PGE2 induced in and released by dying cells functions as an inhibitory DAMP

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Cellular components released into the external milieu as a result of cell death and sensed by the body are generally termed damage-associated molecular patterns (DAMPs). Although DAMPs are conventionally thought to be protective to the host by evoking inflammatory responses important for immunity and wound repair, there is the prevailing notion that dysregulated release of DAMPs can also underlie or exacerbate disease development. However, the critical issue for how resultant DAMP-mediated responses are regulated has heretofore not been fully addressed. In the present study, we identify prostaglandin E2 (PGE2) as a DAMP that negatively regulates immune responses. We show that the production of PGE2 is augmented under cell death-inducing conditions via the transcriptional induction of the cyclooxygenase 2 (COX2) gene and that cell-released PGE2 suppresses the expression of genes associated with inflammation, thereby limiting the cell's immunostimulatory activities. Consistent with this, inhibition of the PGE2 synthesis pathway potentiates the inflammation induced by dying cells. We also provide *in vivo* evidence for a protective role of PGE2 released upon acetaminophen-induced liver injury as well as a pathogenic role for PGE2 during tumor cell growth. Our study places this classically known lipid mediator in an unprecedented context—that is, an inhibitory DAMP vis-à-vis activating DAMPs, which may have translational implications for designing more effective therapeutic regimens for inflammation-associated diseases.

cell death | PGE2 | DAMP | inflammation | tumor regulation

Inflammation is a complex biological response and integral aspect of the immune system for the protection of host tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. However, inflammation can be a two-edged sword. Dysregulated and excessive inflammatory responses are linked to the initiation and/or exacerbation of a variety of diseases (1, 2). A multitude of stimuli that can evoke an inflammatory response have been identified. Whether external or internal in origin, these have been categorized into either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), respectively (3–5). Thus, bacteria-derived lipopolysaccharide (LPS) and virus-derived nucleic acids typically represent PAMPs, which essentially function under infectious conditions (3–5). On the other hand, self-derived high-mobility group box 1 (HMGB1), heat-shock proteins (HSPs), adenosine triphosphate (ATP), and uric acid crystal are among the well-known DAMPs, which are endogenous danger signals capable of triggering inflammatory responses under noninfectious or sterile conditions (5, 6).

DAMPs, each of which usually carries a well-defined intracellular function, are released or exposed following an injury or a variety of stresses that typically induce cell death, and they elicit signals via innate receptors such as Toll-like receptors (TLRs) and distinct classes of cytosolic receptors for the evocation of inflammatory responses (5, 7). Thus, a fine-tuning of the inflammatory responses is essential to accomplish the ideal outcome of inflammation in promoting wound healing and tissue repairs. Of note, the study on DAMPs has gained much attention in the context of inflammation-associated diseases. Accumulating

evidence indicates that DAMPs, continuously released by dying cells, account for persistence of inflammation with structural and functional alterations of tissues that are collectively referred to as chronic inflammatory processes and that such DAMP-mediated inflammation may underlie the development or exacerbation of numerous, seemingly unrelated diseases such as cancer, autoimmunity, and neurodegenerative diseases (8, 9). As such, an interesting issue raised is whether DAMP(s) can also bring to bear inhibition of inflammatory responses.

In the present study, we initially identified in supernatants of dying cells an inhibitory molecule of inflammatory responses. Further characterization, purification, and mass spectrometry analysis revealed the molecule to be prostaglandin E2 (PGE2), a classically known lipid mediator (10). PGE2 is an eicosanoid lipid mediator that can exert various effects including cell proliferation, stem cell expansion, and immunosuppression (11). PGE2 is the main product of two cyclooxygenases, COX-1 and COX-2, of which COX-1 is constitutively expressed and COX-2 is induced by various stimuli such as cytokines and various PAMPs (12, 13). PGE2 is the most abundant eicosanoid lipid in the inflammatory environment, and its receptors EP2 and EP4 and their downstream signaling to cAMP are proposed for the suppression of inflammation, although the precise mechanism is unclear (10, 12, 14–16). COX-2 is also overexpressed in various forms of cancer, and PGE2 produced by COX-2 has been implicated in the exacerbation of cancer (11, 13). Nevertheless, PGE2 released from dying cells and its physiological relevance have not been described previously.

Significance

Inflammation is an integral part of the immune responses for the protection of the host to harmful stimuli. However, because inflammation can also underlie the initiation and/or exacerbation of a variety of diseases, it must be tightly regulated. Upon cell death, self-derived molecules, called damage-associated molecular patterns (DAMPs), are released to activate inflammatory responses. It has been unknown whether inhibitory molecules are also released to balance the magnitude of the inflammatory responses. In this study, biochemical and *in vivo* data demonstrate that prostaglandin E2 is a DAMP that negatively regulates cell death-induced inflammatory responses. These findings reveal an unprecedented facet in the regulation of inflammation via the combined activity of activating and inhibiting DAMPs, which may have clinical implications.

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We show that PGE₂ is induced and released in various forms of cell death and serves as an immunosuppressive DAMP in the context of sterile inflammation and tumor growth in vivo. On the basis of our results, we propose that PGE₂ functions as an inhibitory DAMP (iDAMP) vis-à-vis activating DAMPs (aDAMPs). We discuss the significance of these findings in the regulation of inflammatory responses and translational implications for inflammation-associated diseases.

Results

Inhibition of LPS-Induced Inflammatory Cytokine Production by Dead Cells. We first examined the potential immunomodulatory activity of a dead cell-derived supernatant on macrophage cell line RAW 264.7. Necrosis was induced in mouse Lewis lung cell carcinoma (3LL) cells or embryonic fibroblasts (MEFs) by a freeze-and-thaw method (17), and each of the cell supernatants was examined for its immunostimulatory activities and effect on LPS-stimulated induction of TNF- α in RAW 264.7 cells. As shown in Fig. 1A, necrotic cell supernatants did not strongly induce TNF- α production in this experimental setting. Interestingly, these supernatants inhibited LPS-induced TNF- α production in a dose-dependent manner, albeit with different magnitudes from one another (Fig. 1B). This suppression was also observed in vitro cultured dendritic cells (DCs) stimulated by LPS (Fig. S14). The suppressive effect was also observed with supernatants of the necrotic SL4 cells, a mouse colon carcinoma cell line, and HeLa cells, a human cervical cancer cell line (Fig. S1B).

The supernatant of the necrotic 3LL cells also suppressed LPS-induced mRNA expression for *Tnf* and *Ifnb1*, suggesting the transcriptional inhibition of these genes (Fig. S1C). One interpretation of these data is that necrotic cells release molecules that suppress activities of the dead cell-derived immunostimulatory DAMP molecules under these experimental settings (see the next section).

Characterization of the Immunosuppressive Molecule(s). To characterize further the nature of the immunosuppression, we first treated 3LL necrotic cell supernatants with DNase I or RNase A and observed no effect on the suppressive activity on the LPS-induced production of TNF- α in RAW 264.7 cells (Fig. 1C). Further, neither proteinase K nor trypsin treatment showed any effect on the suppression (Fig. 1D). Because these results indicate the immunosuppressive molecule is neither a nucleic acid nor a protein, we next extracted the lipid fraction from the necrotic supernatant by the Bligh–Dyer method. Interestingly, addition of the lipid cellular fraction reproduced the suppressive effect of the necrotic cell supernatant (Fig. 1E).

Lipid mediators typically have a much lower molecular weight than proteins do, and indeed, size exclusion chromatography revealed that the immunosuppressive activity is found in the less than 6 kDa molecular-weight fraction (Fig. S1D). We also found in these fractions abundant PGE₂ and 6-keto-PGF₁ α by mass spectrometry analysis (Fig. 1F) and PGE₂ by ELISA (Fig. S1E). We then observed that synthetic PGE₂, but not 6-keto-PGF₁ α , suppressed LPS-induced *Tnf* and *Ifnb1* mRNA expression (Fig. 1G and Fig. S1F), an observation consistent with previous reports (10, 12, 14). In addition, the suppressive effect of PGE₂ was also observed for the induction of *Ccl3* and *Cxcl10* mRNA (Fig. S1F). Because PGE₂ did not affect the viability of RAW 264.7 cells (Fig. S1G), the mRNA suppression observed here is likely due to PGE₂-mediated signaling (see Discussion).

Perhaps expectedly, PGE₂ was also found in the supernatants of various necrotic cells (Fig. S1H), and its levels were correlated with the magnitude of TNF- α suppression (Fig. S1B). We next asked whether PGE₂ is released by other forms of cell death. PGE₂ was massively released by peritoneal macrophages after stimulation by ATP and LPS, a combination that induces pyroptosis (Fig. S1I and ref. 18). Further, a similar observation was made when 3LL cells underwent apoptosis by the treatment of cisplatin or etoposide (Fig. S1J). These results suggest that the release of PGE₂ is a common feature of various forms of cell death.

Fig. 1. Suppression of LPS-induced cytokine expression in the presence of a supernatant of dead cells or PGE₂. (A) RAW cells (5×10^4 cells) were treated either with an increasing volume of the supernatant of necrotic 3LL cells or MEFs (the volume of the supernatant is equivalent to 5×10^4 , 3×10^5 , or 1.5×10^6 cells) or with LPS (20 ng/mL) for 24 h. TNF- α protein levels in the cultured media were determined by ELISA. (B) RAW cells were stimulated with LPS and cocultured with the same volumes of supernatant as described in A to examine their combined effect on TNF- α production. (C) TNF- α protein levels determined by ELISA in cultures of RAW cells stimulated with LPS and necrotic supernatant from 3LL cells (equivalent to 3×10^5 cells) following treatment with DNase I or RNase A for 24 h. (D) RAW cells (1.5×10^5 cells) were stimulated with LPS (5 ng/mL) with or without a necrotic supernatant from 3LL cells (9×10^5 cells) pretreated with proteinase K or trypsin. After 2 h of stimulation, *Tnf* mRNA levels were determined by quantitative RT-PCR (qRT-PCR). (E) As described in D except that the lipid-extracted fraction from the necrotic supernatant of 3LL cells (9×10^5 cells) was added to LPS-stimulated cells in lieu of protease treatment. (F) Lipids in the fractionated supernatant (nos. 17–21) in Fig. S1D were analyzed by mass spectrometry. The quantity of each lipid was expressed as a proportion to a corresponding standard sample. (G) RAW cells were stimulated with LPS in the absence (PBS) or presence of PGE₂ (50 nM or 500 nM) for 2 h or in the presence of 6-keto-PGF₁ α . The induction levels for *Tnf* mRNA were then determined. NC; necrotic cell supernatant.

Of the two cyclooxygenase enzymes that catalyze the synthesis of PGE₂, COX-2 is selectively induced under inflammatory conditions (12, 13). We then questioned whether the *Ptgs2* gene is induced under cell death conditions to promote PGE₂ release by dead cells. Interestingly, *Ptgs2* mRNA expression levels were significantly increased in 3LL cells during freeze-thaw treatment (Fig. S1K), suggesting that PGE₂ released by dead cells is, at least in part, a consequence of *Ptgs2* gene induction during the process of cell death. We also examined whether the dead cell's release of PGE₂ involves multidrug resistance-associated protein 4 (MRP4) (19) by treating 3LL cells with the MRP4 inhibitor

Ceefourin 1. However, Ceefourin 1 did not significantly affect PGE2 release from dead 3LL cells (Fig. S1L), supporting the notion that the release is due to a passive leakage as a DAMP during cell death.

Immunosuppressive Function of PGE2 in Innate Immune Signaling. We asked whether PGE2 suppresses inflammatory responses evoked by other pattern recognition receptor (PRR) ligands by stimulating myeloid cells with various ligands for TLRs and cytosolic sensors. We found that PGE2 suppressed *Tnf* mRNA induced by poly I:C (via TLR3), 5'pppRNA (via RLR), or B-DNA (via cGAS)-treated peritoneal macrophages (Fig. S2A). Further, PGE2 also suppressed *Ifnb1* mRNA induced by poly I:C (Fig. S2B) in these cells and the induction of *Tnf* and *Ifnb1* mRNA by CpG-A ODN (via TLR9) or R837 (via TLR7) stimulation of plasmacytoid DCs (Fig. S2C and D). On the other hand, unlike the case for LPS stimulation, PGE2 did not suppress the induction of *Ifnb1* mRNA by 5'pppRNA or B-DNA (Fig. S2B). These results collectively suggest that PGE2 suppresses *Tnf* gene expression induced upon the stimulation of TLRs or cytosolic sensors, whereas the suppression of the *Ifnb1* gene by PGE2 occurs for TLR stimulation but not for the stimulation of cytosolic sensors.

The observations showing that dead cell-derived PGE2 inhibits the LPS-mediated induction of *Tnf* and *Ifnb1* mRNA also prompted us to study the underlying mechanism, which has been poorly understood (12, 14–16). Because the induction of TNF- α by LPS requires activation of NF- κ B and MAPK pathways (3, 4), we next examined the effect of PGE2 on the LPS-mediated activation of canonical NF- κ B and MAPK in RAW 264.7 cells. As shown in Fig. 2A, PGE2 treatment did not affect LPS-mediated phosphorylation levels of I κ B- α , ERK, and JNK. PGE2 treatment did not affect the transcriptional activation of NF- κ B, as monitored by an NF- κ B-driven luciferase reporter assay (Fig. 2B) and by ChIP assay for the binding of p65 to the *Tnf* promoter (Fig. 2C). These results therefore suggest that PGE2 may suppress *Tnf* mRNA induction by yet unknown mechanism (see Fig. 2D).

We also examined the effect of PGE2 on the LPS-induced activation of IRF3 by monitoring its phosphorylation, a hallmark of IRF3 activation and essential for the induction of the *Ifnb1* gene in RAW 264.7 cells (3, 4, 20). As shown in Fig. 2A, IRF3 phosphorylation remained low in the presence of PGE2. The data indicate that PGE2 interferes with the LPS–TLR4–TRIF signaling pathway (3, 4) that activates IRF3 for *Ifnb1* gene induction. Interestingly, when the cells were treated by cycloheximide (CHX) before LPS stimulation, the *Tnf* and *Ifnb1* mRNA induction levels remained essentially unaffected by PGE2 (Fig. 2D and E). These results suggest that the cytokine gene suppression by PGE2 is contingent on de novo synthesis of a protein, the identification of which will require further investigation. It also remains to be examined whether similar inhibitory mechanisms are also operative for other PRR signaling pathways.

Enhanced Immunostimulatory Activities of Necrotic Cells by Depletion of PGE2. A prevailing notion during cell death is that multiple DAMPs are released and activate inflammatory responses (5, 7, 8). However, under our experimental settings, which are essentially identical to those used by others, immune responses are not robust (Fig. 1A and ref. 21). Thus, we envisaged the following scenario: DAMPs that have the potential to evoke inflammatory responses are suppressed by the induction and release of PGE2 by dying cells. To test this concept experimentally, we asked whether inhibition of PGE2 production would convert the necrotic cells to more potent cells in the evocation of inflammatory responses.

We first pretreated 3LL cells with indomethacin, an inhibitor of COX-1 and COX-2 enzymes, and then examined the immunostimulatory activity triggered by the supernatant of indomethacin-treated necrotic cells. PGE2 release was expectedly suppressed in the supernatant of necrosis-induced 3LL cells by the indomethacin treatment (Fig. S3), and concomitantly, the induction of *Tnf* mRNA in peritoneal macrophages was greater

Fig. 2. Effect of PGE2 on the LPS-induced signaling pathways. (A) RAW cells (5×10^6 cells) were stimulated with LPS (20 ng/mL) in combination with PGE2 (500 nM). Whole-cell lysates were subjected to immunoblot analysis. (B) RAW cells (1×10^5 cells) transiently transfected with an NF- κ B luciferase reporter construct were stimulated with LPS (5 ng/mL) with or without PGE2 (50 nM or 500 nM). Luciferase activity was measured and expressed as units relative to the unstimulated control. (C) RAW cells (1×10^7 cells) were stimulated with LPS (5 ng/mL) with or without PGE2 (50 nM) for 1 h, and the cell lysates were subjected to ChIP assay using an anti-p65 antibody and primers amplifying the NF- κ B sites within the promoter of the *Tnf* gene. (D) RAW 264.7 cells (1×10^5 cells) were pretreated with CHX (20 μ g/mL) for 1 h and then stimulated with LPS in the presence or absence of PGE2 for 2 h. Then, the induction levels of *Tnf* mRNA were determined by qRT-PCR. (E) Same as D except that the induction levels of *Ifnb1* mRNA were examined.

compared with untreated cells (Fig. 3A). Similar observations were made with treated extracts from other cell lines (Fig. 3B and C). These data support the view that PGE2 functions as an iDAMP that counteracts the inflammatory responses evoked by aDAMPs.

To obtain a more comprehensive view of the interplay between aDAMP(s) and PGE2, we performed a microarray analysis in which peritoneal macrophages were stimulated with supernatants of mock-treated or indomethacin-treated SL4 necrotic cells. Interestingly, we found that genes that possess antitumor properties were up-regulated by indomethacin-treated supernatants, compared with PBS and mock-treated supernatants (Fig. 3D). For example, *Ccl2* and *Clec5a*, along with *Tnf*, are markers of M1-type macrophages, which are known to be tumoricidal (22, 23), and *Fpr1* is critical to the efficient induction of antitumor immunity in DCs (24). Furthermore, several tumor suppressor genes, such as *Grhl1* and *Mir133b*, are up-regulated by indomethacin-treated supernatants (25). These results suggest that PGE2, functioning as an iDAMP, might modulate macrophage's function and suppress the antitumor effects invoked by aDAMPs (see the next section).

Role of PGE2 as an iDAMP in Vivo. The above observations prompted us to examine the in vivo relevance of PGE2 as an iDAMP in two experimental systems: acetaminophen-induced hepatotoxicity and tumor cell growth. Peritoneal injection of acetaminophen induces massive necrosis of hepatocytes and evokes TNF- α production mediated by aDAMPs (26). Because PGE2 is produced by COX-2 in the liver (27), we hypothesized that this necrotic liver cell-derived PGE2 would function as iDAMP to counteract the activity of

Fig. 3. Enhancement of immunostimulatory activity of necrotic cells by the inhibition of PGE₂ synthesis. (A) Peritoneal macrophages (1.5×10^5 cells) were stimulated with a necrotic supernatant (9×10^5 cells) of 3LL cells pretreated by indomethacin (Indo.) or without treatment (DMSO). After 2 h of stimulation, the *Tnf* mRNA levels were determined by qRT-PCR. (B) Same as A except that SL4 cells were used for the necrotic supernatant. (C) Same as A except that MEFs were used for the necrotic supernatant. (D) Peritoneal macrophages were stimulated with a necrotic supernatant as described in A. After 2 h of stimulation, total RNA was extracted and subjected to microarray analysis. A heat map is depicted in the Left panel. Representative genes whose mRNA expression levels are augmented by indomethacin treatment and may be involved in the regulation of oncogenesis are shown in the Right panel.

aDAMPs by suppressing inflammatory responses. We therefore examined the effect of celecoxib, an inhibitor of COX-2 that is clinically used to treat pain or inflammation. As shown in Fig. 4A, administration of celecoxib resulted in elevated levels of the serum TNF- α in this liver necrosis model. Expectedly, this was accompanied by elevated levels of alanine transaminase (ALT) and aspartate transaminase (AST), the hallmarks of liver damage (Fig. S4A). Thus, these results support the view that PGE₂ functions as iDAMP to suppress the aDAMP-mediated liver inflammation to cause liver damage.

Necrosis is a well-known feature that commonly occurs during in vivo tumor growth (28). Typically, because newly formed blood vessels for tumors are aberrant and have poor blood flow, tumors become hypoxic and many tumor cells undergo cell death by necrosis (29). As such, hypoxia is an event intrinsic to tumor growth, wherein cancer cells undergo genetic and adaptive changes to survive in the hypoxic environment, thereby acquiring a more malignant phenotype (29). In this context, tumor cells often show elevated COX-2 expression (30, 31), suggesting the possibility that the COX-2-mediated production and release of PGE₂ by hypoxia-induced necrotic tumor cells may serve as an iDAMP, thereby affecting the growth of live tumor cells in the in vivo tumor microenvironment. To experimentally address this issue, we first examined PGE₂ production by hypoxia-induced necrosis of SL4 cells in vitro and found that these cells produce PGE₂ at a level about sevenfold higher than that of the cells grown in normoxic conditions (Fig. 4B).

We then created COX-2-deficient SL4 cells by a CRISPR/Cas9-mediated gene deletion system (Fig. S4B). Two independently obtained clones lacking the functional *Ptgs2* gene, designated

as SL4-Cox2d1 and SL4-Cox2d2, were grown in vitro, along with a control clone, SL4-Cox2i, in which the *Ptgs2* gene remained intact, and the parental SL4 cells. As expected, PGE₂ production levels in the supernatant of the hypoxia-treated SL4-Cox2d1 and SL4-Cox2d2 cells were more than fivefold lower than those from the SL4-Cox2i and parental cells (Fig. S4C). Perhaps not surprisingly, these cells all showed similar growth rates in vitro (Fig. 4C). These cells were then s.c. injected into C57BL/6 mice, and their growth was examined. COX-2-deficient SL4-Cox2d1 and SL4-Cox2d2 cells showed marked growth retardation compared with SL4-Cox2i cells (Fig. 4D), which showed a growth rate similar to the parental SL4 cells (Fig. S4D). Further, a similar trend was also found in the lung metastasis model (Fig. 4E). To gain insights into these observations, we isolated immune cells in the tumor-surrounding area of these mice and subjected these cells to flow cytometry analysis. We found no overt differences in terms of the population of T cells, B cells, natural killer (NK) cells, macrophages, neutrophils, and DCs (Fig. S4E). Of note, however, we found that the CD206-expressing macrophages, consistent with an M2 phenotype, are more abundant in the parental SL4 tumor-bearing mice compared with those mice bearing the mutant SL4 cells lacking COX-2 (Fig. 4F).

These results are consistent with the widely accepted notion that PGE₂ skews macrophages toward M2-type macrophages that promote tumor growth in the tumor microenvironment (32). The results also support the scenario that PGE₂ release under hypoxic conditions in vivo is a newly identified mechanism of tumor immune evasion, wherein dying tumor cells assist the growth of surviving tumor cells by modifying the tumor microenvironment. As a means to verify this view, we also examined the effect of dying cells on macrophage polarization by the following in vitro assay. Bone marrow-derived cells were first cultured by M-CSF to generate M0-type macrophages, and subsequently, these cells were further cultured in the presence of either LPS + IFN- γ or IL-4, a well-known protocol for M1 or M2 macrophage polarization, respectively, to examine the effect of dead cell-derived PGE₂ on macrophage polarization. Interestingly, we found that the necrotic supernatant of SL4 cells potentiated M2 polarization, whereas that of indomethacin-treated SL4 cells had the opposite effect (Fig. S4F and Discussion).

Discussion

In recent years, DAMPs have garnered much attention for their role as endogenous danger signals that alert the innate immune system and evoke inflammatory responses typically caused by cell death due to tissue injury or stress (5, 7, 8). Thus, DAMPs are important for maintaining homeostasis of the host; however, excessive and/or sustained DAMP-induced signaling may underlie the development and/or exacerbation of many diseases such as autoimmunity and cancer (8, 33). Given this potential for harm, negative regulatory mechanisms of DAMP-mediated inflammation have undoubtedly evolved. For example, aDAMPs were reported to recruit CD24-Siglec G/10 and Siglec G/10-associated phosphatases, such as SHP1, to repress TLR-mediated or Nod-like receptor-mediated signaling (34). In the present study, however, we show that PGE₂ released upon cell death intrinsically functions to negatively regulate inflammatory responses activated by DAMPs.

Of the numerous DAMPs identified to date, including HMGB1, HSPs, and the products of purine metabolism (e.g., ATP and uric acid), all function as activators of inflammation (5, 6). That PGE₂, when released by dead cells, inhibits inflammation identifies it as a previously unidentified class, which we refer to as iDAMP vis-à-vis immune aDAMPs. Consistent with this role, the inhibition of the PGE₂ synthesis pathway in various cell types renders them more potent to evoke inflammatory responses upon cell death in vitro (Fig. 3 A–C). Further, evidence that PGE₂ released by dead cells protects against acetaminophen-induced hepatotoxicity and enhances tumor cell growth in vivo also lends support to our hypothesis (Fig. 4 A, D, and E).

To our knowledge, this study reveals the first example of the interplay between aDAMPs and iDAMPs in the regulation of

Fig. 4. Potential role of PGE2 as an iDAMP in vivo. (A) C57BL/6 mice were injected with acetaminophen intraperitoneally with or without simultaneous oral administration of celecoxib ($n = 4$ each). After 24 h, serum TNF- α protein levels were determined. (B) SL4 cells (1×10^6 cells) were cultured under hypoxic (1% O₂) or normoxic condition (20% O₂) for 24 h, and then PGE2 levels were determined. At this time point, about 90% of the cells underwent cell death in the hypoxic condition, whereas more than 90% of the cells were alive under the normoxic condition. (C) Cell numbers of parental SL4 cells, SL4-COX2i, SL4-COX2d1, and SL4-COX2d2 grown in vitro. Notably, PGE2 did not affect the viability of SL4 cells at our experimental setting. (D) SL4-COX2i, SL4-COX2d1, or SL4-COX2d2 cells (1×10^6 cells) were transplanted into C57BL/6 mice ($n = 6$ each) by s.c. injection, and the tumor volume was measured. Asterisks show statistical significance ($P < 0.05$) between SL4-COX2i and SL4-COX2d clones. (E) SL4-COX2i or SL4-COX2d1 cells (2×10^5 cells) were injected into C57BL/6 mice intravenously. After 17 d of injection, lungs were subjected to H&E staining. Original magnification, 40 $\times$. (F) SL4-COX2i or SL4-COX2d1 cells (1×10^6 cells) were transplanted into C57BL/6 mice by s.c. injection ($n = 6$ each). After 12 d of transplantation, single-cell suspensions were prepared from the transplanted tumors and subjected to flow cytometry analysis. Expression of CD206 on CD45⁺CD11b⁺F4/80⁺ tumor-infiltrating macrophages (Left) and mean fluorescent intensity (MFI) of CD206 (Right) are shown. Asterisks, $P < 0.01$. APAP, acetaminophen.

inflammatory responses. The significance of this is that although $\sim 10^5$ human cells in our body undergo cell death every second, the vast majority are believed to be silent with respect to immune activation due to their removal by phagocytosis and others (35). Our data, which illustrate the enhanced immunostimulatory activity in the absence of PGE2 that otherwise functions as an iDAMP, may be related to the typical immune-incompetent nature of dying cells. Our observations made in acetaminophen-induced liver inflammation support this view (Fig. 4A). Also consistent with the evidence, mice defective in PGE2 synthesis develop severe peritonitis (36), although a firm connection between the PGE2's function as iDAMP and these inflammatory disorders requires formal demonstration. In the clinic, acetaminophen is often used in the treatment of inflammatory diseases. However, in light of our present findings, new caution may be needed for the use of this and other COX inhibitors.

This study also raises the question of which PGE2 receptor is responsible for the function of PGE2 as a dead cell-derived iDAMP. In this context, there are previous reports showing that PGE2 suppresses LPS-induced gene expression via EP2 and EP4, which are expressed on macrophages (10, 12, 14). As such, we think it is most likely that the immunosuppressive function of PGE2 as an iDAMP is also mediated by these receptors, although we cannot rigorously exclude the involvement of other receptors. How does PGE2 signaling inhibit inflammatory responses? That PGE2 can suppress LPS-induced inflammatory cytokine and type I IFN secretion is known, however the mechanism of these suppressive effects has been controversial (12, 14). One study showed suppression of the NF- κ B pathway by cAMP, a downstream effector molecule of PGE2 signaling, whereas another argued that the NF- κ B pathway is unaffected by cAMP signaling (15, 16). We found no evidence that the NF- κ B pathway is affected by PGE2 signaling (Fig. 2A–C), but de novo protein synthesis is required for the suppression of TNF- α production by PGE2 (Fig. 2D). The suppression of *Ifnb1* gene induction by PGE2 may also involve a similar mechanism (Fig. 2E). Obviously, identification of a target molecule, presumably induced by PGE2 signaling, that mediated direct suppression of these pathways may be an interesting course of research.

An additional ramification of our study is the hitherto unknown facet of PGE2 as an iDAMP in dying tumor cells for promoting growth of live tumor cells. There is emerging recognition that immune system evasion is as a hallmark of cancer progression. PGE2 synthesis and signaling pathways were shown to favor tumor growth in vivo (11, 13, 37). In this context, tumor cell death and release of iDAMPs, due either to hypoxia, loss of cell-cycle control, and so forth, would be predicted to alter the tumor microenvironment to favor immune suppression and tumor survival. Our findings on the reduced growth in vivo of SL4 tumor cells lacking the *Ptgs2* gene offer yet an unidentified mechanism by which tumor cells favor their growth and survival (Fig. 4 *D* and *E*). One may argue that tumor cell death is beneficial for surviving tumor cells as a kind of “altruistic suicide” by the release of PGE2 as an iDAMP. Thus, our present findings may offer an unprecedented rationale for clinical trials evaluating COX-2 inhibitors as chemoprevention and therapeutic agents (38, 39).

There may be multiple mechanisms that underlie the promotion of tumor cell growth by dead cell-derived PGE2. One is our findings that dead tumor cell-derived PGE2 suppresses the gene induction of cytokines critical for coping with tumor growth (Fig. S1C and refs. 40, 41), which would prevent direct attack by these cytokines. SL4 cells, for instance, are known to undergo apoptosis in the presence of TNF- α (42). Another mechanism triggered by PGE2 and supported by our data is a conditioning of the tumor micro-environment in which macrophages are skewed toward a protumor M2 phenotype (43). Conversely, a microarray analysis showed the induction of several genes by an indomethacin-treated necrotic cell supernatant, genes such as M1 macrophage signature genes and tumor suppressor genes that have antitumor effects (Fig. 3D and refs. 24, 25). Although further study is required, such genes would contribute to the suppression of tumor growth. Also, our data do

not exclude additional mechanisms of regulation, which may warrant further investigation.

In conclusion, we revealed that PGE₂ is an iDAMP counteracting the function of aDAMPs. Our findings suggest that PGE₂ may be a promising target, particularly in instances where massive cell death is associated. Further, given that many elements of the hypoxia-response pathway are candidates for therapeutic intervention, our results reveal an unprecedented facet of PGE₂ interference—further support for the possible use of COX inhibitors for cancer therapy (38, 39). It remains to be seen whether additional iDAMPs exist. Given our data showing the enhanced immunostimulatory activity of indomethacin-treated cells, this system may be useful in identifying the nature of aDAMPs.

Materials and Methods

Induction of necrosis was performed as described previously (17, 21). For the preparation of a PGE₂-depleted necrotic supernatant, cells were incubated

with indomethacin (10 μ M) overnight and subjected to a freeze-and-thaw method in the presence of indomethacin (10 μ M). For induction of apoptosis, cells were treated with etoposide (50 μ M) or cisplatin (50 μ M). Peritoneal macrophages were primed with LPS (1 μ g/mL) for 4 h and treated by ATP (5 mM) for 1 h to induce pyroptosis. All animal experiments were done in accordance with guidelines of The University of Tokyo and were approved by the animal research committee of The University of Tokyo.

Additional information can be found in [SI Materials and Methods](#).

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