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Prostaglandin E$_2$ Promotes the Survival of Bone Marrow-Derived Dendritic Cells$^1$

Evros Vassiliou, Vikas Sharma, Huie Jing, Farzad Sheibanie, and Doina Ganea$^2$

Since dendritic cells (DC) participate in both innate and adaptive immunity, their survival and expansion is tightly controlled. Little is known about the mechanisms of DC apoptosis. PGE$_2$, an arachidonic acid metabolite, plays an essential role in DC migration. We propose a novel function for PGE$_2$ as a DC survival factor. Our studies demonstrate that PGE$_2$ protects DC in vitro against apoptosis induced by withdrawal of growth factors or ceramide. DC matured in conditions that inhibit endogenous PGE$_2$ release are highly susceptible to apoptosis and exogenous PGE$_2$ re-establishes the more resistant phenotype. The antiapoptotic effect is mediated through EP-2/EP-4 receptors and involves the PI3K → Akt pathway. PGE$_2$ leads to increased phosphorylation of Akt, protection against mitochondrial membrane compromise, and decreased caspase 3 activity. Macroarray data indicate that PGE$_2$ leads to the down-regulation of a number of proapoptotic molecules, i.e., BAD, several caspases, and granzyme B. In vivo, higher numbers of immature and Ag-loaded CFSE-labeled DC are present in the draining lymph nodes of mice inoculated with PGE$_2$- receptor agonists, compared with animals treated with ibuprofen or controls injected with PBS. This suggests that PGE$_2$ acts as an endogenous antiapoptotic factor for DC and raises the possibility of using PGE$_2$ agonists to increase the survival of Ag-loaded DC following in vivo administration. The Journal of Immunology, 2004, 173: 6955–6964.

Dendritic cells (DC)$^3$ are positioned at the crossroad between innate and adaptive immunity. In response to signaling through TLR, DC become active participants in the innate response through the production and secretion of inflammatory cytokines, chemokines, and cytotoxic agents. TLR and CD40 signaling also results in DC maturation, characterized by increased expression of costimulatory molecules, increased Ag-presenting capacity, and migration to the draining lymph nodes (reviewed in Refs. 1–6). Since DC act as major initiators of the adaptive response and also as significant participants in the innate inflammatory response, their survival and expansion has to be tightly controlled. In vivo, mature DC are eliminated from the draining lymph nodes in a matter of days, presumably by CTL and NK cells (7–10). However, in contrast to DC generation and maturation, little is known about the mechanisms of DC apoptosis. Significant differences have been reported for immature and mature DC in terms of susceptibility to glucocorticoid-, UVB-, and MHC class II ligation-induced apoptosis. Immature DC undergo apoptosis in response to glucocorticoids, exposure to UVB, and are resistant to MHC class II ligation (11, 12). In contrast, mature DC undergo apoptosis upon MHC II ligation and are resistant to treatment with glucocorticoids or UVB (12–15). Mature DC are also resistant to apoptosis induced by death receptors such as Fas, TNFR1, and TRAIL, whereas immature DC are partially sensitive to Fas- and TRAIL-mediated apoptosis (7, 16–20). The mechanisms that operate in immature and mature DC leading to differences in the susceptibility to apoptosis are not understood.

PG are arachidonic acid metabolites generated by cyclooxygenase (Cox) 1/Cox2 and PG synthases in response to physical, chemical, hormonal, and inflammatory signals (reviewed in Ref. 21). Cells involved in innate immunity, i.e., monocytes/macrophages, PMN, and DC, are an important source of endogenous PG in inflammatory conditions through the up-regulation of Cox2 expression (reviewed in Ref. 21). Among PG, PGE$_2$ is one of the best characterized in terms of immunomodulation. Depending on the nature of the maturation signals, PGE$_2$ has different and sometimes opposite effects on DC function.

In conjunction with TNF, IL-1$\beta$, and IL-6, PGE$_2$ was reported to contribute to the maturation of human monocyte-derived DC (22, 23). Subsequent studies established that the major function of PGE$_2$ in the context of an inflammatory cytokine milieu consists in promoting DC migration and that this effect is mediated through the EP-4 receptor (24–27). In addition, EP-4-deficient mice were shown to be resistant in a model of rheumatoid arthritis (28). These studies strongly suggest that PGE$_2$ acts as a proinflammatory agent and support the beneficial effects of the Cox1/Cox2 inhibitors in some autoimmune diseases, particularly rheumatoid arthritis.

In contrast, PGE$_2$ was shown to inhibit the release of proinflammatory cytokines (TNF, IL-6, IL12p70) (23, 29, 30) and of proinflammatory chemokines (CCL3, CCL4, CXCL10) (31, 32) in LPS-stimulated human monocyte-derived DC and murine bone marrow-derived DC (BM-DC). Also, EP-4-deficient mice were shown to develop severe colitis (33), in agreement with the observation that nonsteroidal anti-inflammatory drugs often trigger and exacerbate inflammatory bowel disease in humans (34). These studies suggest an anti-inflammatory role for PGE$_2$, in agreement with previously reported antiproliferative effects on activated T cells (reviewed in Ref. 35).

We would like to propose a novel function for PGE$_2$ in the biology of DC. The present study demonstrates that PGE$_2$ promotes the survival of BM-DC. In vitro, PGE$_2$ protects BM-DC

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$^3$Abbreviations used in this paper: DC, dendritic cell; BM, bone marrow; PKB, protein kinase B; PI, phosphoinositide; Cox, cyclooxygenase; PKA, protein kinase A; dbcAMP, dibutyl cAMP.

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against apoptosis induced by withdrawal of growth factors or ceramide. The antiapoptotic effect is mediated through EP-2/EP-4 receptors and involves the PI3K → Akt (protein kinase B (PKB)) pathway. In vivo, we observed increased viability of DC in the draining lymph nodes upon administration of an exogenous EP receptor agonist.

Materials and Methods

**Mice**

Male B10.A mice 6–8 wk old were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the animal facility at Rutgers-Newark under pathogen-free conditions.

**Reagents**

LPS (Escherichia coli O26:56) and PGE₂ were purchased from Sigma-Aldrich (St. Louis, MO). Butaprost, misoprostol, sulprostone, SC530, NS398, and ibuprofen (Ibup) were purchased from Cayman Chemical (Ann Arbor, MI). Recombinant murine GM-CSF and TNF-α were purchased from PeproTech (Rocky Hill, NJ). The following Abs were used for FACS analysis: FITC-conjugated anti-CD11c mAb, FITC-conjugated anti-active caspase 3 mAb (BD Pharmingen, San Diego, CA), mouse monoclonal anti-Akt and rabbit polyclonal anti-phospho-Akt (Cell Signaling Technology, Beverly, MA), and FITC-conjugated F(ab’2) goat anti-rabbit IgG and FITC-conjugated F(ab’2) goat anti-mouse IgG (Sigma-Aldrich). The annexin V-FITC detection apoptosis kit II was purchased from BD Pharmingen. Wortmannin and dibutyryl cAMP (dbcAMP) were obtained from Calbiochem (San Diego, CA) and H89 was obtained from NovaScreen V-FITC detection apoptosis kit II was purchased from BD Pharmingen. The purity of the sorted cells was determined by FACS analysis (>=96% CD11c+).

**Generation and purification of BM-DC**

DC were generated in vitro from B10.A BM as described previously (30, 31). BM cells (2 x 10⁶) were cultured in 100-mm petri dishes containing 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, 50 mM 2-ME, and 20 ng/ml murine GM-CSF. After 3 days, another 10 ml of fresh medium containing 20 ng/ml GM-CSF was added to each dish. At day 7, the nonadherent cells were harvested and used as immature DC. The percentage of CD11c+ DC in the nonadherent population averaged 70% by FACS analysis. CD11c+ DC were purified by immunomagnetic sorting using anti-CD11c-coated magnetic beads and the AutoMACS system (Miltenyi Biotech, Bergish Gladbach, Germany). The purity of the sorted cells was determined by FACS analysis (>96% CD11c+).

**Stimulation of DC and RNA preparation**

Purified CD11c+ DC were cultured in petri dishes at a concentration of 0.5 x 10⁶ cells/ml and stimulated with LPS and Ibup in the presence or absence of PGE₂, for 24 h. Cells were collected and total RNA was isolated with the Absolutely RNA reagent (Stratagene, La Jolla, CA) as recommended by the manufacturer.

**Apoptosis assay (annexin V/propidium iodide (PI) staining)**

BM-DC cultured in 2% serum were collected at 48 h, washed, and adjusted to 1 x 10⁶ cells/ml in staining buffer. Staining was performed as recommended by BD Pharmingen and the cells were analyzed immediately by flow cytometry. Annexin V has high affinity for phosphatidylserine that is translocated to the outer leaflet of the plasma membrane during apoptosis. PI intercalates in the DNA of apoptotic cells with compromised plasma membrane.

**Determination of mitochondrial membrane potential and permeability by flow cytometry**

BM-DC treated for 48 h with LPS and Ibup in the presence or absence of PGE₂, were stained with the JC-1 reagent (5,5′,6,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolocarbocyanin iodide; Molecular Probes), commonly known as JC-1, which fluoresces orange upon accumulation in an aggregated form in the mitochondria of viable cells and green when present in monomeric form in the cytosol of cells with compromised mitochondria (apoptosis). Cells were resuspended after centrifugation in 0.5 ml of JC-1 reagent solution and incubated for 15 min in 5% CO₂ at 37°C. After washing twice, cells were resuspended in assay buffer and analyzed using flow cytometry for red and green fluorescence.

### Proliferation assay

Proliferation was assessed using the Vybrant CFDA SE Cell Tracer kit (Molecular Probes). The kit utilizes the dye CFSE that diffuses through cell membranes. Intracellular esterases cleave the acetate groups, yielding highly fluorescent amine-reactive carboxyfluorescein succinimidyl ester that reacts with intracellular amines forming stable fluorescent conjugates. Cells were incubated at 37°C in PBS containing the dye for 15 min, washed, incubated an additional 30 min in prewarmed medium to ensure complete modification, washed twice, and cultured for 72 h. Flow cytometric analysis was performed using the FL1 detector for fluorescence emission.

### FACS analysis

Cells were subjected to analysis by using a three-color FACSCalibur (BD Biosciences, Mountain View, CA) after staining with annexin-FITC and PI. For intracellular active caspase 3 staining, the Cytofix/Cytoperm kit (BD Pharmingen) was used according to the manufacturer’s instructions. For mitochondrial membrane integrity, cells were stained with the JC-1 dye. Double staining assays and mitochondrial staining data were collected after appropriate compensation and analyzed using the CellQuest software from BD Biosciences.

### PKB (Akt) assay

To determine the amount of total and phosphorylated intracellular Akt, we used intracellular flow cytometry. Briefly, the cells were fixed and permeabilized, stained with rabbit polyclonal anti-phospho-Akt (Cell Signaling Technology) or mouse monoclonal anti-Akt (Cell Signaling Technology) Abs for 45 min, followed by the appropriate secondary Abs for 30 min. Control rabbit and mouse IgG were used to confirm specificity. After extensive washing, cells were analyzed by FACS analysis.

### Apoptosis macroarray

Two micrograms of poly(A)+ mRNA isolated from LPS + Ibup and LPS + Ibup + PGE₂, was reverse transcribed in the presence of 2 µl of 20 µg [α-33P]dCTP, 1 µl each of 333 µM dATP/dGTP/dTTP/dTTP, 2 µl of 50 U AMV reverse transcriptase, 0.5 µl of 20 U RNase inhibitor, 6 µl of 1X reverse transcriptase buffer, and 4 µl of mouse apoptosis cDNA labeling primers in a total volume of 30 µl. Primer annealing was conducted by preheating the mixture to 90°C for 2 min, followed by 20-min ramping to 42°C. Reverse transcription was conducted at 42°C over 3 h. Unincorporated radiolabeled nucleotides were removed using a Sephadex G-25 gel filtration spin column. Hybridization of radiolabeled cDNA was performed in roller bottles in a hybridization oven overnight at 65°C in 5 ml of hybridization buffer.

### Table 1. Effects of PGE₂ on DC apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Early Apoptosis</th>
<th>% Late Apoptosis</th>
<th>% Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium (10% serum)</td>
<td>9 ± 5</td>
<td>16 ± 3</td>
<td>75 ± 6</td>
</tr>
<tr>
<td>Ceramide</td>
<td>28 ± 4</td>
<td>32 ± 6</td>
<td>39 ± 3</td>
</tr>
<tr>
<td>Ceramide + PGE₂</td>
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<td>22 ± 3*</td>
<td>70 ± 7*</td>
</tr>
<tr>
<td>Fas agonist</td>
<td>10 ± 4</td>
<td>18 ± 4</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>Fas agonist + PGE₂</td>
<td>8 ± 3</td>
<td>14 ± 3</td>
<td>78 ± 4</td>
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<tr>
<td>Medium (2% serum)</td>
<td>36 ± 6</td>
<td>46 ± 4</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Ibup</td>
<td>41 ± 5</td>
<td>46 ± 2</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Exogenous PGE₂</td>
<td>25 ± 4*</td>
<td>28 ± 3*</td>
<td>43 ± 2*</td>
</tr>
<tr>
<td>LPS</td>
<td>22 ± 3</td>
<td>23 ± 2</td>
<td>51 ± 5</td>
</tr>
<tr>
<td>LPS + Ibup</td>
<td>38 ± 4</td>
<td>56 ± 4</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>LPS + Ibup + exogenous PGE₂</td>
<td>30 ± 3*</td>
<td>21 ± 4*</td>
<td>47 ± 4*</td>
</tr>
</tbody>
</table>

*Upper portion: DC (5 x 10⁶ cells/ml) cultured in medium containing 10% serum and GM-CSF were treated with 50 µM ceramide or with the agonistic Fas Ab Jo-2 (1 µg/ml) in the absence or presence of 10⁻⁶ M PGE₂, for 24 h. Lower portion: DC (5 x 10⁶ cells/ml) were cultured in medium containing 2% serum without GM-CSF and treated with Ibup (10⁻⁵ M), PGE₂ (10⁻⁶ M), LPS (1 µg/ml), LPS + Ibup and LPS + Ibup + PGE₂ for 48 h. Viability was assessed using PI and annexinV. Results are expressed as the mean ± SD of triplicate cultures. Similar results were obtained in three independent experiments. *p < 0.01; **p < 0.05."
The arrays were washed twice with washing buffers I and II at 65°C for 20 min, air dried for 5 min, and subjected to autoradiography using a phosphor imager (Bio-Rad, Hercules, CA).

**In vivo experiments**

Purified CD11c+ BM-DC were labeled with 2.5 μM CFSE (Molecular Probes) and cultured overnight. Mice were preinjected s.c. in the footpads with TNF-α (100 ng/animal; PeproTech) followed 6 h later by 1 × 10^6 BM-DC s.c. in the same footpad. At different times the draining and non-draining popliteal lymph nodes were harvested, cut into small pieces, and digested with 1 mg/ml collagenase D (Sigma-Aldrich) at 37°C for 45 min. Single-cell suspensions were prepared, and the cells were washed with PBS and counted. Viability was assessed by trypan blue exclusion. The presence of CFSE-labeled cells was assessed by flow cytometry.

**Statistical analysis**

The results were expressed as mean ± SD of at least three independent experiments. Where indicated, the Student’s t test was used to compare control and experimental groups. Statistical significance was based on a value of p < 0.05.

**Results**

**PGE2 protects DC from apoptosis**

Immature and mature DC differ in the ability to undergo apoptosis and the molecular mechanisms responsible for these differences are largely unknown. Maturation of DC following signaling through TLR results in the production of endogenous PGE2, reported to play an important role in the directed migration of DC to lymph node T cell areas. We propose that endogenous PGE2 also plays a role in the increased survival of DC. Purified CD11c+ BM-DC were treated with ceramide or the Fas agonistic Ab Jo-2, and early and late apoptosis were determined 24 h later by double staining with annexin V and PI. As previously reported, DC are quite resistant to apoptosis following Fas signaling (Table I). In contrast, ceramide induces apoptosis and exogenous PGE2 results in a significant level of protection (70 vs 39% viable cells in the presence and absence of PGE2; Table I). Since DC are resistant to extrinsic apoptosis induced through death receptors such as Fas and TNFR1, we focused on intrinsic apoptosis caused by withdrawal of growth factors. Apoptosis in immature DC cultured in 2% serum is high and exogenous PGE2 has a significant protective effect (18 vs 43% viable cells, respectively; Table I). Since immature DC do not produce PGE2, the Cox1/2 inhibitor Ibup does not affect survival (13 vs 18% viable cells; Table I). In contrast to immature DC, DC matured by LPS treatment are less sensitive to apoptosis (51 vs 18% viable cells; Table I). To evaluate the role of...
endogenous PGE$_2$ in the protection against apoptosis, we matured DC with LPS in the presence of Ibup. A substantial decrease in viability is observed (5 vs 51%, respectively; Table I). The effect of Ibup is indeed mediated through the blockage of endogenous PGE$_2$ production, since exogenous PGE$_2$ re-establishes protection (Table I). Specific Cox 1 (SC530) and Cox 2 (NS398) inhibitors also decrease the viability of LPS-stimulated DC, but less than Ibup (results not shown). The protective effect of PGE$_2$ is time dependent and dose dependent, with significant protective effects as early as 12 h and with maximal effects for $10^{-7}$–$10^{-6}$ M PGE$_2$ (Fig. 1).

The protective effect of PGE$_2$ is mediated through specific receptors

To establish the nature of the PGE$_2$ receptors involved in protection from apoptosis, we used receptor agonists. Sulprostone, an agonist for EP-1 ($K_i = 21$ nM) and EP-3 ($K_i = 0.6$ nM), does not inhibit apoptosis (Fig. 2). In contrast, butaprost, an EP-2 agonist, inhibits apoptosis, and misoprostol, which binds EP-3 and EP-4 at low concentrations ($K_i = 67$ nM) and EP-1 and EP-2 at higher concentrations ($K_i = 120$ and 250 nM for EP-1 and EP-2, respectively), is also inhibitory. These findings suggest that the inhibitory effect of PGE$_2$ on apoptosis is mediated through EP-2 and possibly EP-4 receptors.

PGE$_2$ inhibits the generation of activated caspase 3 and the disruption of mitochondrial permeability in DC

Both intrinsic and extrinsic apoptosis result in the activation of downstream effector caspases, specifically caspase 3. We assessed the effects of PGE$_2$ on the activation of caspase 3 by intracellular staining for active caspase 3. In DC matured with LPS in the absence of endogenous PGE$_2$ (Ibup treatment), the percentage of cells expressing intracellular active caspase 3 is increased (25 vs 8%; Fig. 3A). In contrast, addition of exogenous PGE$_2$ significantly reduces the number of cells expressing active caspase 3

![Figure 3](http://www.jimmunol.org/)
These results are in agreement with the protective role of PGE2 on DC apoptosis (see above).

In the intrinsic apoptotic pathway, mitochondria play the major role in the activation of effector caspases. Changes in mitochondrial potential and permeability leads to the release of cytochrome c and apoptosis-inducing factor, essential apoptosis mediators. We assessed the effects of PGE2 on mitochondrial integrity in DC by using the cationic fluorescent dye JC-1. In live cells, the dye enters the negatively charged mitochondria, aggregates, and fluoresces orange. In apoptotic cells with collapsed mitochondrial membranes and lost electrochemical potential, the dye diffuses to the cytosol in a monomeric form that fluoresces green. DC matured in the absence of endogenous PGE2 (LPS + Ibup) consist of an equal mix of cells with intact and disrupted mitochondria (Fig. 3B). Addition of exogenous PGE2 shifts the balance in favor of cells with intact mitochondria (Fig. 3B). The effect of PGE2 in maintaining mitochondrial integrity in DC is in agreement with the protective effect against apoptosis.

**PGE2 inhibits DC proliferation**

The high numbers of viable DC in cultures containing PGE2 could result from protection against apoptosis and/or increased cell proliferation. To assess the effects of PGE2 on proliferation, we first
cultured CFSE-labeled DC in 10% serum + GM-CSF in the presence and absence of LPS, Ibup, and PGE2. DC cultured in medium alone divide, whereas proliferation is significantly reduced in the presence of PGE2 (Fig. 4A). DC treated with LPS show two distinct peaks, which indicates that more than one-half of the cells divided. In the presence of Ibup, there is a strong shift toward

![Figure 5](image)

**FIGURE 5.** PGE2 inhibits mRNA expression of a variety of proapoptotic agents. DC were treated with LPS + Ibup in the presence or absence of PGE2 (10^{-6} M). Cells were collected at 24 h and mRNA was extracted, reverse transcribed, and used in the apoptosis macroarray assay as described in Materials and Methods. Numbers represent fold reduction. One representative experiment of two is shown.

![Figure 6](image)

**FIGURE 6.** PGE2 enhances Akt phosphorylation. DC were treated with or without PGE2 (10^{-6} M), collected at different time points (0, 30, and 60 min), washed, fixed, permeabilized, and subjected to intracellular staining for total Akt and phospho-Akt. Markers were established based on appropriate isotypes. One representative experiment of three is shown.
division, indicating that DC divide at an accelerated pace in the absence of endogenous PGE2. Addition of exogenous PGE2 suppresses proliferation (Fig. 4A). These results indicate that both endogenous and exogenous PGE2 inhibit DC proliferation, at least for cells cultured in optimal serum conditions. The experiments were repeated with DC cultured in 2% serum without GM-CSF, the conditions in which we observed the antiapoptotic effect of PGE2. No DC proliferation was observed, either in the presence or absence of LPS, Ibup, and PGE2 (Fig. 4B). These experiments indicate that the PGE2-induced increase in the number of viable DC results from a direct antiapoptotic effect and not increased cell proliferation.

PGE2 inhibits the expression of several proapoptotic factors

Although many apoptosis-related molecules are activated through posttranslational modifications, proapoptotic and antiapoptotic factors are also controlled in terms of transcription. To compare gene expression in the presence and absence of PGE2, we used an apoptosis-related macroarray for LPS + Ibup and LPS + Ibup + PGE2-treated DC. We observed significant down-regulation of a number of proapoptotic molecules in PGE2-treated DC (Fig. 5). mRNA encoding the BH3-only proapoptotic BAD protein was down-regulated 80-fold. Expression of BAK was decreased ~8-fold. In addition, significant reductions (4-fold or higher) in the expression of caspases 1, 2, 3, 6, 7, 12, and 14 and of granzyme B were observed (Fig. 5).

The PI3K pathway is involved in the protective effect of PGE2 in DC apoptosis

cAMP generated through the activation of adenylate cyclase is the classical intracellular mediator for EP-2/EP-4 signaling. Similar to PGE2, dbcAMP protects DC against apoptosis, but H89 (a protein kinase A (PKA) inhibitor) does not reverse the protective effect of PGE2 (Table II). These results suggest that although cAMP plays a role in the protective effect of PGE2, PKA does not appear to be the next intracellular mediator. The major player in the survival of many cell types is Akt (PKB). Akt acts primarily through the phosphorylation and inactivation of BAD and is, in turn, phosphorylated and activated by PI3K. Therefore, we determined first that wortmannin, a PI3K inhibitor, reverses the protective effect of PGE2 (Table II). In addition, we assessed the levels of phosphorylated Akt in DC treated with or without PGE2. PGE2 induces a significant increase in the percentage of cells expressing phosphorylated Akt (from 2.4 to 67.2%), without affecting total Akt levels, as early as 30 min (Fig. 6). These results suggest that PGE2 promotes survival of DC primarily through the PI3K → Akt → P-BAD pathway.

PGE2 plays a role in the survival of DC in vivo

To determine the in vivo effect of PGE2 on DC survival, we injected CFSE-labeled BM-DC in combination with Ibup or misoprostol in B10.A mice. The mice were inoculated in the right footpads with TNF 6 h before the administration of DC. The TNF treatment has been reported to increase DC migration to lymph nodes (36) and was chosen to minimize the effects of PGE2 on migration. CFSE-labeled DC were administered into the same footpad along with Ibup (to inhibit endogenous PGE2 release) or misoprostol (to provide an exogenous source of stable EP receptor agonist). The numbers of CFSE-DC were determined at days 2, 6, and 12 in both the draining popliteal lymph node (right hind leg) and in the popliteal node from the left hind leg. Controls included animals not pretreated with TNF or pretreated with TNF and injected with CFSE-DC and PBS instead of Ibup/misoprostol. On day 2, small numbers of DC reached the draining lymph nodes in the absence of TNF pretreatment. In contrast, much higher numbers were detected in animals preinjected with TNF and treated with PBS, Ibup, or misoprostol (Fig. 7). No CFSE-labeled cells were found in the nondraining popliteal lymph nodes (left hind leg). The fact that similar numbers were observed in Ibup- and

<table>
<thead>
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<th>% Late Apoptosis</th>
<th>% Viable Cells</th>
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</thead>
<tbody>
<tr>
<td>Medium (2% serum)</td>
<td>35 ± 5</td>
<td>44 ± 5**</td>
<td>20 ± 3*</td>
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<tr>
<td>PGE2 (10⁻⁶ M)</td>
<td>11 ± 2</td>
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<td>65 ± 4</td>
</tr>
<tr>
<td>dbcAMP (10⁻³ M)</td>
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<td>61 ± 4</td>
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<td>PGE2 + wortmannin (10⁻⁷ M)</td>
<td>22 ± 3</td>
<td>48 ± 3**</td>
<td>28 ± 4**</td>
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</table>

*DC (5 × 10⁵ cells/ml) were cultured in medium containing 2% serum and treated with PGE2 (10⁻⁶ M), dbcAMP (10⁻³ M), PGE2 + H89 (10⁻³ M), and PGE2 + wortmannin (10⁻⁷ M). Viability was assessed at 48 h. Controls consisted of DC treated with medium alone, H89, or wortmannin in the absence of PGE2. Results are expressed as the mean ± SD of triplicate cultures. Similar results were obtained in three experiments. **, p < 0.01 (between groups indicated by brackets).
misoprostol-treated animals indicates that in the conditions used in these experiments, endogenous PGE2 or the exogenous EP receptor agonist do not play an essential role in DC migration and facilitated the conclusions regarding the PGE2 role in DC survival in the lymph nodes.

Significant differences between the Ibup- and misoprostol-treated animals became apparent starting with day 6. A significant decrease in the number of DC occurred on day 6, and no CFSE-labeled cells were detected from day 12 on in the PBS- or Ibup-treated group. In contrast, there was only a slight reduction on day 6 and significant numbers of CFSE-labeled DC were still observed on day 12 in the group treated with misoprostol. These results suggest that, similar to the in vitro observations, endogenous or exogenous PGE2 increases DC viability in vivo.

PGE2 promotes the survival of DC loaded with Ag in vitro and in vivo

To determine whether PGE2 exerts a similar effect on the survival of Ag-loaded DC, we generated BM-DC and loaded them with OVA. In preliminary experiments, we established that a 1-h incubation of DC with OVA-FITC is sufficient for maximal loading; all DC become loaded, with a majority of cells exhibiting high levels of fluorescence (Fig. 8A). The next experiments were performed with similar concentrations of nonfluorescent OVA. DC were loaded with OVA and exposed to growth factor withdrawal by culture in 2% serum with LPS (1 μg/ml) or medium (control) in the presence or absence of PGE2 (10^{-7} M). Viability was assessed at 48 h. One representative experiment of three is shown. C, DC (5 × 10^5 cells/ml) were loaded with OVA (0.5 mg/ml OVA for 1 h) and labeled with CFSE. Mice (groups of four) were inoculated with TNF, followed by OVA-DC with PBS, Ibup, or misoprostol (Misp.) as described in Fig. 7. The draining lymph nodes were harvested on days 2, 4, and 6 and cell suspensions were obtained and analyzed as described in Fig. 7. One experiment of two is shown.
at day 4 a significant difference was observed between the misoprostol-treated mice and the controls (Fig. 8C). This suggests that, similar to the effect on immature DC, PGE$_2$ enhances the in vivo survival of Ag-loaded DC. However, in contrast to immature DC which were still present on day 12 in the misoprostol-treated group, we did not detect CFSE-labeled OVA-loaded DC on day 6 or later, suggesting that, in vivo, Ag-loaded DC are more susceptible to apoptosis.

**Discussion**

Cyclooxygenases (Cox1/Cox2) and downstream PGE$_2$ synthases, both constitutive and inducible, contribute to the generation of PGE$_2$, one of the major arachidonic acid metabolites. Activated APC, including DC, produce and secrete endogenous PGE$_2$ primarily through the rapid induction of Cox2. In vitro studies show different effects of PGE$_2$ on immature and mature DC. PGE$_2$ was reported to contribute to DC maturation and migration to draining lymph nodes (22–26). The role of endogenous PGE$_2$ was confirmed in EP-4-deficient mice, where DC migration to the lymph nodes is severely reduced (27). In contrast, when DC are matured through strong TLR signals, PGE$_2$ inhibits the production of proinflammatory cytokines (IL12p70, TNF, IL-6) and chemokines (CCL3, CCL4, CXCL10) both in vitro and in vivo (29–32).

In the present study, we present evidence for a new biological role for PGE$_2$ in promoting the survival of DC. Immature and mature DC have been reported to differ in terms of susceptibility to apoptosis (?11–20). We propose that endogenous PGE$_2$ released during DC maturation is responsible for at least some of these differences. Our results indicate that DC matured with LPS in the presence of the Cox1/Cox2 inhibitor Ibup revert to the high susceptibility of serum-starved immature DC and that addition of exogenous PGE$_2$ re-establishes the more resistant phenotype characteristic for mature DC. The in vivo experiments indicate that injected CFSE-DC survive significantly longer in the draining lymph nodes of mice treated with a PGE$_2$ receptor agonist in comparison to animals treated with Ibup or controls injected with PBS. PGE$_2$ increases the survival of both immature and Ag-loaded DC injected into naive hosts.

Among the four PGE$_2$ receptors, EP-2 and EP-4 mediate most, if not all, of the PGE$_2$ effects on DC (23, 30, 31). We reported previously that both EP-2 and EP-4 are expressed at mRNA level and as surface proteins on murine BM-DC (30). In agreement with these reports, the present study indicates that PGE$_2$ promotes DC survival through EP-2/EP-4 receptors.

The major signaling molecule related to the EP-2/EP-4 receptors is cAMP (37). Many of the observed effects of PGE$_2$ on DC have been connected to the cAMP-dependent activation of PKA. However, recently, PGE$_2$ was shown to contribute to PKB activation in monocyte-derived DC (26), and a new intracellular cAMP receptor EPAC was identified as the mediator of the PI3K-dependent activation of PKB (38). Therefore, the possibility exists that increased levels of cAMP generated by EP-2/EP-4 signaling lead to the activation of PKB (Akt) in DC. We showed that indeed PGE$_2$ increases PKB phosphorylation and that wortmannin, a PI3K inhibitor, reverses the antiapoptotic effect of PGE$_2$ in DC. The major mechanism involved in the antiapoptotic effect of PKB is the phosphorylation of BAD, which is then sequestered in the cytosol and prevented from interacting with Bcl-2/Bcl-x and from disrupting the mitochondrial membrane (39, 40). Our results are in agreement with this mechanism, since PGE$_2$ preserves the integrity of the mitochondrial membranes in DC. Interestingly, we also observed a significant reduction (80-fold) in the expression of BAD in PGE$_2$-treated DC. The down-regulation of various antiapoptotic molecules, including some of the caspases and granzyme B, suggests that in addition to the posttranslational modifications typical for apoptosis, the mechanisms for cell survival/apoptosis also involve transcriptional regulation.

The PGE$_2$-dependent increased survival of DC occurs also in vivo. Since PGE$_2$ promotes DC migration, we needed a system that could distinguish between DC migration and survival in response to PGE$_2$. Therefore, we chose an experimental system in which migration is induced by TNF rather than PGE$_2$ (36). In these conditions, similar numbers of DC arrive to the draining lymph nodes of mice treated with Ibup (a Cox inhibitor that reduces the levels of endogenous PGE$_2$), misoprostol (a stable EP receptor agonist with a relatively large specificity spectrum), and PBS, used as control. However, differences between the PBS/Ibup and the misoprostol group become apparent at later time points, when the misoprostol-treated mice continued to exhibit significantly higher numbers of DC in the draining lymph nodes. This suggests that the administration of PGE$_2$ receptor agonists increases the survival of DC in vivo. However, we cannot exclude the possibility that the higher numbers of DC in the draining lymph nodes of misoprostol-treated mice is due to sustained DC migration, in situ DC proliferation, or even CFSE transfer from exogenous to endogenous DC. We would argue against an increased in situ proliferation since our in vitro studies indicate that PGE$_2$ suppresses DC proliferation. Also, it is unlikely that PGE$_2$ causes increased migration, since s.c. administered DC migrate to the draining lymph nodes in ~48 h and similar numbers of DC have been observed in all three groups on day 2. Transfer of CFSE to endogenous DC could follow apoptosis of the administered exogenous DC. However, since PGE$_2$ promotes DC survival in vitro, it is unlikely that misoprostol-treated DC will exhibit enhanced apoptosis. Similar conclusions were reached regarding the effects of PGE$_2$ on Ag-loaded DC. In vitro, both immature and OVA-loaded DC were susceptible to apoptosis induced by growth factor withdrawal and PGE$_2$ protected them to the same degree. In vivo, administration of misoprostol enhanced the number of OVA-loaded DC in the draining lymph nodes of naive mice compared with PBS- or Ibup-treated controls. However, even in the presence of misoprostol, the Ag-loaded DC survived for a shorter period of time compared with immature DC.

What is the biological significance of the antiapoptotic effect of PGE$_2$? Our experiments indicate that exogenous PGE$_2$ protects immature and Ag-loaded DC against growth factor withdrawal-induced apoptosis and that the increased resistance of LPS-stimulated DC is mediated by endogenous PGE$_2$. We propose that PGE$_2$ released locally at inflammatory loci contributes to the survival of DC. By increasing the survival and migration of DC, PGE$_2$ augments their potential as initiators of adaptive immunity. In addition, the fact that PGE$_2$ receptor agonists increase the number and possibly survival of Ag-loaded DC in the draining lymph nodes is exciting as a potential treatment in cancer vaccination. However, it remains to be ascertained whether PGE$_2$ also acts on Ag-loaded DC in immunized hosts, a model which is more relevant to the use of DC in therapeutic vaccine trials.

**References**


