation, leading to a delayed, but rapid attenuation of DC cytokine production.

**IL-10 acts early to reduce BMDC apoptosis in response to S. pneumoniae**

In the next set of experiments, we wished to determine whether the IL-10 signaling leading to the subsequent reduction in bacteria-induced BMDC apoptosis (see Fig. 3) occurred during the same time frame following bacterial activation, as we observed earlier for the IL-10 effects on cytokine secretion (see Fig. 5). Thus, in an analogous set of experiments, various doses of IL-10 (0.1, 1.0, and 10 ng/ml) were added at various times after coculture of S. pneumoniae with IL-10−/− and/or IL-10*/+ BMDC (200:1 bacteria:BMDC ratio), and the percentage of hypodiploid (apoptotic) cells was enumerated 72 h after initiation of culture (Fig. 7A). Unpulsed IL-10−/− BMDC, even in the presence of exogenous IL-10, demonstrated minimal apoptosis at 72 h. Addition of S. pneumoniae induced ~20% of the IL-10*/+ BMDC to undergo apoptosis, whereas in contrast 85% of IL-10−/− BMDC were apoptotic at this point. Addition of 10 ng/ml IL-10 1 h before pulse of IL-10−/− BMDC with S. pneumoniae resulted in a level of apoptosis (~20%) comparable to that observed for pulsed IL-10*/+ BMDC alone. Lower concentrations of IL-10 resulted in progressively higher levels of apoptosis. Delaying the addition of IL-10 by adding it at either 0 or 1 h led to a progressive increase in apoptotic cells among the IL-10−/− BMDC population, an effect that was also dose dependent. Addition of any dose of IL-10 at 3 h after initiation of culture of IL-10−/− BMDC with S. pneumoniae had no effect at all on the percentage of apoptotic cells. Thus, IL-10 must act on BMDC no later than 1–3 h after initial contact with bacteria to have a significant impact on the subsequent onset of bacteria-induced apoptosis.

In a complementary study, we wished to determine the duration of time that was required in order for autocrine IL-10 to down-regulate BMDC apoptosis in response to S. pneumoniae. Thus, neutralizing anti-IL-10 mAb was added on different times after coculture of IL-10*/+ BMDC with S. pneumoniae and apoptotic cells were enumerated 72 h after initiation of culture (Fig. 7B). Once again, unpulsed BMDC underwent minimal apoptosis in the presence of either anti-IL-10 mAb or isotype-matched control mAb. Likewise, pulsed BMDC treated with an isotype-matched control mAb exhibited ~20% apoptotic cells similar to what was illustrated in Fig. 6A. In contrast, addition of anti-IL-10 mAb from 1 h before to 1 h after initiation of culture led to the generation of 50–65% apoptotic BMDC. Delay of addition of anti-IL-10 mAb by 3 h allowed for sufficient IL-10 action during this first 3 h of
culture to reduce the percentage of apoptotic cells to ~30%, whereas anti-IL-10 mAb added at 6 h showed a percentage of apoptotic cells (~20%) similar to that observed with IL-10+/− BMDC treated with control mAb. Collectively, these results indicate that bacteria activate BMDC to undergo a sequence of interrelated responses including cytokine secretion, followed by phenotypic maturation, and then finally apoptosis. During a short frame of time, between 2 and 6 h of contact with the bacteria, activated BMDC become susceptible to the modulatory action of autocrine and paracrine IL-10.

**IL-10 can potentiate BMDC induction of antibacterial Ig isotype responses in vivo**

A 24-h pulse of BMDC with *S. pneumoniae* is much less efficient than a 5-h pulse in stimulating protein- and polysaccharide-specific IgG isotype production upon transfer of the pulsed BMDC into naive mice (see Fig. 1), and this is most likely due to the onset of BMDC apoptosis (>24 h after initial contact with bacteria (see Fig. 2A). Our current results demonstrating that IL-10 can reduce BMDC apoptosis in response to *S. pneumoniae*, while still allowing for early bacterial uptake and BMDC activation, led us to hypothesize that autocrine or paracrine IL-10 will enhance the ability of BMDC, pulsed for 24 h with *S. pneumoniae*, to induce protein- and polysaccharide-specific IgG isotype production upon transfer into naive mice. To determine this, we pulsed IL-10+/− and IL-10−/− BMDC with *S. pneumoniae* for 24 h in the presence of neutralizing anti-IL-10 mAb or IL-10, respectively. Serum was collected 14 days after BMDC transfer for determination of anti-PspA, anti-PC, and anti-Cps14 Ig isotype titers. As shown in Fig. 8, treatment of IL-10−/− BMDC with IL-10 significantly enhanced their capacity to induce both antiprotein and antipolysaccharide IgG isotype responses. Nevertheless, IL-10−/− BMDC pulsed for 24 h in the presence of IL-10 were still somewhat less effective than IL-10+/− BMDC pulsed for 5 h in the absence of IL-10. In a similar vein, treatment of IL-10−/− BMDC, pulsed for 24 h with anti-IL-10 mAb, further diminished their ability to induce Ag-specific IgG isotype responses. These data strongly suggest that apoptosis of BMDC consequent to induction of activation and maturation in response to bacteria will lead to a termination of their ability to act as APCs for induction of humoral immunity. Furthermore, the data support the novel view that autocrine and paracrine IL-10 do not directly inhibit DC function, but rather attenuate the response of DC to prolonged bacterial signaling, thus potentially enhancing DC longevity and APC function, while limiting the negative effects of excessive release of proinflammatory mediators.

**Apoptotic BMDC are ineffective inducers of antibacterial IgG responses in vivo**

We recently demonstrated that the pneumolysin released by live *S. pneumoniae* rapidly induces BMDC apoptosis (22), with kinetics similar to that occurring after treatment of BMDC with a primary inducer of apoptosis, Act D (see Fig. 2B). To determine whether apoptotic BMDC are ineffective inducers of antibacterial IgG responses in vivo, we pulsed BMDC with heat-killed *S. pneumoniae* in the presence or absence of pneumolysin-containing supernatant from live *S. pneumoniae* cultures. Pneumolysin-containing supernatant induced apoptosis in 30% of the treated BMDC by 6 h and >80% by 20 h (data not shown). As shown in Fig. 9A, BMDC exposed to pneumolysin during a 5-h pulse with heat-killed bacteria showed a striking reduction in their ability to induce IgG anti-PspA and significantly reduced ability to induce IgG antipolysaccharide responses in vivo, upon transfer into naive recipients relative to BMDC pulsed with heat-killed bacteria in the absence of pneumolysin. These results strongly support the notion that apoptotic BMDC are ineffective inducers of humoral immunity to *S. pneumoniae*.
pneumoniae. These results also suggest that apoptosis by itself accounts for the reduced APC function of BMDC subjected to long periods of pulse with heat-killed bacteria.

Finally, to directly demonstrate that the IL-10 potentiation of BMDC-induced antibacterial Ig isotype responses was associated with its ability to delay the induction of apoptosis, we treated BMDC obtained from IL-10−/− mice with an irreversible inhibitor of caspase-10 during a 24-h pulse with heat-killed bacteria that led to an almost complete inhibition of BMDC apoptosis (22). As shown in Fig. 9B, treatment of IL-10−/− BMDC with caspase-10 inhibitor significantly enhanced their capacity to induce both antiprotein and antipolysaccharide IgG responses, in a manner similar to that following treatment with IL-10 (see Fig. 8). Moreover, the responses induced by IL-10−/− BMDC pulsed during 24 h in the presence of caspase-10 inhibitor were not significantly different to those induced by IL-10+/− BMDC pulsed during 5 h either in the presence or absence of the inhibitor (data not shown). Because these experiments were conducted in the absence of endogenous or exogenous IL-10, they strongly suggest that the potentiation of the antibacterial Ig isotype responses in vivo by IL-10 was largely due to their antiapoptotic effects. Recovery of APC function in vivo was also observed for wild-type BMDC treated with caspase-10 inhibitor after a 24-h pulse with heat-killed bacteria (data not shown).

Discussion

These data elucidate three major related issues regarding DC regulation of antibacterial humoral immunity. First, they demonstrate that nonviable bacteria stimulate a maturational program in DC that is associated with apoptosis. Second, DC undergoing apoptosis lose their ability to induce an in vivo antibacterial Ig response, suggesting that the processing of incoming apoptotic DC loaded with bacteria, by viable DC resident within secondary lymphoid organs, is not a major pathway for delivery and presentation of bacterial Ags. Finally, these data strongly suggest that IL-10, in contrast to being a direct inhibitor of DC function, rather modulates the activation of DC by limiting the time frame during which they can respond to continued exposure to bacteria. Specifically, by delaying the development of apoptosis induced by prolonged bacterial stimulation of DC, combined with its ability to attenuate sustained proinflammatory cytokine production, IL-10 may promote prolonged interactions of activated DC and T cells, required for effective T cell priming, while minimizing inflammatory tissue injury, in the context of sustained bacterial infection. Thus, these data support the novel concept that bacterial PAMPs and IL-10 provide balanced positive and negative signaling, respectively, in DC that may be critical for optimizing DC function in vivo.

Although a number of bacteria have been shown to induce apoptosis in macrophages through the direct action of specific mediators such as pneumolysin (24), NO (25), and the Yersinia pestis virulence factor, Ag V (26), very little is known regarding the ability of bacteria to induce apoptosis in DC. One report demonstrated the ability of L. monocytogenes to rapidly induce DC apoptosis via the action of hemolysin (14), and we recently reported that pneumolysin from S. pneumoniae had similar effects (22). Additionally, injection of mice with LPS induced a rapid onset (within several hours) of DC apoptosis following DC migration from the splenic marginal zone to the T cell zone, but the mechanism by which this occurred was not delineated (11). This report further expands upon our recent results (22) demonstrating that DC exposure to a heat-killed bacterial pathogen can trigger a maturational program that terminates in apoptosis.

A substantial increase in the percentage of apoptotic cells was observed beginning 24 h following exposure to heat-killed S. pneumoniae and increased to >80% by 72 h, whereas DC cultured in medium alone showed minimal apoptotic cells at this latter time point. These kinetics are in distinct contrast to the far more rapid induction of apoptosis (~80% by 10 h) observed for direct apoptosis inducers such as Act D (this study) as well as for live S. pneumoniae containing pneumolysin (30% by 6 h and 80% by 24 h), but not a pneumolysin-deficient isogenic mutant (22). Pneumolysin-containing supernatant from fresh cultures of live S. pneumoniae also induced this rapid onset of apoptosis. The induction of apoptosis in response to heat-killed S. pneumoniae, while relatively delayed, required only 5 h of contact with bacteria, an exposure time that was also sufficient to induce significant cytokine release and phenotypic maturation by DC. Indeed, bacterial dose-response studies demonstrated that the extent of induction of DC apoptosis closely paralleled the degree of initial cytokine release and subsequent phenotypic maturation. In this regard, endogenous
TNF-α appeared, at best, to play only a modest role in the induction of DC apoptosis. Thus, whereas macrophages are highly sensitive to the proapoptotic effects of TNF-α, we observed only a very modest reduction in DC apoptosis upon addition of a neutralizing anti-TNF-α mAb, consistent with the known resistance of DC to TNF-α-mediated cell death (27, 28). Collectively, these data indicate that bacteria can induce either the rapid onset of DC apoptosis through the direct action of bacterial toxins (e.g., hemolysin or pneumolysin) or, in a delayed fashion, trigger apoptosis most likely as an end-stage event of cellular maturation. In both situations, the outcome is a reduced ability of the DC to function as APC for induction of humoral immunity, which most likely promotes bacterial pathogenicity.

We previously demonstrated that necrotic DC loaded with bacteria were not competent to induce IgG antiprotein or IgM and IgG antipolysaccharide responses when transferred into naive mice, despite their ability to enter the spleen in numbers comparable to that seen with viable DC (8). This was somewhat surprising given the ability of necrotic cells to stimulate innate immunity through expression of adjuvant molecules such as endogenous heat-shock proteins. However, these data did not rule out a possible role for apoptotic bacteria-loaded DC in stimulating in vivo humoral immunity. Indeed, DC have been reported to be able to cross-prime CTL through uptake of virally infected apoptotic cells (16). Furthermore, DC can internalize Salmonella-infected macrophage-apoptotic bodies for presentation of bacteria-encoded Ag on MHC class I and MHC class II molecules (17). Our results demonstrate that BMDC undergoing apoptosis following bacterial stimulation are ineffective at eliciting an in vivo antibacterial humoral immune response. Given their relatively high expression of B7-1 and B7-2, CD40, and MHC class II, and the reported stability (i.e., several days) of MHC class II-peptide complexes on the surface of mature DC (29, 30), it is unlikely that the defective induction of Ig by these cells was due simply to an intrinsic lack of T cell-activating ligands. These data instead suggest that Ag-loaded DC are required to play an active role in the induction of an antibacterial humoral immune response, and that APC function is lost when DC become apoptotic. Thus, BMDC pulsed for only 5 h with heat-killed S. pneumoniae undergo rapid apoptosis and become ineffective as APC in vivo, similar to that observed for BMDC pulsed with heat-killed bacteria alone for 24 h. These data strongly suggested that apoptosis also accounted for the defective in vivo Ig responses after adoptive transfer of BMDC treated with heat-killed S. pneumoniae for 24 h, and not to changes in the phenotype or subset selection occurring during their culture in vitro. Direct confirmation of this notion is provided by our observation that the inhibition of BMDC caspase-10 activity, which almost completely blocks BMDC apoptosis induced by heat-killed S. pneumoniae (22), restores the ability of BMDC pulsed for 24 h with heat-killed bacteria to induce antibacterial Ig responses to the level obtained after only a 5-h pulse with bacteria.

We further demonstrate that both autocrine and exogenous IL-10 dramatically reduces DC apoptosis in response to bacterial stimulation, although it has no effect on apoptosis when added to

![Figure 9](image-url)
DC cultured in medium alone. Consistent with our hypothesis that Ag-loaded apoptotic DC are ineffective at eliciting antibacterial Ig responses in vivo, IL-10 restores the ability of pulsed DC from extended in vitro cultures to elicit Ig responses upon transfer into naive mice. The full antiapoptotic effect of IL-10 is observed only when it acts over the first 4–6 h following exposure to bacteria, and if IL-10 addition to pulsed IL-10-treated DC cultures is delayed by as little as 2–4 h, it has no antiapoptotic effect. Thus, the time frame for the antiapoptotic action of IL-10 is strikingly similar to that required to obtain significant induction of cytokine release and maturation in response to bacteria. Indeed, the down-regulation of apoptosis in response to IL-10 is directly paralleled by a corresponding decrease in DC cytokine secretion and phenotypic maturation, consistent with our hypothesis that DC apoptosis is a result of terminal maturation.

Numerous studies have demonstrated the ability of IL-10 to inhibit the release of proinflammatory mediators during the innate phase of the immune response (31–33), suggesting that IL-10 is a potent inhibitor of adaptive immunity. However, our data suggest that this view may require some revision. Specifically, we observe that IL-10 does not directly inhibit DC activation in response to heat-killed S. pneumoniae, in that DC responsiveness to the cytokine-inducing effects, as well as DC uptake, of the bacteria, during the first 4 h of culture is completely unaffected by the presence of IL-10. However, as a result of IL-10 action, DC responsiveness to the bacteria significantly declines at latter time points as reflected by a progressive diminution in ongoing cytokine release despite continual bacterial exposure. In distinct contrast, in the absence of IL-10, DC continue to respond to bacteria over a 7-h period, as evidenced by progressively increasing cytokine secretion. Thus, IL-10 is acting not as a direct inhibitor of DC activation, but rather by limiting the initial time period during which DC can respond to bacteria. This then serves to effect a balanced level of cytokine release, bacterial uptake, maturation, and a consequent enhancement in DC life span, despite prolonged bacterial exposure. Given the requirement for sustained DC-T cell interaction for effective CD4+ T cell priming (9), IL-10 acting at the level of the DC may ultimately help to promote the subsequent adaptive response.

Of interest, in contrast to IgG, the in vivo IgM, antipolysaccharide response was not reduced by prolonged culture of bacteria-pulsed BMDC before transfer into naive mice. We previously demonstrated that the IgM and IgG antipolysaccharide responses to S. pneumoniae are T cell independent and dependent, respectively (20, 34). Given that the majority of transferred BMDC were viable immediately before transfer, but were poised to rapidly undergo apoptosis thereafter, the data suggest that induction of the IgM antipolysaccharide response either requires a brief period of active DC help than that needed for optimal induction of IgG, or that apoptotic DC may indeed be stimulatory in this context. In support of the former notion, an active role for DC in mediating direct help for activated B cells has been reported (35) and may thus be particularly important for DC induction of the T cell-independent polysaccharide-specific IgM response. In contrast, the requirement for more prolonged DC viability for induction of IgG antiprotein and IgG antipolysaccharide responses may reflect the additional need for prolonged DC interactions with T cells for effective T cell priming.

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