Introduction

Prostaglandin E2 (PGE2) is generated by the sequential metabolism of arachidonic acid by cyclo-oxygenase and prostaglandin E synthase (1, 2). This lipid mediator has pleiotropic actions in a range of tissues, including the immune system (3). Within the immune system, PGE2 modulates the functions of cell populations, such as T cells and macrophages, which are critical to the immune response. For example, PGE2 suppresses proliferation of human T cells (4, 5). In macrophages, PGE2 inhibits production of cytokines such as TNF-α, and IL-12 (6, 7) and alters antigen presentation by inhibiting expression of MHC class II proteins (8). Thus, the overall actions of PGE2 on in vitro models of cellular immune responses tend to be inhibitory and suppressive (9). Along with its actions to inhibit cellular functions, PGE2 may also affect the overall character of an immune response. PGE2 may polarize cellular response toward a Th2 phenotype enhancing IL-4 and IL-5 production (10, 11) and facilitating immunoglobulin class switching to IgE (12).

These actions of PGE2 can dramatically alter the outcome of immune responses in the intact organism. For instance, PGE2 has inhibitory and protective effects in autoimmune disease. In murine lupus models, administration of PGE2 and its analogues improves survival (13, 14). This improvement in survival is accompanied by reduced auto-Ab production and a substantial reduction in immune-mediated kidney injury. Similarly, PGE2 may delay or prevent allograft rejection. In a rat model of kidney transplantation, administration of PGE1 markedly prolonged graft survival and reduced systemic cellular alloimmune responses (15). Analogous effects of PGE2 to ameliorate rejection have been observed in animal models of heart, intestinal, and skin transplantation (16–19). In human renal transplant recipients, a reduced number of kidney allograft rejection episodes has also been reported with PGE2 analogues (20).

Production of prostaglandin E2 (PGE2) is enhanced during inflammation, and this lipid mediator can dramatically modulate immune responses. There are four receptors for PGE2 (EP1–EP4) with unique patterns of expression and different coupling to intracellular signaling pathways. To identify the EP receptors that regulate cellular immune responses, we used mouse lines in which the genes encoding each of the four EP receptors were disrupted by gene targeting. Using the mixed lymphocyte response (MLR) as a model cellular immune response, we confirmed that PGE2 has potent antiproliferative effects on wild-type responder cells. The absence of either the EP1 or EP3 receptors did not alter the inhibitory response to PGE2 in the MLR. In contrast, when responder cells lacked the EP2 receptor, PGE2 had little effect on proliferation. Modest resistance to PGE2 was also observed in EP4−/− responder cells. Reconstitution experiments suggest that EP2 receptors primarily inhibit the MLR through direct actions on T cells. Furthermore, PGE2 modulates macrophage function by activating the EP4 receptor and thereby inhibiting cytokine release. Thus, PGE2 regulates cellular immune responses through distinct EP receptors on different immune cell populations: EP2 receptors directly inhibit T cell proliferation while EP2 and EP4 receptors regulate antigen presenting cells functions.

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Receptors for prostaglandin E2 that regulate cellular immune responses in the mouse

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Production of prostaglandin E2 (PGE2) is enhanced during inflammation, and this lipid mediator can dramatically modulate immune responses. There are four receptors for PGE2 (EP1–EP4) with unique patterns of expression and different coupling to intracellular signaling pathways. To identify the EP receptors that regulate cellular immune responses, we used mouse lines in which the genes encoding each of the four EP receptors were disrupted by gene targeting. Using the mixed lymphocyte response (MLR) as a model cellular immune response, we confirmed that PGE2 has potent antiproliferative effects on wild-type responder cells. The absence of either the EP1 or EP3 receptors did not alter the inhibitory response to PGE2 in the MLR. In contrast, when responder cells lacked the EP2 receptor, PGE2 had little effect on proliferation. Modest resistance to PGE2 was also observed in EP4−/− responder cells. Reconstitution experiments suggest that EP2 receptors primarily inhibit the MLR through direct actions on T cells. Furthermore, PGE2 modulates macrophage function by activating the EP4 receptor and thereby inhibiting cytokine release. Thus, PGE2 regulates cellular immune responses through distinct EP receptors on different immune cell populations: EP2 receptors directly inhibit T cell proliferation while EP2 and EP4 receptors regulate antigen presenting cells functions.

couple to different signaling pathways including G_α, G_β, and calcium. The existence of this family of EP receptors coupled to distinct intracellular signals provides a molecular basis for the diverse physiological actions of PGE_2. EP receptor isoforms are expressed by the major cellular constituents of the immune system (3). However, the precise EP receptor isoforms that mediate the immunoregulatory actions of PGE_2 are not known. Thus, the objective of our studies was to define the expression of EP receptors by immune cell populations in the mouse and to determine their contribution to the regulation of cellular immune responses. Using a combination of pharmacological and genetic approaches, we find that the actions of PGE_2 to suppress antigen-specific proliferation are complex. To a significant extent, these actions are mediated by EP2 receptors on T cells and by EP2 and EP4 receptors on macrophages.

**Methods**

**Animals.** The production of mouse lines with targeted disruptions of the four EP receptor genes are described elsewhere (23–26). The EP1-deficient line was produced on an inbred DBA/1 background using an embryonic stem (ES) cell line derived directly from DBA/1 mice (25). Thus, controls for the EP1 experiments were age-matched DBA/1 mice that were purchased from The Jackson Laboratories (Bar Harbor, Maine, USA). Mice with targeted mutations of the EP2 and EP3 genes were produced on a 129/SvEv background. Controls for these studies were wild type 129/SvEv littermates. On inbred backgrounds, most EP4-deficient mice die within 24 hours from complications of patent ductus arteriosus (24). However, by selective breeding on a mixed background, EP4-deficient lines have been produced in which the ductus closes and the animals survive normally (24). EP4−/− mice from these selected mixed breedings were used in our experiments. Controls for these studies are wild type littermates. Animals were bred and maintained in the animal facility of the Durham VA Medical Centers under the NIH guidelines.

**Identification of EP receptor mRNA expression by RT-PCR.** Expression of EP receptor mRNA was assessed by RT-PCR as described (27). Splenocyte suspensions were prepared from wild-type and EP-deficient mice by gently grinding the spleen between glass slides. The cells were washed once in PBS and then resuspended in ice-cold PBS containing 10 mM Tris HCl (pH 7.4), 1% BSA. Splenic T cells were isolated using a commercial separation column (R&D Systems Inc., Minneapolis, Minnesota, USA), splenic B cells were isolated by panning using a polyvalent anti-mouse IgG Ab, and splenic macrophages were isolated by plastic adherence. Purity of the cell populations was confirmed by cytofluorometry. Total RNA was isolated from these cell preparations using Tri-Reagent (Sigma Chemical Co., St. Louis, Missouri, USA), and 0.5 µg was reverse-transcribed using oligo-dT primers. EP receptor cDNA was amplified in PCR reactions using the following primers: EP1 sense 5′-TTAACCCTGAGCC-

**Mixed lymphocyte responses.** Primary one-way mixed lymphocyte responses (MLRs) were performed as described previously (27). Splenocyte suspensions and isolated T cells were prepared as described above. Suspensions of responder splenocytes or T cells were reconstituted at various concentrations and were mixed with irradiated stimulator splenocytes from H-2 disparate mice at the indicated ratios. Fifty microliters of each cell suspension was added to individual wells of a 96-well plate along with various concentrations of EP agonists or vehicle. Plates were incubated at 37°C in a humidified incubator containing 5% CO₂. After varying periods in culture, cells were pulsed with 0.5 µCi of ³H-thymidine per well for the final 18 hours of culture. The amount of ³H-thymidine incorporated in cells was assessed by harvesting cells onto a glass fiber filtermat using an automated cell harvester (Tomtek, Hamden, Connecticut, USA). Filter-bound radioactivity was measured using a scintillation counter. Values are expressed as specific counts per minute, which are calculated from counts in wells with responders alone subtracted from counts in wells with responders and stimulators. Within each experiment, individual conditions were examined in triplicate or quadruplicate samples.

In some experiments using isolated T cell responders, purified populations of macrophages were added to the cultures. To prepare purified cultures of macrophages, bone marrow was harvested from EP2-deficient and control mice. B cells and natural killer (NK) cells were removed from the suspension by sequential panning, with anti-Ig for B cells and anti-asialo GM1 for NK cells, followed by complement lysis. The resulting cell population was highly enriched for macrophages as determined by cytofluorometry with MAC-1 and F480 Abs. These pure populations of macrophages were used to reconstitute responder populations in MLR by adding 6 × 10⁴ macrophages to cultures containing 4 × 10⁵ T cells. Splenic T cells were isolated using a commercial separation column (R&D Systems Inc.). Allopecific proliferative responses were then assessed as described above.

**Prostanoid compounds used in MLR experiments.** Prostanoid compounds including PGE₂, misoprostol, and sulprostone were obtained from Cayman Chemical (Ann Arbor, Michigan, USA) as crystalline solids of greater than or equal to 99% purity. Based on the manufacturer’s recommendations, stock solutions were prepared in organic solvent (ethanol) and were stored...
at -20°C between experiments. The compounds are stable in these solutions at this temperature for up to 6 months. For the studies, fresh dilutions were prepared in media on the day of study to produce the desired experimental concentrations. A similar concentration of ethanol vehicle alone was added to the control cultures.

**LPS-stimulated production of TNF-α and IL-12.** Macrophages were prepared by culturing bone marrow from EP2−/−, EP4−/−, and wild-type mice in Petri dishes with media supplemented with GM-CSF (30% L929 supernatants). Nonadherent cells were discarded from these plates after 3 days, and cultures were maintained in GM-CSF–enriched media for an additional 4–5 days. Macrophages predominate in these cultures as adherent cells. Purity was confirmed by cytofluorometry using MAC-1 and F480 Ab’s. Macrophages (4 × 10⁵) were cultured in 96-well tissue culture plates in the presence 10 nM LPS along with vehicle or PGE2 in the indicated concentration. Supernatants were removed from these wells after 8 and 24 hours. TNF-α production was assessed using a bioassay and IL-12 production was measured by ELISA.

**Statistical analysis.** The values for each parameter within a group are expressed as the mean plus or minus the SEM. For comparisons between EP-deficient and wild-type groups, statistical significance was assessed using an unpaired t test for normally distributed data. A paired t test was used for comparisons within groups. For non-parametric analyses, a Mann-Whitney U test was used.

**Results**

**EP receptor isoform mRNA expression in murine lymphocyte subsets.** Because there is limited information regarding expression of EP receptor isoforms by various immune cell populations in the mouse, we used RT-PCR to examine EP receptor mRNA in cell populations isolated from mouse spleen. Using this RT-PCR assay, expression of EP1 receptor mRNA was easily detected in T and B lymphocytes, as well as macrophages (data not shown). Similarly, expression of mRNA for EP2 and EP4 receptors was present in all three splenocyte subsets (not shown). While all three isoforms of the EP3 receptors were detected in B cells and macrophages, the α and β isoforms could not be reproducibly amplified from splenic T cells (not shown). Thus, with the exception of α and β isoforms of the EP3 receptor in T cells, mRNA from all of the known EP receptors was detected in the major immune cell populations in mouse spleen.

**PGE2 analogues suppress proliferative responses of lymphocytes to alloantigens.** To begin to define the actions of specific EP receptors to regulate immune responses, we first examined the effects of PGE2 and several of its analogues upon the MLR, a model of the cellular alloimmune response. The MLR is designed to mimic the conditions that might occur in a transplanted organ when recipient immune cells are activated by recognition of foreign MHC antigens expressed on the donor tissue. One-way MLRs were performed using responders from wild-type (129SvEv) H-2b mice and irradiated stimulators from wild-type (C3H-HeJ) H-2k animals. Proliferative responses were compared between cultures containing PGE2 analogues and those that received vehicle alone. The various PGE2-agonists differ in their affinities for EP receptor isoforms (21). PGE2 binds with high affinity to all four EP receptor subtypes. Misoprostol, at lower concentrations, exhibits relative specificity for EP3 receptors (Kᵢ = 67 nM), but it may also activate EP1 and EP2 receptors at higher concentrations (Kᵢ = 120–250 nM) (21). Sulprostone, on the other hand, is a relatively specific agonist for the EP1 (Kᵢ = 21 nM) and EP3 receptors (Kᵢ = 0.6 nM) (21).

As depicted in Figure 1, PGE2, among the compounds tested, caused the most potent inhibition of the cellular immune response; concentrations of 5–10 nM caused 50% inhibition of proliferation. Although misoprostol also inhibited the MLR, it was significantly less potent than PGE2, requiring almost log dose-higher concentrations to produce 50% inhibition. In contrast, concentrations of sulprostone up to 50 µM did not substantially affect the vigor of the proliferative response. These data confirm the actions of PGE2 to suppress cellular immune responses and suggest that EP2 and/or EP4 receptors may mediate these effects.

**Identification of EP receptors that inhibit MLR using genetically altered mice.** To more precisely identify the EP receptor isoforms that regulate proliferation in MLR, we compared the actions of PGE2 upon responder cell populations derived from mice with targeted disruption of each of the individual EP receptor genes. Figure 2 summarizes the results of experiments with EP1-deficient (Figure 2a), EP3-deficient (Figure 2b), and EP4-deficient (Figure 2c) responders. PGE2 caused potent inhibition of allo-specific proliferation by the EP1- and EP3-deficient splenocytes, and this effect was virtually identical to that observed in their respective wild-type
controls. In the lower concentration range, the inhibitory actions of PGE2 on EP4-deficient cells were also similar to controls. However, at concentrations of 100 nM and above, the absence of EP4 receptors was associated with a modest, but significant reduction in sensitivity to the antiproliferative effects of PGE2. By contrast, as depicted in Figure 3, EP2-deficient responders were markedly resistant to the inhibitory actions of PGE2. At the lowest concentrations, PGE2 had no effect on proliferation of the EP2-deficient T cells. Compared with the mixed population of EP2-deficient splenocytes, EP2-deficient T cells had a more complete resistance to PGE2. The difference in PGE2 responsiveness between EP2−/− mixed splenocytes and isolated T cells suggests a contribution of other (non-T cell) populations to the regulatory actions of PGE2 in the MLR.

**Distinct actions of PGE2 on T cells and macrophages.** To determine whether EP receptors on macrophages might contribute to the regulation of cellular immune responses by PGE2, we performed reconstitution experiments with macrophages and isolated T cells. MLR cultures were set up with T cells from EP2-deficient splenocytes in the presence of exogenous PGE2 (Figure 5). Some of the cultures were reconstituted with macrophages derived from wild-type or EP2-deficient mice. As shown previously, EP2-deficient T cells alone were completely resistant to the actions of PGE2. Addition of syngeneic EP2+/+ macrophages restored some sensitivity to the antiproliferative actions of MLR with purified T cells. In our initial experiments, we studied responder cells consisting of a mixed population of splenocytes including T cells, B cells, and macrophages. Since all of these cell populations express various EP receptor isoforms, including EP2, EP-receptor–mediated effects in any or all of these cells might contribute to the actions of PGE2 to modulate the cellular response to alloantigens. To determine whether PGE2 influences allospecific proliferation by direct effects on T cells and to examine the role of the EP2 receptor in these actions, we performed additional MLR experiments using isolated T cells as responders. As shown by Figure 4 and similar to its actions on mixed splenocyte responders, PGE2 caused significant inhibition of the MLR when the responders were a purified population of T cells isolated from wild-type mice. In marked contrast, concentrations of PGE2 up to 30 µM had no effect on proliferation of isolated EP2-deficient T cells. Compared with the mixed population of EP2-deficient splenocytes, EP2-deficient T cells had a more complete resistance to PGE2. The difference in PGE2 responsiveness between EP2−/− mixed splenocytes and isolated T cells suggests a contribution of other (non-T cell) populations to the regulatory actions of PGE2 in the MLR.

![Figure 2](image_url)

**Figure 2**
PGE2 inhibits proliferation in MLR with EP1-, EP3-, and EP4-deficient lymphocytes. MLR experiments were performed comparing responders that were lacking (a) EP1, (b) EP3, and (c) EP4 receptors with their respective wild-type controls. On the x-axis the effects of various concentrations of PGE2 upon proliferation were determined. The data are expressed as a percentage of the control response in MLR with vehicle alone. *P = 0.008 vs. EP4+/−.

![Figure 3](image_url)

**Figure 3**
EP2-deficient lymphocytes are resistant to the antiproliferative actions of PGE2 in the MLR. MLR experiments were performed comparing responders that were lacking EP2 receptors with their respective wild-type controls. On the x-axis, the effects of various concentrations of PGE2 upon proliferation were determined. The data are expressed as a percentage of the control response in MLR with vehicle alone. *P = 0.004 vs. EP2+/+; **P < 0.0001 vs. EP2+/+. 
PGE2. Inhibitory actions of PGE2 were also conferred by EP2–/– macrophages, but the magnitude of inhibition was less prominent than with EP2+/+ macrophages (13% vs. 27%; \( P = 0.03 \)). These data suggest that EP receptors on autologous macrophages contribute to the regulation of the cellular alloimmune response.

**Discussion**

The actions of PGE2 to regulate immune responses have been long recognized. PGE2 modulates a wide range of T cell functions, and these actions are largely suppressive or inhibitory (3). For example, PGE2 inhibits antigen-induced proliferation, cytokine production, and cell surface expression of cytokine receptors (9). A variety of effector functions are also inhibited by PGE2, including the development of antigen-specific cytotoxic T cells (28). The capacity of PGE2 to suppress many of the responses that are triggered by T cell receptor activation suggests that it acts at a central site in the biochemical cascade associated with T cell receptor stimulation. In this regard, Paliogianni and associates found that PGE2 antagonizes the actions of calcineurin phosphatase to stimulate the transcription of cytokine genes (29), such as the IL-2 and IFN-\( \gamma \) genes, that are an important part of the genomic activation program in T cells. The inhibition of IL-2 and IFN-\( \gamma \) by PGE2 may also contribute to its ability to skew cellular responses toward a Th2 phenotype.

These inhibitory actions of PGE2 upon T cell functions can impact immune responses in the whole animal. In murine models of autoimmune disease, chronic administration of PGE2 has dramatic effects to ameliorate the manifestations of autoimmunity and to prevent immunemediated end organ injury (13, 14). Likewise, PGE2 and its analogues dramatically suppress rejection of organ and tissue transplants (15–19). In some models, long-term allograft survival can be induced by treatment with PGE2 and no other immunosuppressive therapy. Beneficial effects of E-series prostanoids in human transplant recipients have also been reported (20, 30, 31). Nonetheless, the precise role of individual EP receptor isoforms in mediating these actions is not clear. Because of the potent actions of PGE2 to inhibit transplant rejection, we chose to first examine the role of EP receptor isoforms in the MLR, an in vitro model of the cellular alloimmune response.
Our studies using genetically manipulated mice indicate that the regulatory actions of PGE2 in cellular immunity are complex, involving more than one receptor and different immune cell populations. Our data show that the EP2 receptor is a dominant mediator of the inhibitory actions of PGE2 in the MLR. In T cells, absence of the EP2 receptor confers a resistance to PGE2 that is virtually absolute. It is possible that the deletion of the EP2 receptor may have uncovered stimulatory actions of other EP receptors, such as EP3, that might contribute to the apparent resistance of EP2-deficient cells to PGE2. However, no exaggerated effect of PGE2 was observed in the EP3-deficient cells, which would be expected if the EP3 receptor has a major effect to antagonize EP2 signaling in this circumstance. Thus, despite the presence of multiple EP isoforms in T cells, the Gs-linked EP2 receptor alone seems to mediate the inhibition of antigen-specific proliferation by PGE2. This finding is consistent with previous work showing that many of the inhibitory actions of PGE2 upon T cells can be reproduced by cAMP or maneuvers that increase intracellular cAMP concentration (15, 29, 32). Although EP4 receptors are also expressed by murine T cells and these receptors also couple to adenyl cyclase, our studies indicate that EP4 receptors do not directly modulate antigen-stimulated proliferation of T cells. At this point, our findings are most clearly relevant to murine systems. It is possible that there may be differences in expression and functions of EP receptors in human leukocytes.

While recognition of antigens by T cells initiates and drives cellular immune responses, macrophages also play several critical roles. They can efficiently present peptide antigens to the T cell in the context of MHC class II proteins and provide additional costimulatory signals that are required for T cell activation. In addition, macrophages produce a variety of cytokines and other mediators that shape the T cell response. PGE2 has the capacity to inhibit or suppress many of these macrophage functions. For example, PGE2 inhibits expression of MHC class II proteins (8) and thus may interfere with antigen-presenting functions of the macrophage. Cytokine production is likewise inhibited by PGE2 (6, 7). Our studies have demonstrated that the actions of PGE2 to suppress cytokine production by macrophages are mediated almost exclusively by the EP4 receptor; PGE2 has no effect on LPS-stimulated cytokine release in EP4-deficient macrophages. In MLR, enhanced production of cytokines such as TNF-α and IL-12 contributes to the vigor of the proliferative response (33, 34), and therefore suppression of monokine production by PGE2 might contribute to the overall actions of PGE2 to reduce proliferation. The modest resistance of EP4-deficient mixed splenocytes to PGE2 may be explained by a failure to inhibit TNF-α and/or IL-12 production by macrophages. If so, the apparent contribution of this pathway to the overall actions of PGE2 in the MLR is relatively small. However, in other circumstances where monokine production has a more critical role, the inhibitory actions of EP4 receptors may have more profound consequences. Furthermore, IL-12 production by antigen-presenting cells drives CD4+ T cell differentiation toward the Th1 phenotype (35). Accordingly, inhibition of IL-12 production by PGE2 may contribute to its propensity to promote Th2 responses.

Along with regulation of cytokine release, the reconstitution MLR experiments suggest other actions of PGE2 in the macrophage that modulate cellular immunity. In these studies (Figure 5), the addition of macrophages to the cultures of EP2-deficient T cells partially restored the inhibitory actions of PGE2 on alloantigen-induced proliferation. These findings are most consistent with active suppression and suggest that PGE2 may induce a factor in macrophages that inhibits the MLR. As the magnitude of suppression was less with EP2-deficient compared with wild-type macrophages, the EP2 receptor seems to contribute to this activity. Because of the mixed genetic background of the EP4-deficient mice, an analogous reconstitution experiment to determine the contribution of the EP4 receptor could not be carried out. Nonetheless, our findings suggest that the macrophage plays a complex role in mediating inhibitory actions of PGE2 in cellular immunity. Furthermore, in the macrophage as in the T cell, EP2 and
EP4 receptors have divergent functions despite their similar intracellular signaling pathways. A mechanism to explain this apparent compartmentalization of signal-effector coupling remains to be defined.

Our studies have identified distinct actions of different EP receptor isoforms to regulate cellular immune responses. These receptors, EP2 and EP4, therefore represent potential targets for immunomodulatory therapies. Although the immunosuppressive actions of PGE2 are well recognized, problematic side effects related to its broad biological activities along with difficult pharmacokinetics have made it impractical to use as a therapeutic agent. Identification of small molecules that interact specifically with immunoregulatory EP receptor isoforms may allow a wider, more practical exploitation of these pathways in the therapy of autoimmune diseases and transplant rejection. The distinct separation of functions of these receptors within individual cells provides a potential mechanism for modulating immune responses with relatively fine specificity affecting a range of immunological functions.

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