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Aspirin Inhibits In Vitro Maturation and In Vivo Immunostimulatory Function of Murine Myeloid Dendritic Cells[1]

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Aspirin is the most commonly used analgesic and antiinflammatory agent. In this study, at physiological concentrations, it profoundly inhibited CD40, CD80, CD86, and MHC class II expression on murine, GM-CSF + IL-4 stimulated, bone marrow-derived myeloid dendritic cells (DC). CD11c and MHC class I expression were unaffected. The inhibitory action was dose dependent and was evident at concentrations higher than those necessary to inhibit PG synthesis. Experiments with indomethacin revealed that the effects of aspirin on DC maturation were cyclooxygenase independent. Nuclear extracts of purified, aspirin-treated DC revealed a decreased NF-κB DNA-binding activity, whereas Ab supershift analysis indicated that aspirin targeted primarily NF-κB p50. Unexpectedly, aspirin promoted the generation of CD11c$^+$ DC, due to apparent suppression of granulocyte development. The morphological and ultrastructural appearance of aspirin-treated cells was consistent with immaturity. Aspirin-treated DC were highly efficient at Ag capture, via both mannose receptor-mediated endocytosis and macropinocytosis. By contrast, they were poor stimulators of naive allogeneic T cell proliferation and induced lower levels of IL-2 in responding T cells. They also exhibited impaired IL-12 expression and did not produce IL-10 after LPS stimulation. Assessment of the in vivo function of aspirin-treated DC, pulsed with the hapten trinitrobenzenesulfonic acid, revealed an inability to induce normal cell-mediated contact hypersensitivity, despite the ability of the cells to migrate to T cell areas of draining lymphoid tissue. These data provide new insight into the immunopharmacology of aspirin and suggest a novel approach to the manipulation of DC for therapeutic application. ***The Journal of Immunology,*** **2001, 166: 7053–7062.**

Aspirin (acetylsalicylate) is the most commonly used analgesic and antiinflammatory substance (reviewed in Ref. 1). Discovery of its ability to inhibit PG synthesis provided a mechanistic basis for the antiinflammatory effects of salicylates (2). However, the finding that therapeutic plasma levels of aspirin exceeded those necessary to inhibit PG synthesis in patients with chronic inflammatory diseases (1–3 mM (3, 4)) raised the possibility that aspirin might also target other, PG-independent pathways of inflammation. In recent years, evidence has accumulated that aspirin exerts a broad spectrum of pharmacological actions, including inhibition of activation of the transcription factor NF-κB (5–7) and other molecular pathways of inflammation (8–10).

The cellular targets of aspirin in the immune system are poorly understood. Several groups have shown that aspirin, or its metabolite salicylate, acts on key steps in granulocyte-mediated inflammation (9, 11–13). With respect to lymphocytes, a suppressive effect on ex vivo lymphocyte transformation (14) and a cytotoxic effect on B cell chronic lymphocytic leukemia cells at very high aspirin concentrations (50% inhibitory concentration, >5–10 mM) have been reported (15). Recently, it has been observed that aspirin inhibits inducible NO synthase (16) and IL-12 production by monocytes/macrophages (17).

Dendritic cells (DC)[3] are rare, ubiquitously distributed, migratory APC, derived from CD34$^+$ bone marrow (BM) stem cells. They are uniquely well equipped to activate naive T lymphocytes. Mature DC are powerful stimulators of cellular and humoral immune responses (reviewed in Ref. 18). To acquire naive T cell-stimulatory ability, DC must undergo maturation. This involves up-regulation of surface MHC class II and costimulatory molecules during their migration from the periphery to T cell areas of secondary lymphoid tissue (reviewed in Ref. 19).

In this study, we have analyzed the influence of physiological concentrations of aspirin on BM-derived DC maturation and function, including effects on morphology, macropinocytosis, and receptor-mediated endocytosis; expression of MHC class I, II, and costimulatory molecules; IL-12p40 and p70 production; and stimulatory capacity for T cell proliferation and IL-2 production in

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[3] Abbreviations used in this paper: DC, dendritic cell(s); TNCB, 2,4,6-trinitrochlorobenzene; TNBS, 2,4,6-trinitrobenzenesulfonic acid; BM, bone marrow; COX, cyclooxygenase; SEM, scanning electron microscopy; TEM, transmission electron microscopy; rm, recombinant murine.

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responding T cells. Our results indicate that aspirin is a potent inhibitor of myeloid DC maturation. In vivo data show that although aspirin-treated DC migrate to T cell areas of secondary lymphoid tissue, they fail to induce normal cell-mediated contact hypersensitivity responses. These findings provide new insight into the immunopharmacology of aspirin. Moreover, exposure to this readily available drug provides a simple, inexpensive, and highly effective means to manipulate the immunostimulatory capacity of DC. Due to the critical role of these professional APC in the initiation and regulation of immune responses and the ready availability of aspirin, our findings may have important implications for the manipulation of DC function for potential therapeutic application.

Materials and Methods

Animals

Male 8- to 12-wk-old C57BL/10J (B10; $H2K^b$, IA^b) and BALB/c ($H2K^d$, IA^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in the specific pathogen-free central animal facility of the University of Pittsburgh Medical Center.

Reagents

Recombinant mouse (rm) GM-CSF was provided by Dr. S. K. Narula (Schering-Plough, Kenilworth, NJ). rmIL-4 and rmM-CSF were purchased from R&D Systems (Minneapolis, MN). Aspirin and indomethacin, as well as FITC-dextran (m.w. 42,000), FITC bovine albumin (FITC-albumin), LPS (*Escherichia coli* serotype 026:B6), and brefeldin A were purchased from Sigma (St. Louis, MO). FITC- or PE-conjugated mAbs used to detect cell surface expression of CD11c (HL3), CD40 (HM40-3), CD54 (ICAM-1; 3E2), CD80 (16-10A1), CD86 (GL1), IA^b β-chain (25-9-17), $H2K^b$ (AF6-88.5), or GR-1 (Ly6G) or intracellular expression of IL-12p40 (C15.6) or IL-10 (JES5-16E3) by flow cytometry, as well as isotype-matched control mAbs and streptavidin-CyChrome, were purchased from PharMingen (San Diego, CA). Biotinylated anti F4/80 (CI-A3-1) mAb was purchased from Bachem Laboratories (San Carlos, CA).

Generation of BM-derived DC and macrophages

BM-derived DC were generated as described previously in detail (20), with minor modifications. Briefly, BM cells were removed from femurs and tibias of B10 mice, filtered through nylon mesh, and depleted of RBC by hypotonic lysis using 0.83% w/v ammonium chloride. Erythroid precursors, T and B lymphocytes, NK cells, and granulocytes were removed by complement depletion using a mixture of mAbs. After complement lysis, the cells were cultured in RPMI 1640, supplemented with 10% v/v heat-inactivated FCS, L-glutamine, nonessential amino acids, sodium pyruvate, penicillin-streptomycin, HEPES, 2-ME (all from Life Technologies, Gaithersburg, MD), rmGM-CSF (1000 U/ml), and rmIL-4 (1000 U/ml), referred to subsequently as complete medium. On day 0, 5×10^6 cells were seeded per 75-cm^2 flask and incubated at 37°C in 5% CO_2 in air. On days 2, 4, and 6, 75% of the culture supernatant was aspirated and replaced with complete medium ($\pm$ aspirin/indomethacin; final concentrations of 0.5–2.5 mM and 5 μM, respectively; pH 7.4). On day 4, the nonadherent cells were discarded. Total numbers of viable cells were determined after trypan blue staining (Sigma) and counted in an improved Neubauer hemocytometer. BM-derived macrophages were cultured in the presence of rmM-CSF (1000 U/ml) as described (21). Briefly, after an initial adherence step to remove resident mature BM macrophages and BM stromal cells, progenitor cells were expanded over 7 days.

Flow cytometric analysis

Cells (5×10^5) were first blocked with 10% v/v normal goat serum for 15 min at 4°C and then stained with mAb for 30 min at 4°C. Cells stained with the appropriate isotype-matched Ig were used as negative controls. After staining, the cells were fixed with 2% w/v paraformaldehyde and analyzed using an EPICS Elite flow cytometer (Beckman Coulter, Hialeah, FL). For intracellular cytokine staining, the cells were treated with brefeldin A (10 μg/ml, 4 h, 37°C), washed with 1% v/v FCS-PBS (staining buffer), stained with FITC-conjugated anti-CD11c mAb, and fixed in 4% w/v paraformaldehyde (15 min, 4°C). Subsequently, the cells were washed twice in staining buffer, permeabilized in 100 μl 0.1% saponin, 1% FCS-PBS (permeabilization buffer), and incubated with PE-conjugated anti-IL-12p40 or anti-IL-10 mAb (30 min, 4°C). Appropriate isotype-matched, irrelevant mAbs served as negative controls.

Endocytosis assay and analysis of phagocytosis

Cells (5×10^5) were incubated with 5 μg/ml FITC-albumin or 0.1 mg/ml FITC-dextran at either 37°C or 4°C for 1 h. Endocytosis was stopped by three washes in ice-cold 0.1% sodium azide-1% FCS-PBS. The cells were stained for surface CD11c, as described above. Solid particle phagocytosis was analyzed by adding 2×10^5/ml yellow-green-fluorescent polystyrene beads ($d = 4$ μm; Molecular Probes, Eugene, OR) for 2.5 h at 37°C or 4°C (negative control) to the cells. Free particles were washed away with PBS buffer, and a total of 200 cells were counted under the microscope as described (22). Cells were scored positive if they had phagocytosed at least one bead. In the negative controls, the percentage of positive cells was $\leq$6.

Immunomagnetic bead sorting of DC

To obtain highly purified populations for subsequent analyses, DC were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Auburn, CA) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. DC purity of 91–95% was consistently achieved.

Preparation of nuclear protein fractions and EMSA

Nuclear extracts of purified DC were prepared as described elsewhere in detail (20). Protein concentrations were measured by using the Bio-Rad protein assay, with BSA as a standard. The NF-κB binding sequence from the Ig κ light chain enhancer (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was used as a probe. DNA probes were end labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (Boehringer Mannheim, Indianapolis, IN) and purified on Sephadex G-50 columns (Sigma) equilibrated with 10 mM Tris, 1 mM EDTA, 100 mM NaCl as described by the manufacturer (Sigma). A portion (5 μl (10 μg)) of each sample of nuclear protein was incubated with $\sim 10^5$ cpm of ^{32}P-labeled consensus NF-κB oligonucleotide (0.5 ng) for 45 min at room temperature. The nuclear proteins and NF-κB probe were incubated in buffer (10 mM Tris, pH 7.5; 10% glycerol; 0.2% Nonidet P-40). Poly(dI-dC) (Boehringer Mannheim) was included as nonspecific competitor DNA. Protein-DNA complexes were resolved on 4% nondenaturing polyacrylamide gels in running buffer (450 mM Tris borate, 1 μM EDTA, pH 8.0). After electrophoresis, gels were dried and subjected to autoradiography. Ab supershift experiments included the addition of 1 μl anti-p50, anti-p65, or anti-Stat1 polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

ELISA

Murine IL-12 p70 and IL-2 were measured using ELISA kits (Quantikine M; PharMingen) according to the manufacturer's instructions. Murine IL-10 was measured with reagents purchased from PharMingen (capture mAb clone JES5-2A5, detection mAb clone SXC-1) using the procedure recommended by the manufacturer. The detection limits for IL-12 p70, IL-2, and IL-10 were 7.8, 15.8, and 30 pg/ml, respectively.

Mixed leukocyte reaction

BALB/c splenic T cells were enriched by passage through nylon wool columns and then used as responders (2×10^5 cells/well in round bottom 96-well plates) against graded numbers of γ-irradiated (20 Gy), bead-sorted B10 DC. To prevent carryover of pharmaceutical substances, DC were washed three times before using them as stimulators in 72-h primary MLR, as described (20).

DC morphology and ultrastructure

Bead-sorted B10 DC were used for light or electron microscopy. For light microscopy, DC were spun onto glass slides (5 min at 230 $\times$ *g*) using a cytocentrifuge (Shandon, Astmoor, U.K.), fixed in methanol, air-dried, and stained with Giemsa. DC were processed for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) as described (23).

Cell-mediated immunity (contact hypersensitivity)

Sensitization phase. Bead-sorted B10 DC were pulsed with 0.1% w/v TNBS (2,4,6-trinitrobenzenesulfonic acid; Sigma) in PBS for 15 min at 37°C. After three washes in PBS, the cells were counted, and viability was assessed by trypan blue exclusion. One million cells were injected s.c. in the dorsal base of the ear of each B10 mouse. As a positive control, the abdomen of animals was shaved and painted with 7% w/v 2,4,6-trinitrochlorobenzene (TNCB; a gift from Dr. K. Rock, University of Massachusetts, Worcester, MA) diluted in acetone-olive oil, 4:1 v/v (vehicle). Negative controls included animals injected with unpulsed DC (without hapten) and animals treated with vehicle alone.

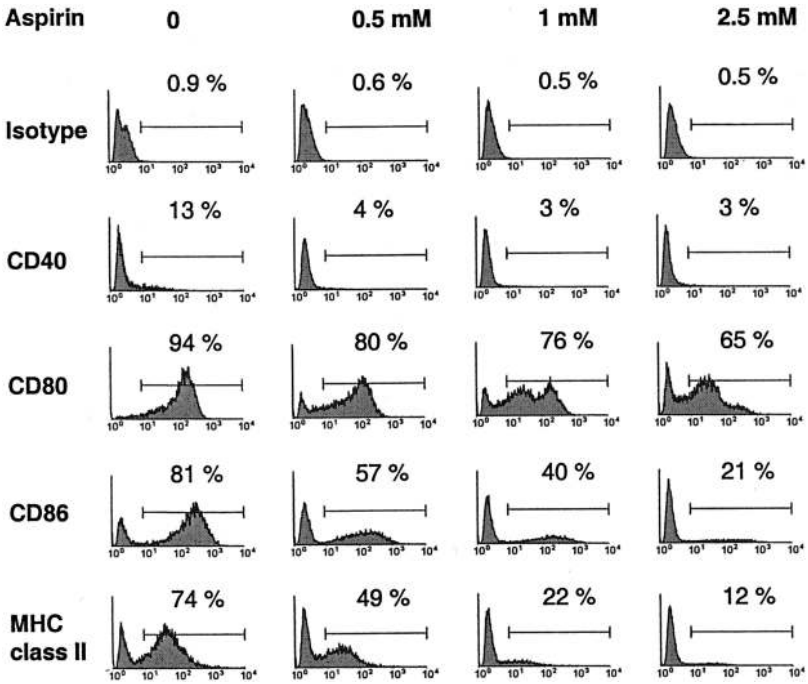

FIGURE 1. Aspirin inhibits the expression of CD40, CD80, CD86, and MHC class II on BM-derived murine myeloid DC in a dose-dependent manner. BM-derived DC were generated as described in *Materials and Methods* (± the indicated concentrations of aspirin). At day 7, cells were harvested and analyzed by two-color flow cytometry. Cells were gated on CD11c. The incidence of $CD11c^+$ cells expressing the Ag of interest is indicated within each histogram. Results are from one representative experiment of three performed.

Elicitation phase. Seven days after sensitization, mice were painted on the dorsal and ventral side of the left ear with 10 μl 1% w/v TNCB in vehicle. The thickness of the left (challenged) and the right (control) ear was measured after 24 and 48 h by using an engineer's spring-loaded micrometer (Mitutoyo, Chicago, IL). The percentage increase in ear thickness was calculated using the formula 100 × [(thickness of challenged ear − thickness of unchallenged ear)/thickness of unchallenged ear].

DC migration in vivo

Bead-sorted B10 DC were labeled with PKH67-GL green-fluorescent dye (Sigma) according to the manufacturer's instructions. Cells were washed twice in serum-free PBS, pulsed with TNBS, and injected as described above. Control experiments included the injection of BM-derived macrophages. After 24 h, the auricular lymph nodes were removed and imaged using a multiphoton laser scanning confocal microscope system comprising a titanium-sapphire ultrafast tunable laser system (Coherent Mira model 900-F), Olympus Fluoview confocal scanning electronics, an Olympus IX70 inverted system microscope, custom-built input power attenuation, and external photomultiplier tube detection systems (Olympus, Melville, NY). Dual photon excitation was at 870 nm, with fluorescence emission detected using a HG510/50 steep passband emission filter (Chroma, Brattleboro, VT).

Immunofluorescence staining of lymphoid tissue

Lymph nodes were embedded in Tissue-Tek OCT (Miles Laboratories, Elkhart, IN), snap frozen in isopentane-liquid nitrogen, and stored at −80°C. Cryostat sections (8 μm) were air-dried, fixed in acetone (10 min at 4°C), and incubated with 1) normal goat serum, 2) avidin blocking solution (Vector Laboratories, Burlingame, CA), and 3) biotin conjugated anti-CD3ϵ or biotin anti-CD19 mAb (PharMingen). After rinsing, the sections were incubated with Cy3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, PA). Nuclei were counterstained with 4,6-diamidino-2-phenylindolole (Molecular Probes). Sections were fixed in 2% w/v paraformaldehyde and mounted with glycerol-PBS (pH 7.0). Slides were examined by fluorescence microscopy, and images were acquired as described (20).

Statistics

Statistical analysis was performed using a two-tailed Student t test; $p < 0.05$ was considered significant. Normal distribution of values, a prerequisite for using the Student t test, was proved by using the Kolmogorov-Smirnov test. Results are expressed as means ± SD. All statistical analyses were performed using the SPSS software version 8.0 (SPSS, Chicago, IL).

Results

Aspirin inhibits the maturation of murine BM-derived DC in a dose-dependent manner

To investigate the effect of different physiological concentrations of aspirin on DC maturation, B10 BM-derived cells were cultured from day 2 in the presence of 0.5–2.5 mM aspirin, as described in *Materials and Methods*. This dose range was selected because in vivo concentrations of 1–3 mM aspirin have been shown to be therapeutic for the treatment of autoimmune diseases, including rheumatoid arthritis (3, 4). As shown in Fig. 1, 0.5 mM aspirin was sufficient to reduce the expression of CD40, CD80, CD86, and MHC class II on $CD11c^+$ cells on day 7. The inhibitory effect of aspirin was dose dependent and targeted primarily the expression of CD86 and MHC class II, which were down-regulated markedly at 2.5 mM (Fig. 1). Stimulation of cells with LPS from day 6 resulted in up-regulation of CD40, CD80, CD86, and MHC class II expression within 24 h. Aspirin-treated DC consistently expressed lower levels of these surface markers compared with untreated cells (Table I).

Table I. *Aspirin markedly inhibits the expression of MHC class II and costimulatory molecules on LPS-stimulated $CD11c^+$ DC*[a]

Surface Ag	% Positive Cells (MFI[b])	
	Normal DC	Aspirin DC
CD40	62 (42)	25 (21)
CD80	99 (309)	72 (121)
CD86	95 (466)	51 (193)
MHC class II	94 (241)	55 (112)

[a] BM-derived DC were generated as described in *Materials and Methods* (± 2.5 mM aspirin). On day 6, maturation was induced by stimulation with LPS (50 ng/ml, 24 h). Two-color flow cytometry was used to determine the level of Ag expression on $CD11c^+$DC.

[b] MFI, Mean fluorescence intensity. Data are from one experiment representative of five performed.

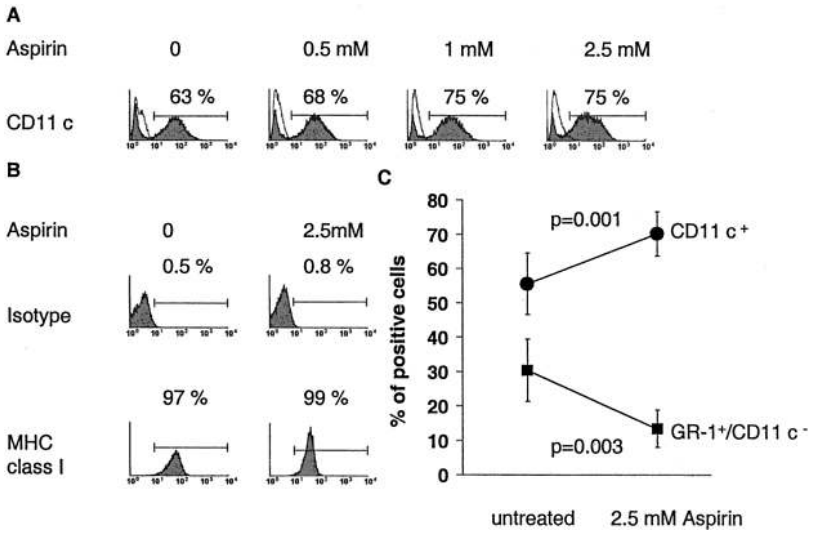

FIGURE 2. Aspirin enhances growth of CD11c$^+$ DC in vitro and inhibits the generation of granulocytes. BM-derived DC were generated as described in *Materials and Methods* (± the indicated concentrations of aspirin) and analyzed at day 7 by flow cytometry. DC were stained for CD11c (*A*), CD11c and MHC class I (*B*), and CD11c and GR-1 (*C*). The percentage within each histogram represents the incidence of CD11c$^+$ cells (*A*) or CD11c$^+$ cells expressing MHC class I (*B*). Percentages of DC (CD11c$^+$) and granulocytes (GR-1$^+$/CD11c$^-$) were analyzed in paired cultures, and differences were compared using the *t* test for paired samples (C). Data are means (±SD). Aspirin treatment did not affect total cell numbers or cell viability (trypan blue staining). The results are representative of three (*A, B*) and seven (*C*) separate experiments.

Aspirin promotes relative increases in CD11c$^+$ BM-derived DC in vitro

To analyze whether the apparent inhibitory effect of aspirin on DC maturation was mediated simply by drug toxicity or by interference with the generation of DC, we analyzed the effects of aspirin on cell viability and the numbers of CD11c$^+$ cells in parallel cultures. Interestingly, aspirin consistently increased the relative proportion of CD11c$^+$ DC, compared with untreated cultures (Fig. 2, *A* and *C*). Neither the expression of MHC class I (Fig. 2 B), nor cell viability (>95%) nor the total number of cells was affected in treated (2.5 mM aspirin) vs untreated cultures. Thus, the increase in incidence of CD11c$^+$ cells shown in Fig. 2*C* reflected a moderate increase in DC yield (2.47×10^6/flask ± 0.47 vs 1.84×10^6/flask ± 0.51; $n = 5$ experiments). The "DC-promoting" effect was accompanied by an equally pronounced inhibition in the incidence of granulocytes, as determined by flow cytometry using GR-1 mAb (Fig. 2*C*).

Aspirin inhibits the maturation of BM-derived DC via a cyclooxygenase (COX)-independent pathway

The finding that aspirin inhibited the maturation of murine BM-derived DC at concentrations that exceeded those necessary for COX-1 and -2 isoform inhibition (24) indicated that the observed effects were COX independent. To confirm this, we analyzed the influence of the COX inhibitor indomethacin on DC maturation. Indomethacin was used at a concentration of 5 μM that has been shown to be sufficient for both COX-1 and -2 isoform inhibition (16, 24). In parallel cultures, and compared with aspirin, indomethacin could not reproduce the strong inhibitory effect of aspirin on DC maturation (Fig. 3*B*), nor did it enhance the incidence of CD11c$^+$ DC (Fig. 3*A*). Interestingly, the expression of CD54 (ICAM-1) was not substantially affected by either aspirin or indomethacin (Fig. 3*B*).

Aspirin decreases p50 NF-κB nuclear DNA-binding activity in myeloid DC

There is recent evidence that aspirin and sodium salicylate can inhibit NF-κB (5, 6). Because NF-κB activation is an important event underlying DC maturation (20, 25), we examined the effects of aspirin on nuclear NF-κB DNA binding activity in purified DC using EMSA, as described in *Materials and Methods*. As shown in Fig. 4, aspirin (2.5 mM) decreased nuclear NF-κB DNA-binding activity in myeloid DC compared with untreated DC grown in parallel cultures (Fig. 4). Classical NF-κB is a heterodimeric molecule formed of two subunits, NF-κB1 (p50) and Rel A (p65). To determine the identity of the nuclear NF-κB proteins primarily targeted by aspirin, Abs specific for different NF-κB proteins were included in the DNA binding reaction. Ab supershift analysis indicated predominantly nuclear NF-κB p50 in untreated DC (Fig. 4), which was markedly decreased in aspirin-treated DC (Fig. 4).

DC exposed to aspirin are poor stimulators of allogeneic T lymphocytes and induce lower levels of IL-2 in responding T cells

Next, we investigated the ability of washed, aspirin-treated DC to stimulate the proliferation of naive allogeneic BALB/c T cells in a 72-h MLR. As shown in Fig. 5, untreated DC were ~3- to 4-fold more efficient T cell stimulators (Fig. 5*A*) and induced ~2-fold

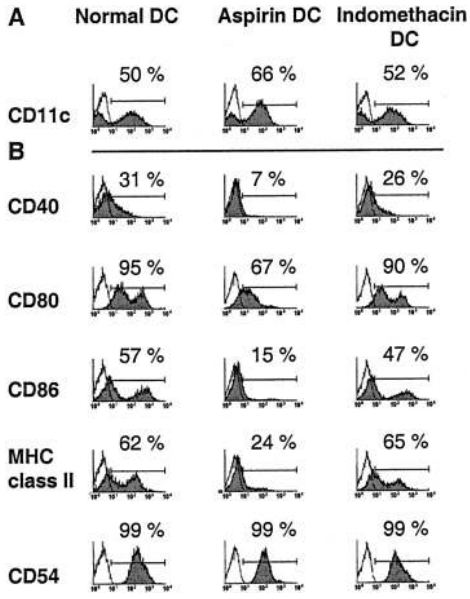

FIGURE 3. Effects of aspirin on DC maturation are COX independent. BM-derived DC from identical animals were generated as described in *Materials and Methods* (±2.5 mM aspirin or 5 μM indomethacin). At day 7, cells were harvested and analyzed by two-color flow cytometry. Cells were gated on CD11c. The percentage of CD11c$^+$ cells expressing the surface Ag of interest is indicated within each histogram. Results show one representative experiment of five performed.

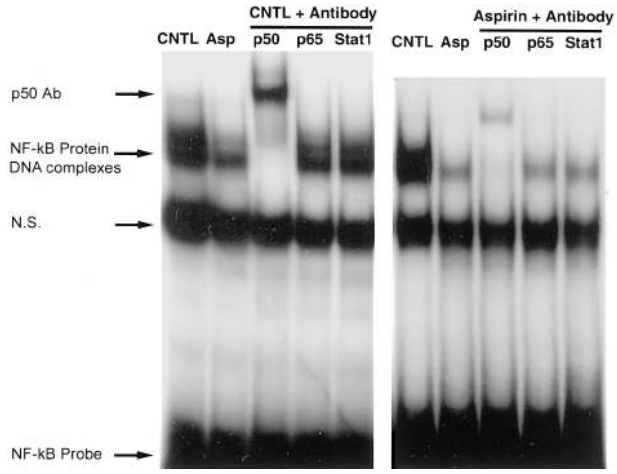

FIGURE 4. Aspirin treatment decreases p50 NF-κB nuclear DNA binding activity in GM-CSF + IL-4-stimulated BM-derived DC, generated as described in *Materials and Methods*. The experiment depicted illustrates the nuclear protein DNA complex from untreated control (CNTL) or aspirin-treated (2.5 mM, Asp) nuclear protein extracts from purified DC (day 7) in gel shift assays using a consensus NF-κB oligonucleotide. Aspirin treatment markedly reduced the amount of protein-DNA complex. Only Ab to p50 NF-κB protein eliminated and supershifted the protein-DNA complex. Abs to p65 NF-κB or Stat1 proteins failed to alter the protein-DNA complex. Data are representative of three separate experiments performed, with independent sets of DC. N.S., nonspecific complex.

more IL-2 production than aspirin-treated DC (Fig. 5*B*). By contrast, indomethacin treatment did not affect the allostimulatory capacity of DC (Fig. 5, *A* and *B*). Importantly, maturation induced by LPS stimulation (24 h, 50 ng/ml) strongly promoted the allostimulatory capacity of untreated or indomethacin-treated DC, whereas exposure to LPS only marginally affected the allostimulatory capacity of aspirin-treated DC (Fig. 5*C*). This observation indicates that aspirin-treated DC were at least partially maturation resistant.

Aspirin-treated DC exhibit an immature phenotype but display characteristic DC morphology

Purified, $CD11c^+$ DC (±2.5 mM aspirin; ±50 ng/ml LPS for the last 24 h of culture) were analyzed in cytospin preparations after Giemsa staining and by TEM and SEM (Fig. 6). Aspirin-treated DC displayed a more immature phenotype, with a round nucleus (Fig. 6, *C* and *G*) and a prominent endocytic compartment (Fig. 6*G*). Untreated DC showed more indented/reniform nuclei and a less prominent endocytic compartment (Fig. 6, *A* and *E*). Cytoplasmic projections were visible on untreated (Fig. 6, *E* and *I*) and aspirin-treated DC (Fig. 6, *G* and *K*) and appeared mainly as needle-like processes. LPS stimulation promoted the development of typical cellular protrusions on aspirin-treated (Fig. 6, *D, H*, and *L*) and untreated DC (Fig. 6, *B, F,* and *J*), but aspirin-treated DC maintained a less lobulated nucleus and a more prominent endocytic compartment (Fig. 6, *D* and *H*) than untreated DC (Fig. 6, *F* and *J*). The immature phenotype of aspirin-treated DC is in accordance with the functional data obtained using endocytosis assays (see below) and similar to the appearance of sorted, immature myeloid DC (A. E. Morelli, unpublished observations). Thus, besides the fact that these cells expressed high levels of the DC lineage marker CD11c (see above) and were nonadherent, they met typical morphological criteria for DC, consistent with an immature phenotype.

Aspirin-treated DC are not macrophages

To address the question whether treatment of DC with aspirin altered the progenitor composition leading to a more macrophage-like population, we cultured adherent BM-derived macrophages in response to M-CSF and compared solid particle phagocytosis, expression of F4/80 (macrophage marker) and CD11c (dendritic cell marker) directly with BM-derived DC (±2.5 mM aspirin). As shown in Table II, macrophages expressed much higher levels of F4/80 and showed a much greater ability to phagocytose solid particles compared than DC (±2.5 mM aspirin). BM-derived macrophages also expressed only very low levels of CD11c, similar to background levels. Thus, aspirin-treated DC are unlikely to be macrophages, and CD11c appears to be a reliable surface marker for murine myeloid DC.

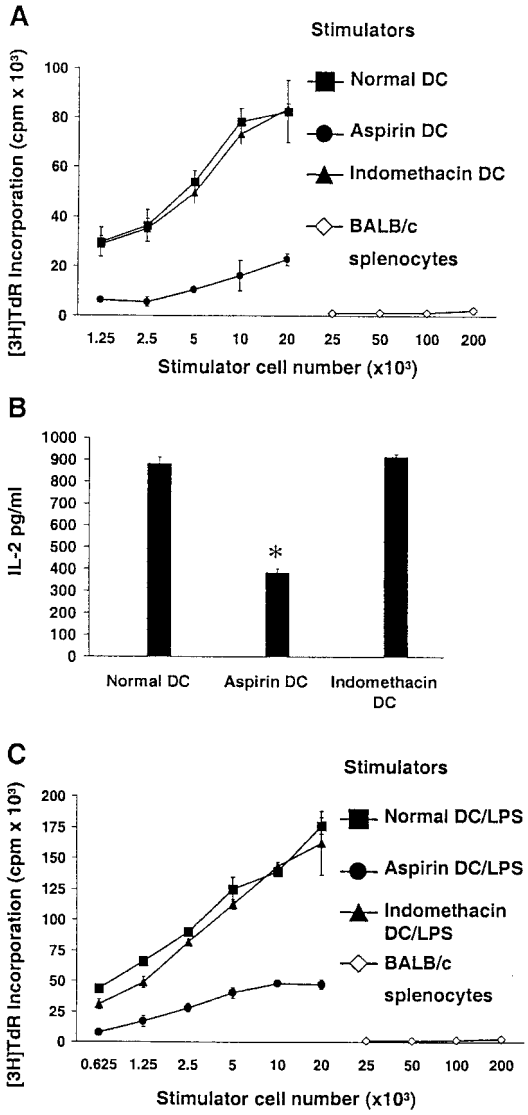

FIGURE 5. BM-derived DC generated in the presence of aspirin are poor stimulators of naive allogeneic T cells. B10 ($H2K^b$) BM-derived DC (±2.5 mM aspirin, ± 5 μm indomethacin) were generated as described in *Materials and Methods*, harvested at day 7, washed extensively, purified, and used as stimulators of naive allogeneic BALB/c ($H2K^d$) T cells in one-way MLR (*A–C*). Syngeneic bulk spleen cells were used as control stimulators (*A, C*). *B,* Analysis of IL-2 levels in 48-h MLR supernatants (stimulator-responder ratio, 0.1) by ELISA. Data represent the means (±SD) of three separate experiments. *, $p \leq 0.01$ (Student's t test for independent samples). In C, DC were further stimulated with LPS (50 ng/ml) for the last 24 h of culture before used as stimulators. Results were obtained from 72-h MLR and are the means (±SD) from triplicate cultures (*A, C*). Data are representative of three separate experiments (*A–C*).

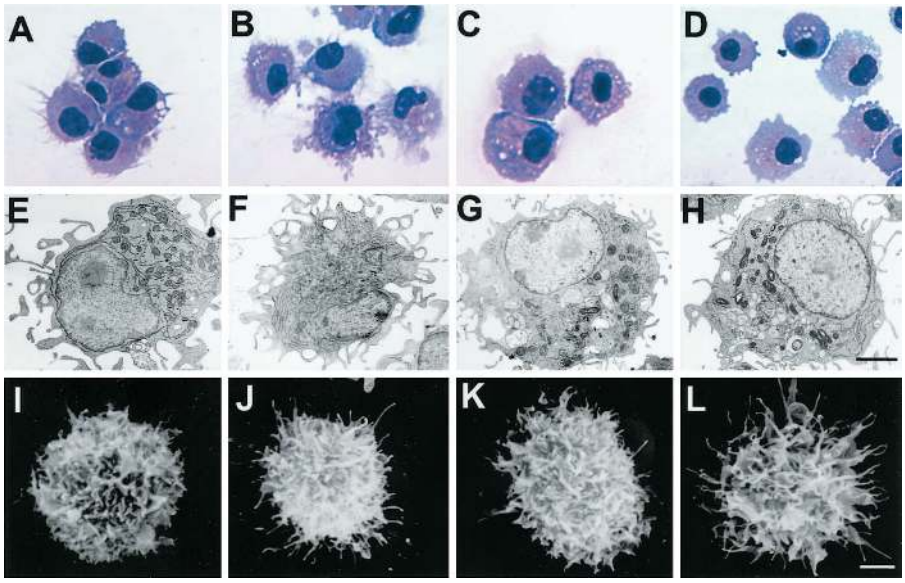

FIGURE 6. Compared with controls (*A, E, I*), aspirin-treated DC (*C, G, K*) show morphological features characteristic of immature DC. BM-derived DC were generated as described in *Materials and Methods* (±2.5 mM aspirin; ± 50 ng/ml LPS for the last 24 h of culture), purified on day 7 using immunomagnetic beads, and analyzed by light microscopy, TEM, and SEM. Aspirin-treated DC exhibited short, needle-like protrusions evident at the EM level (*G, K*), a rounded nucleus (*C, G*), and a prominent endocytic compartment (*G*), whereas untreated DC showed a more reniform nucleus (*A, E*), a less prominent endocytic compartment (*E*), and short protrusions (*I*). LPS stimulation further promoted the development of cytoplasmic projections, in both aspirin-treated (*D, H, L*) and untreated DC (*B, F, J*), but aspirin-treated DC retained a rounded nucleus (*D, H*) with an enlarged endocytic compartment (*H*). *A--D*, Giemsa staining; *E–H,* TEM, ×4000; *I–L,* SEM ×3500; bar, 3 μm.

Aspirin-treated DC are functionally active, immature cells with high endocytic capacity

The flow cytometry and MLR data indicated that exposure to aspirin profoundly inhibited the maturation and allostimulatory activity of in vitro-generated myeloid DC. However, these results did not exclude the possibility that aspirin caused a general inhibition of DC physiological functions. Consequently, we investigated the ability of aspirin-treated DC to endocytose FITC-dextran as well as to exhibit macropinocytosis of FITC-albumin. Aspirin-treated DC showed unequivocally higher endocytic capacity for both FITC-dextran and FITC-albumin than did untreated DC (Fig. 7), again indicating that they were functionally active, immature DC.

Aspirin-treated DC show impaired IL-12 production and do not secrete IL-10

Secretion of bioactive IL-12p70 requires the coordinate expression of two subunits, p35 and p40, that are encoded by two separate genes and regulated independently (26). Because IL-12p40, in contrast to IL-12p35, is secreted in substantial amounts as a monomer and because p40 homodimer can inhibit IL-12p70-mediated functions (27), we analyzed both IL-12p40 and bioactive IL-12p70 production by aspirin-treated DC. As shown in Fig. 8*A*, intracellular staining of FITC-labeled CD11c$^+$ DC with PE-labeled IL-12 p40 or IL-10 mAbs revealed that aspirin-treated DC (2.5 mM) expressed lower amounts of IL-12p40 compared with untreated DC, whereas IL-10 was not detectable. When supernatants where analyzed by ELISA (detection limit, 30 pg/ml), IL-10 was also not detectable after LPS (50–1000 ng/ml) stimulation. The inhibitory effect of aspirin on IL-12p40 expression was independent of LPS concentration (50–500 ng/ml) and was even detected in unstimulated DC (Fig. 8*A*). Analysis of IL-12p70 production by ELISA showed only low cytokine levels (≤50 pg/ml) when DC were stimulated with LPS. Aspirin-treated DC, however, produced somewhat higher cytokine levels (Fig. 8*B*). We reasoned that the cytokines used during the BM-culture (GM-CSF/IL-4) were important for high IL-12p70 production. Interestingly, combined stimulation with GM-CSF + IL-4 plus LPS markedly enhanced IL-12p70 production by control DC but not by aspirin-treated DC (Fig. 8*B*), indicating that exposure to aspirin impaired the capability of DC to produce high amounts of bioactive IL-12p70 (Fig. 8*B*).

Table II. *Phenotypical and functional comparison of BM-derived DC and BM-derived macrophages*[a]

Function/Marker	Control DC (%)	Aspirin DC (%)	Macrophages (%)
Solid particle phagocytosis	19.0 (±3.9)	23.5 (±2.3)	60.8 (±5.1)
F4/80	20.9 (±0.4)	25.1 (±2.1)	84.8 (±3.4)
CD11c	53.6 (±8.3)	64.1 (±1.7)	3.3 (±2.8)

[a] BM-derived DC (±2.5 mM aspirin) and macrophages were generated as described in *Materials and Methods.* Data represent the mean (±SD) percentages of positive cells of three separate experiments.

Aspirin-treated DC fail to induce a normal cell-mediated immune response

A single s.c. injection of 10^6 TNBS-pulsed, purified DC induced a strong contact hypersensitivity response visualized after rechallenge of the animals with the model hapten 7 days later. By contrast, aspirin-treated DC failed to elicit a significant immune response (Fig. 9). In fact, the responses of animals sensitized with TNBS-pulsed, aspirin-treated DC were similar to those of unsensitized animals. Control groups injected with either unpulsed DC

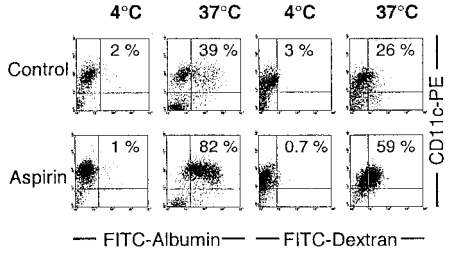

FIGURE 7. Aspirin-treated DC exhibit increased endocytotic activity. BM-derived DC were generated as described in *Materials and Methods* (±2.5 mM aspirin) and harvested on day 7; macropinocytosis (FITC-albumin) and receptor-mediated endocytosis (FITC-dextran) were analyzed on CD11c-PE-positive DC by flow cytometry. Numbers indicate the percentage of CD11c$^+$ cells that were positive for either FITC-albumin or FITC-dextran. Results are representative of two separate experiments that gave similar results.

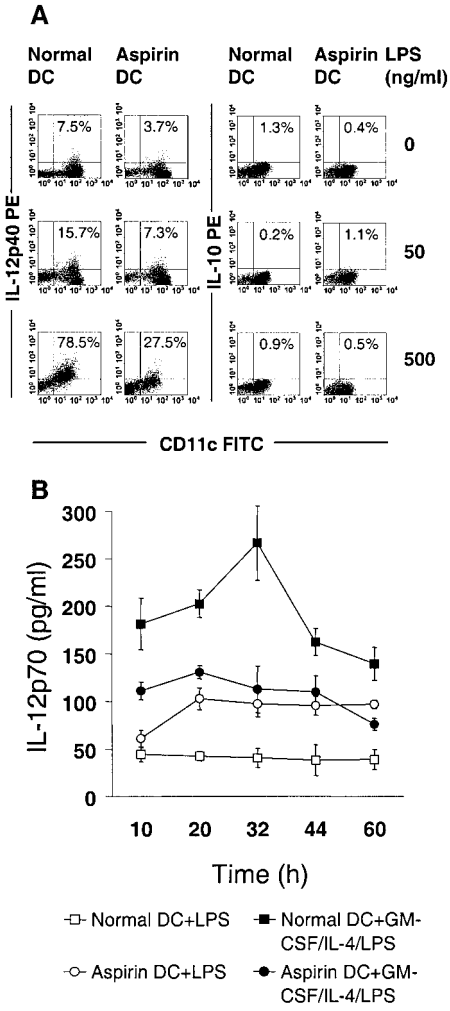

FIGURE 8. Analysis of IL-12 and IL-10 production in BM-derived DC (±2.5 mM aspirin) *A,* Analysis of IL-12p40 and IL-10 expression in $CD11c^+$ DC by intracellular cytokine staining after LPS stimulation (24 h). Numbers indicate percentages of $CD11c^+$ cells expressing IL-12 p40 or IL-10. Results are representative of three separate experiments. *B,* Analysis of IL-12p70 production by magnetic bead-purified DC (0.5×10^6/ml) over time (10–60 h) after LPS stimulation (500 ng/ml) and combined stimulation with GM-CSF (1000 U/ml)/IL-4 (1000 U/ml) plus LPS (500 ng/ml) using ELISA. Data represent the means (±SD) of two separate experiments.

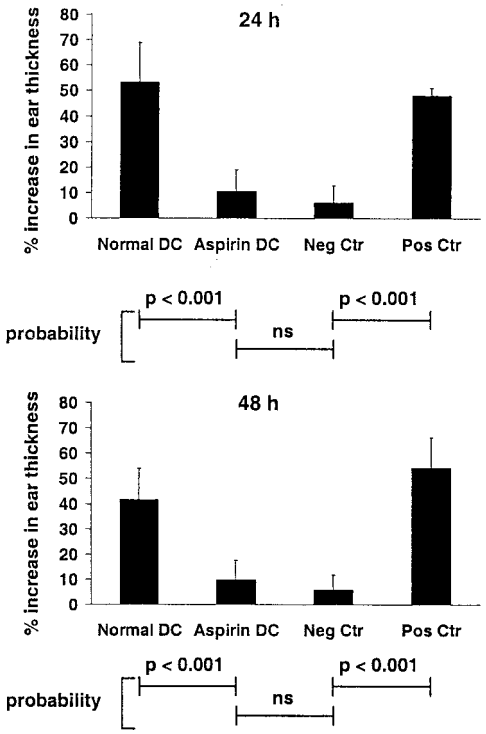

FIGURE 9. Aspirin-treated DC fail to induce a normal cell-mediated immune response. One million purified DC cultured in the presence or absence of aspirin (2.5 mM) were pulsed with 0.1% w/v TNBS and injected s.c. on day 0, as described in *Materials and Methods*. In control groups, DC were either not TNBS-pulsed (Neg Ctr) and injected s.c., or animals were shaved and the skin of their abdomen painted with 7% w/v TNCB (Pos Ctr). After 7 days, left ears were challenged epicutaneously with 1% w/v TNCB, and ear thickness was measured after 24 and 48 h. Results represent mean (±SD) percentage increase of ear swelling for six to nine animals in the treatment groups and three to six animals in the control groups. Treatment with vehicle alone did not induce a swelling response. Values of *p* were calculated by Student's *t* test for independent samples.

(without hapten; negative control) or animals sensitized by epicutaneous application of the model hapten (positive control) proved that the immune response was Ag specific. (Fig. 9).

Locally injected, aspirin-treated DC migrate to T cell areas of draining lymphoid tissue

To ascertain whether the substantial impairment of immune responses was due to inability of aspirin-treated DC to migrate to draining lymphoid tissue, we labeled haptenized DC (±aspirin) with a green cell tracker dye (PKH67) and treated the animals in the same way as for induction of the contact hypersensitivity responses. Spatial analysis of native tissue by multiphoton confocal laser microscopy demonstrated that both aspirin-treated DC and control DC migrated in vivo to the draining lymphoid tissue within 24 h of s.c. injection (Fig. 10, *A* and *B*). When BM-derived macrophages were injected, we observed only very rare green-labeled cells in the draining lymphoid tissue (Fig. 10*C*). Immunofluorescence staining of cryostat sections, revealed that aspirin-treated DC were localized in T cell areas (Fig. 10, *D* and *E*).

Discussion

This is the first report of which we are aware concerning the effects of aspirin on the generation and maturation of murine BM-derived DC. It is also the first study in which DC exposed to aspirin have been tested for homing ability and capacity to sensitize recipients for cell-mediated immune responses. While we were completing this report, Matasic et al. (28) observed that aspirin inhibited the maturation of human monocyte-derived DC. There are several differences between this latter study and the present report. In addition to the species difference, we propagated myeloid DC from BM progenitors, in contrast to circulating blood monocytes. We also performed functional assays (endocytosis, solid particle phagocytosis) to ascertain the function of aspirin-treated DC and in addition conducted in vivo assessment of the trafficking ability of the cells and their capacity to prime T cell-mediated responses. The results indicate that the effects of aspirin were COX independent and involved primarily the suppression of NF-κB p50 activation. To ensure that the observed effects of aspirin could be ascribed to DC and not to contaminating cells present in BM-derived cell cultures, DC were purified (>90%) before analysis in each of the assays undertaken.

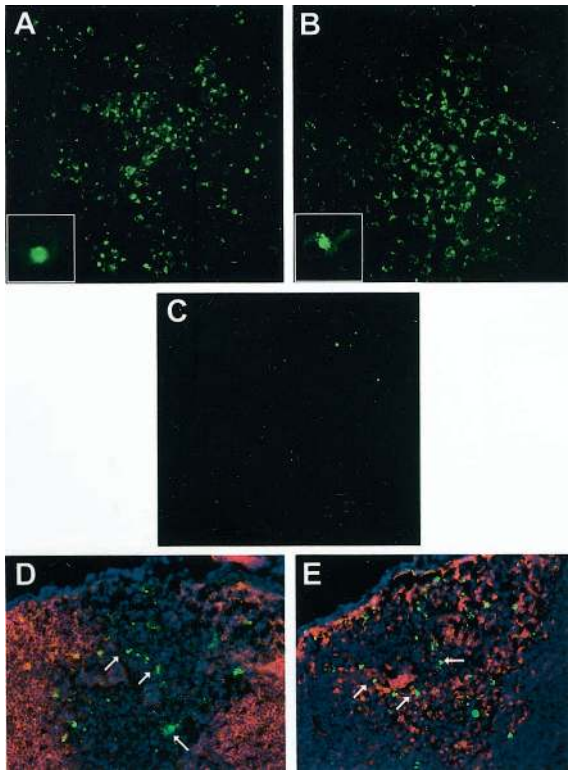

FIGURE 10. Aspirin-treated DC migrate to T cell areas of draining lymphoid tissue. One million purified DC cultured in the presence or absence of aspirin (2.5 mM) were labeled with green cell tracker dye PKH-67GL and 0.1% w/v TNBS, then injected s.c. as described in *Materials and Methods*. Control animals were injected with 1×10^6 BM-derived macrophages. After 24 h, the animals were killed, and the draining auricular lymph nodes were removed. Whole, unsectioned lymph nodes were imaged using a multiphoton laser scanning confocal microscope system (*A–C*). In depth spatial view of lymphoid tissue from mice injected with untreated (*A*) or aspirin-treated DC (*B*) or macrophages (*C*). Field of view, 350 μm (*A–C*). Main panel images are composed of maximum brightness image projections of *z*-series fluorescence multiphoton confocal images (*A–C*). Projections were constructed from a *z* series spanning the same depth (52.2 μm) into the tissue, with a *z*-axis step size of 1.8 μm (*A, B*). *Insets* in *A* and *B* are higher magnification single plane images of single cells (field of view, ~20 μm). *D* and *E,* Localization of aspirin-treated DC (green) in draining lymph nodes by three-color immunofluorescence on serial cryostat sections. B cells were labeled with Cy3 anti-CD19 mAb (in red, *D*), and T cells were labeled with Cy3 anti-CD3ϵ mAb (in red, *E*). Cell nuclei were labeled with 4,6-diamidino-2-phenylindolole (in blue, *D* and *E*) as described in *Materials and Methods*. Aspirin-treated DC, labeled with PKH67 (in green, some DC are indicated by arrows; *D* and *E*) were identified predominantly in T cell areas. Original magnification, ×100.

Aspirin inhibited the maturation of myeloid DC in a dose-dependent manner, without impairing the differentiation of progenitor cells into $CD11c^+$ DC. This contrasts with the effects of corticosteroids. Woltman et al. (29) reported recently that dexamethasone and prednisolone suppressed the generation of monocyte-derived DC in vitro. Indeed, our results indicate that aspirin moderately promotes the growth of myeloid $CD11c^+$ DC in GM-CSF + IL-4-stimulated cultures by limiting the expansion of contaminating granulocytes. Growth of contaminating granulocytes is a long recognized problem associated with the use of GM-CSF to promote generation of DC from BM cultures and can limit the yield of DC (30, 31). In addition to the fact that aspirin strongly inhibited DC maturation, we found, unexpectedly, a novel and effective means to restrict the growth of granulocytes and to improve the expansion of DC in vitro. It is important that the "DC-promoting" and granulocyte-inhibiting effects of aspirin were more pronounced when no cells where discarded during the culture period (data not shown).

The extent of maturation inhibition by aspirin was similar to or greater than that reported for IL-10 (32, 33), TGF-β (34, 35), corticosteroids (29, 36, 37), cyclosporine (38), 1,25-dihydroxyvitamin D_3 (39–42), and mycophenolate mofetil (43). It may be argued that the strong suppressive effects of aspirin on DC maturation are attributable to a general, nonspecific inhibitory effect on energy-dependent cellular functions. We addressed this question by analyzing the capacity of aspirin-treated DC to internalize FITC-albumin or FITC-dextran via macropinocytosis or mannose receptor-mediated endocytosis, respectively. Both mechanisms are complex, energy-dependent processes, that require the coordinate action of the actin cytoskeleton and are characteristic and distinctive properties of immature vs mature DC (44, 45). The endocytic activity of aspirin-treated DC both with respect to internalization of FITC-dextran or FITC-albumin was strongly increased, as judged by flow cytometry. Similar findings have been reported with other inhibitors of DC maturation, like dihydroxyvitamin D_3 (41), glucocorticoids (37), and IL-10 (46, 47), and these data further support the view that exposure to aspirin promoted the generation of functionally active, immature DC. On the basis of morphological appearance, nonadherence, high CD11c expression, and low F4/80 expression, together with low activity to phagocytose solid particles, these aspirin-treated DC are unlikely to represent macrophages.

It has been reported that aspirin inhibits bioactive IL-12 production by macrophages (17) and IL-12p40 secretion by monocyte-derived DC (28). Our results confirm the inhibitory effect of aspirin on IL-12p40 expression. With respect to IL-12p70 production, we found substantial cytokine levels in control DC only when the cells were stimulated with LPS plus the cytokines used during the BM culture (GM-CSF/IL-4). This finding is in agreement with reports showing substantially increased IL-12 production by PBMC (48) or DC (49) stimulated with bacteria and IL-4 that had been pretreated (conditioned) with IL-4 or IL-4/GM-CSF, respectively. The results further confirm recent data of Hochrein et al. (50) showing that IL-4 is a major regulatory cytokine promoting bioactive IL-12 production by murine and human DC. Interestingly, our data indicate that exposure to aspirin impaired the production of bioactive IL-12p70 in BM-derived DC stimulated with LPS plus GM-CSF/IL-4.

On the basis of our in vitro observations, we hypothesized that aspirin-treated DC would exhibit impaired ability to stimulate naive T cells in vivo and to initiate a cell-mediated immune response. This question was addressed by examining the in vivo trafficking and function of DC pulsed with the model hapten TNBS. It was demonstrated recently that as few as 10^5 TNBS-pulsed murine BM-derived DC could induce a strong contact hypersensitivity response (51). Using the same model system, we confirmed that haptenized, BM-derived DC injected s.c. were potent inducers of T cell-mediated immune responses. Interestingly, we observed that s.c. injection of 10^6 TNBS-pulsed, aspirin-treated DC did not induce a significant contact hypersensitivity reaction, despite the fact that these cells could be detected in T cell areas of draining lymphoid tissue. These results indicate that the decreased T cell-stimulatory capacity of aspirin-treated, BM-derived DC is not readily reversed after removal of aspirin and is sustained in vivo.

The present study is the first account on the effects of aspirin on the generation and maturation of murine BM-derived myeloid DC. At physiological concentrations, and without impairing the generation of $CD11c^+$ cells, aspirin proved to be a potent inhibitor of

DC maturation. The inhibitory effect of aspirin on DC maturation is COX independent and is associated with suppressed activation of NF-κB p50. An additional novel aspect of our findings is that the T cell-stimulatory capacity of DC preexposed to aspirin is markedly diminished in vivo. This report may encourage further evaluation of the in vivo function of aspirin-treated DC in relation to their potential regulatory influence on allo- or autoimmune responses, in which immature DC have been shown to exhibit tolerogenic properties (52–54). Because DC maturation can be regulated in a cost-effective manner by exposure to aspirin, this approach may find therapeutic application.

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