Apoptotic Cells, through Transforming Growth Factor-β, Coordinate Induce Anti-inflammatory and Suppress Pro-inflammatory Eicosanoid and NO Synthesis in Murine Macrophages*

Celio G. Freire-de-Lima1,2, Yi Qun Xiao3,4, Syhra J. Gardai3, Donna L. Bratton5, William P. Schiemann5, and Peter M. Henson1,3

From the 1Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro RJ 21944-970, Brazil and 2Program in Cell Biology, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206

Apoptotic cells are rapidly engulfed by adjacent tissue cells or macrophages before they can release pro-inflammatory/pro-immunogenic intracellular contents. In addition, recognition of the apoptotic cells is actively anti-inflammatory and anti-immunogenic with generation of anti-inflammatory mediators such as transforming growth factor-β (TGF-β) and anti-inflammatory eicosanoids. Here, we have investigated the role played by the induction of TGF-β in the coordinate expression of anti-inflammatory eicosanoids or peroxisome proliferator-activated receptor-γ and in the suppression of pro-inflammatory lipid mediators and nitric oxide (NO). By use of a dominant negative TGFβIII receptor, TGF-β signaling was blocked, and its participation in the consequences of apoptotic cell stimulation was determined. The induction of TGF-β itself could be attributed to exposed phosphatidylserine on the apoptotic cells, which therefore appears to drive the balanced inflammatory mediator responses. Arachidonic acid release, COX-2, and prostaglandin synthase expression were shown to be significantly dependent on the TGF-β production. On the other hand, a requirement for TGF-β was also shown in the inhibition of thromboxane synthase and thromboxanes, of 5-lipoxygenase and sulfidopeptide leukotrienes, as well as of inducible nitric-oxide synthase and NO. TGF-β-dependent induction of arginase was also found and would further limit the NO generation. Finally, apoptotic cells stimulated production of 15-lipoxygenase and 15-hydroxyeicosatetraenoic acid, a potentially anti-inflammatory pathway acting through peroxisome proliferator-activated receptor-γ, and lipoxin A4 production, which were also up-regulated by a TGF-β-dependent pathway in this system. These results strongly suggest that the apoptotic cell inhibition of pro-inflammatory mediator production is pleiotropic and significantly dependent on the stimulation of TGF-β production.

Recognition and clearance of apoptotic cells by phagocytes play pivotal roles in development, maintenance of tissue homeostasis, control of the immune response, and resolution of inflammation (1, 2). Apoptotic cells are removed by professional phagocytes, members of the mononuclear phagocyte system such as macrophages and immature dendritic cells, or by nonprofessional phagocytes such as fibroblasts, endothelial, epithelial, smooth muscle, or stromal cells (3). Uptake of the apoptotic cell is by a specialized and highly conserved form of phagocytosis termed efferocytosis (4, 5). As a cell becomes apoptotic, it is generally removed in situ by near-neighbor cells or macrophages in a quiet, almost invisible fashion, i.e. the process does not induce a local tissue reaction. In fact, recognition and removal of apoptotic cells are normally both anti-inflammatory and anti-immunogenic (6–9).

The interaction and recognition are triggered by surface changes on the apoptotic cells. Two widely distributed surface ligands on apoptotic cells are phosphatidylserine (PS)4 (10, 11) and calreticulin (5), which become associated in patches together on the cell surface. Indirect effects of the collectin family of molecules or direct action of calreticulin leads to stimulation of low density lipoprotein receptor-related protein (LRP) on the phagocytosing cell (5, 12). However, LRP activation seems to induce production of pro-inflammatory mediators (13). On the other hand, there is considerable evidence to implicate PS as the main stimulus for the anti-inflammatory or anti-immunogenic effects (6–8, 14–16). We suspect that these two stimuli, acting through different signaling pathways, are balanced, with a normal bias toward the anti-inflammatory. Unfortunately, the receptor(s) that recognizes PS (PS recognition structures, PSRS) that is responsible for this effect is unknown, although it does seem to distinguish between stereoisomeric forms of the phosphoserine head group (10, 14) and does seem to react with an activating IgM antibody mAb217 (17) whose binding is blocked by PS. The antibody binds to and

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1 Both authors contributed equally to this work.

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3 To whom correspondence should be addressed: Program in Cell Biology, Dept. of Pediatrics, National Jewish Medical and Research Center, 1400 Jackson St., Denver, CO 80206. E-mail: hensonp@njc.org.

4 The abbreviations used are: PS, phosphatidylserine; PSRS, phosphatidylserine recognition structures; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; LPS, lipopolysaccharide; IFN-γ, interferon-γ; COX, cyclooxygenase; PG, prostaglandin; NO, nitric oxide; LO, lipoxygenase; PPARγ, peroxisome proliferator-activated receptor-γ; iNOS, inducible nitric-oxide synthase; LXA4, lipoxin A4; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco’s modified Eagle’s medium; LRP, low density lipoprotein receptor-related protein.
activates cells and mimics exactly the effects of PS on apoptotic cells in contributing to uptake and on the generation of anti-inflammatory mediators (7, 18), and it has been used here along with apoptotic cells to stimulate macrophages for production or suppression of eicosanoids.

A major anti-inflammatory mediator induced in response to apoptotic cells, mAb217, or PS liposomes is TGF-β (6, 8, 16). Blockade of TGF-β has been shown to reverse the suppressive effects of apoptotic cells or PS in vivo on either inflammation or adaptive immunity (7, 8). On the other hand, earlier studies also suggested induction of other candidates such as IL-10 (19), PGE₂ (6), and even platelet-activating factor (6), although the last two can have both pro- or anti-inflammatory effects. The ability of apoptotic cell recognition to alter the production of eicosanoids had first been noted for thromboxane (20, 21) and its ability of apoptotic cell recognition to alter the production of prostaglandins in the apoptotic cell enhancement of Trypanosoma cruzi growth in macrophages (22). Therefore, a key issue is whether apoptotic cell-induced TGF-β, acting in an autocrine/paracrine fashion, mediates the alterations in eicosanoid generation. By use of a dominant negative TGF-β receptor construct, we have been able to show that apoptotic cells stimulate via their induction of active TGF-β, a coordinate production of generally anti-inflammatory and simultaneous inhibition of generally pro-inflammatory, eicosanoids. The effect is mediated by effects on the synthases for these mediators. Additional coordinate effects were seen on related proteins, including iNOS, which was down-regulated, and PPARγ or arginase, which were induced, i.e. combining to reduce NO production and also potentially in keeping with the anti-inflammatory balance.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—TGF-β was purchased from R&D Systems. Lipopolysaccharide (LPS, Escherichia coli 0111:B4) was from List Biological Laboratories, Inc. Recombinant murine interferon-γ was from BD Biosciences. Human factor Va was from Hematologic Technologies Inc. Cyclosporin A and protease inhibitor mixture set I were from Calbiochem. Anti-arginase 1 antibody was from Santa Cruz Biotechnology. Antibodies against COX-2, human PGD-2 synthase, murine PGE-1 synthase, 1, PGI synthase, thromboxane synthase, 5-LO, 15-LO II, PPARγ1, iNOS, and indomethacin were from Cayman Chemical. Anti-β-actin antibody was from Cell Signaling. mAb217 is an IgM monoclonal antibody that was originally raised against PS-recognizing macrophages. It was obtained from concentrated hybridoma supernatants. Unfortunately, attempts to label, fragment, or convert the antibody to an IgG isotype have universally resulted in loss of activity. [5,6,8,9,11,12,14,15,16-H]Arachidonic acid (200 Ci/mol) was from American Radiolabeled Chemical. Lipofectamine Plus reagent was from Invitrogen.

**Induction of Apoptotic Cells**—Jurkat T cells were exposed to UV irradiation at 254 nm for 10 min. Jurkat T cells were cultured in RPMI 1640 with 10% fetal calf serum (Gemini Bio-Products) for 3 h at 37 °C in 5% CO₂. The cells were generally ≥80% apoptotic by nuclear morphology and maintained intact cell membranes that excluded trypan blue (6).

**Cell Culture, Stimulation and Measurement of Pro-inflammatory Mediators by ELISA**—Murine peritoneal macrophages were obtained from BALB/c mice 4 days after intraperitoneal injection of 1 ml of thioglycollate. Murine RAW 264 cells were obtained from the American Type Culture Collection. RAW-V and RAW-TβRII were made by stable transfection of empty vector and truncated TGF-β receptor II constructs, respectively. The cells (1 × 10⁶ cells/well) were plated in each well of a 24-well tissue culture plate and were cultured in DMEM supplemented with 10% heat-inactivated endotoxin-free fetal bovine serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin under a humidified 5% CO₂ atmosphere at 37 °C for 24 h. Then the cells were cultured in serum-free DMEM in the absence or presence of LPS, IFN-γ, factor Va, or cyclosporin A for 18 h with 3 × 10⁶ apoptotic Jurkat cells (apoJ), viable Jurkat cells (ViableJ), 50 μg/ml mAb217, 50 μg/ml control isotype IgM, or 100 μM liposomes (containing 30:70 molar ratios of P5:PC or PC alone). The supernatants were collected and measured for TGF-β, TNF-α, PGE₂, PGF1α, 15-HETE, LXA₄, and leukotrienes by ELISA according to the manufacturer’s instructions (ELISA TECH, Aurora, CO). In some experiments, to inhibit pro-inflammatory mediators, the macrophages were treated with stimuli cited before and 100 ng/ml LPS or LPS plus 40 units/ml IFN-γ.

**NO Production Assay**—NO levels produced by RAW 264 cells, RAW-TβRII cells, and murine peritoneal macrophages were measured by reducing the nitrate accumulated over 18 h to nitrite with nitrate reductase (23) and measuring the nitrite concentration by the method of Green et al. (24). The nitrite concentrations were quantified by using a double three-point standard curve of NaNO₂ concentrations (in a linear range between 1 and 80 μM).

**Measurement of Arachidonate Release**—Arachidonic acid release was measured as described (25). Briefly, the cells were cultured in 24-well plates to incorporate arachidonate by incubation for 24 h in 1 ml of DMEM containing 10% fetal bovine serum and [³H]arachidonic acid (1 μCi/ml) for 24 h. Then the cells were washed and incubated in serum-free DMEM containing 0.1% human serum albumin and stimulated with LPS or apol) or ViableJ or mAb217 or isotype control IgM. The medium was removed 2 h after stimulation and centrifuged at 500 × g for 10 min, and the amount of radioactivity (arachidonate and arachidonate products) in the supernatant was determined.

**Transient Cell Transfection and Reporter Gene Assay**—The p3TP-luc (26) luciferase reporter gene construct was transfected into RAW-TβRII and RAW-V cells using Lipofectamine Plus reagent according to the manufacturer’s instructions. pSV-β-galactosidase vector (Promega) was co-transfected as an internal control to measure differences in transfection efficiency. Luciferase and β-galactosidase activities were measured 18 h after TGF-β stimulation using the luciferase assay system (Promega) and Galacto-Light (Tropix), respectively.

**Immunoblotting Analysis**—Immunoblotting analysis was carried out as described previously with some modification (27). Briefly, cells (3 × 10⁶ cells/well) were plated in each well of a 12-well tissue culture plate and incubated overnight. Following stimulation, the cells were lysed in lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 0.5% Triton
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**RESULTS**

**Apoptotic Cells or Antibody to PSRS on Murine Macrophages Stimulate Production of TGF-β and Concomitant Blockade of LPS-induced TNF-α, NO, and iNOS—Murine macrophages (peritoneal or RAW 264, 1.0 × 10^6 cells/ml) were stimulated with LPS (100 ng/ml) as positive control or mAb217 (50 μg/ml) or apoptotic Jurkat T cells (3 × 10^6 cells/ml) for 18 h. These stimuli each induced TGF-β production in both types of macrophages (Fig. 1A). Isotype control IgM or viable Jurkat T cells were inactive. These data are in accordance with previous reports (6, 16). The stimulation of TGF-β production by apoptotic cells in this system was blocked by preincubation of the targets with the PS-binding protein factor Va (Fig. 1B) as had been shown earlier with annexin V (29). PS liposomes also stimulated the production of TGF-β but less efficiently. This is probably because the presentation to the PSRS from PS exposed on the apoptotic cell is from a quite different environment compared with a lymphocyte. However, PS liposomes increased the production of TGF-β in the presence of LPS or cyclosporin A (Fig. 1C). These findings suggest that PS liposomes themselves may up-regulate TGF-β translation when TGF-β message has been induced by other stimuli (17, 30). Classically activated macrophages (LPS and IFN-γ stimulation) exhibit release of TNF-α and NO as well as up-regulation of iNOS. As shown in Fig. 1, D and E, these three responses to stimulation with LPS and IFN-γ were inhibited by exposure of the macrophages to apoptotic cells or mAb217. Previous studies implicated the TGF-β produced, in the suppression of TNF-α induction, and might be expected to serve the same role for suppression of iNOS and NO.

**Statistical Analysis**—All data are presented as means ± S.E. from three or more separate experiments. The means were analyzed using analysis of variance for multiple comparisons. When analysis of variance indicated significance, the Tukey-Kramer honestly significant difference test for all pairs was used to compare groups. All data were analyzed using JMP statistical software (version 5; SAS Institute) for the Macintosh computer.
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To demonstrate this presumed requirement for TGF-β in the suppression, a dominant negative form of the TGFβRII was employed. Transfection of RAW 264 cells with this construct was shown to block the ability of TGF-β to signal for 3xPPT-luc reporter (which contains three consecutive 12-O-tetradecanoyl phorbol-13-acetate-response elements and a portion of the plasmogen activator inhibitor (PAI-1) promoter region) gene activation (Fig. 2A). Because TGF-β can induce its own synthesis, the effect of the dominant negative receptor was also examined on the production of TGF-β itself after stimulation with apoptotic cells, mAb217, or LPS. As shown in Fig. 2B, this treatment blocked 60–70% of the TGF-β produced by each of the stimuli, i.e. supporting an additional autocrine/paracrine effect of TGF-β on its own induction in these systems.

Transfection of the truncated TGFβRII was found to completely reverse the suppression of TNF-α and NO production caused by apoptotic cells or PSRS stimulation and also restored the up-regulation of the iNOS protein (Fig. 2, C–E). In keeping with the suppression of NO production by blocking up-regulation of iNOS, exposure of macrophages to apoptotic cells or PSRS stimuli also led to increases in intracellular levels of arginase 1 (Fig. 2F), which could further reduce the production of nitric oxide.

Macrophages with Truncated TGFβRII Are Defective in Prostaglandin Production and Prostaglandin Synthase Expression in Response to Stimulation with Apoptotic Cells or LPS—In the original studies of anti-inflammatory effects of apoptotic cells, PGE₂ was also shown to be generated, and it too seemed to play a role in suppression of inflammatory mediators (27). Accordingly, we next examined the effect of apoptotic cells and stimulation with mAb217 on induction of potentially anti-inflammatory prostaglandins as well as the role of TGF-β in their regulation. The original studies did not address the probable induction of PGI₂ (detected as PGI₂α) along with PGE₂, and accordingly, this was included in the analysis. Supernatants from the cell culture were collected 18 h after stimulation and analyzed for PGE₂ and PGI₂α. The cell lysates were collected, and the levels of synthases for PGE₂ (PGES₁), PGD₂, and PGI₂ were determined by Western blotting. As expected, the two stimuli induced production of PGE₂ and PGI₂α, starting at 2 h earlier and extending out to 18 h of incubation (Fig. 3, A–C). Importantly, they also increased the intracellular levels of the prostaglandin synthases (Fig. 3D).

It has been reported previously that TGF-β can induce prostaglandin production (22, 31, 32), and in data not shown, the direct addition of active TGF-β to the macrophage cultures did stimulate production of PGE₂ and PGI₂α. When the macrophage response to TGF-β was blocked with the dominant negative receptor, induction of PGE₂ and PGI₂α by either apoptotic cells or mAb217 was prevented (Fig. 3, A and B). Interestingly, LPS-induced PGE₂ and PGI₂α were also reduced by about 80%. We suspect that the lack of complete blockade with this stimulus reflects the possible use of alternative pathways not involving TGF-β. In keeping with the data on the prostaglandin themselves, the truncated receptor also reduced up-regulation of the synthases (Fig. 3D), although not to as great an extent as seen for the secreted prostaglandins.

These prostaglandins are lipid mediators that like TGF-β have been reported to have pro- or anti-inflammatory properties in different circumstances. For example, we earlier showed PGE₂ to decrease TNF-α production from macrophages (6). A
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FIGURE 3. Prostaglandin production and prostaglandin synthase expression in response to apoptotic cells or mAb217 stimulation is TGF-β-dependent. A and B, RAW-V or RAW-TβRII cells were incubated in the presence of LPS, mAb217, isotype IgM, apoJ, or ViableJ for 18 h. PGE₂ or PGF₁α concentrations in the conditioned medium were analyzed by ELISA. *, significantly different from RAW-V. C, PGE₂ and PGF₁α time course. RAW cells were incubated in the presence of the stimuli, and the supernatant was collected after 30 min and 2, 4, 8, and 18 h. D, PGE-1, human PGD and PGI synthase protein levels in the total cell lysate were analyzed by Western blot. E, RAW cells were stimulated with mAb217, isotype IgM, apoJ, or ViableJ in the presence or absence of indomethacin (indo) for 18 h. TGF-β concentration was analyzed by ELISA.

possible contributory effect of prostaglandins to themselves to TGF-β production is depicted in Fig. 3E, wherein indomethacin was shown to be able to reduce the amount of TGF-β produced in response to apoptotic cells and mAb217 (see also Ref. 6). This, along with the autostimulation of TGF-β by TGF-β (see above), further shows the extensive feedback responses inherent in these systems.

TGF-β-dependent Suppression of Thromboxane Synthesis—Early studies of responses to apoptotic cells showed that thromboxane production was decreased (6, 20, 21). This suggests coordinate up-regulation of potentially anti-inflammatory prostaglandins along with down-regulation of pro-inflammatory thromboxane and raises the question of whether TGF-β is responsible for both effects. Because macrophages do not produce TXA₂ spontaneously, and did not do so at any time after incubation with apoptotic cells or mAb217 alone (data not shown), they were activated with LPS to demonstrate the suppressive effect of co-stimulation with either apoptotic cells or mAb217 (Fig. 4A). Almost complete inhibition of TXA₂ (measured as thromboxane B₂) was seen, and transfection of the macrophages with the dominant negative TGF-β receptor reversed this inhibition. Because LPS also induces TGF-β production, enhancement of the LPS effect on thromboxane production might have been seen if this TGF-β was blocked. However, this was not observed, probably because the generation of active TGF-β following LPS occurred after the majority of the thromboxane had already been produced. Once again, the effect of the TGF-β appeared to be at the level of the synthase. Thromboxane synthase levels in the macrophages were suppressed by apoptotic cells and mAb217, but not after transfection of the truncated TGF-β receptor (Fig. 4B).

Stimulation of Macrophages with mAb217 or Apoptotic Cells Induced COX-2 Expression and Arachidonic Acid Release through TGF-β-dependent Signaling—The demonstrated reciprocal effect of TGF-β on the prostaglandin synthases raised the possibility that the induction of PGE₂ and PGI₂ reflected a diversion of the precursor PGH₂ from utilization by thromboxane synthase. However, because the prostaglandins were increased directly in macrophages that did not express the thromboxane synthase, it seemed likely that the apoptotic cells also led to increased levels of one or other PGH synthases (COX enzymes) and thereby increased production of PGH₂. No evidence was found for altered amounts of COX-1 (data not shown), but both apoptotic cells and mAb217, as well as LPS as expected, did increase the intracellular amounts of COX-2 (Fig. 5A). In keeping with the theme of this study, macrophages expressing the truncated TGF-β receptor did not show up-regulation of COX-2 in response to the apoptotic cells and markedly reduced that stimulated by LPS.

To initiate eicosanoid production, a source of free arachidonate must be available and, presumably, must be initiated by the apoptotic cell or mAb217 stimulus. This is shown in Fig. 5B where free [³H]arachidonate (including its metabolites) was measured in the supernatant after prior incorporation into macrophage phospholipids before stimulation. In this case, blocking the TGF-β effects with the dominant negative receptor reduced the amounts of arachidonate released by about 50% in the case of all three stimuli. This presumably reflects either a timing issue as noted for the LPS-induced thromboxane or the availability of preformed phospholipases that were stimulated by either the LPS or PSRS engagement in addition to an effect from TGF-β. TGF-β has been reported to induce prostaglandin...
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production (22, 31, 32), which would necessitate its ability to initiate released arachidonic acid, an effect that was shown here in Fig. 5B.

**Acting through TGF-β, Apoptotic Cells or mAb217 Decreased LPS-enhanced 5-Lipoxygenase and Leukotrienes but Increased 15-LO, 15-HETE, and LXA₄ as Well as PPARγ—** The pattern of TGF-β induction of potentially anti-inflammatory eicosanoids, but the suppression of pro-inflammatory eicosanoids, was further explored by examining the effects on lipoxygenases and leukotrienes. Our earlier study (6) had shown suppression of leukotriene release from macrophages by apoptotic cells. Stimulation of macrophages with apoptotic cells or mAb217 by themselves did not induce cysteinyl leukotrienes at 18 h and suppressed that induced by priming with LPS (Fig. 6A). However, a time course study (Fig. 6B) did show an early production at 2 h but none subsequently. Similarly, LTB₄ was seen only 2 h after stimulation, after this time the production was down-regulated (data not shown). Examination of the key upstream enzyme 5-lipoxygenase revealed, as expected, that its levels were increased after priming with LPS but that this did not occur in the presence of the apoptotic cells or mAb217 (Fig. 6C). In the presence of the truncated TGF-β receptor, the LPS-primed increase in levels of 5-LO was no longer prevented (Fig. 6C). We attribute the early induction of leukotrienes to an effect of endogenous 5-LO acting on early released arachidonate before the TGF-β effect has time to kick in.

By contrast, when 15-LO was examined, the apoptotic cell stimulus directly increased intracellular levels of the enzyme and production of 15-HETE (Fig. 6, D–F). The 15-HETE production was seen 4–8 h after stimulation and peaked at 18 h (Fig. 6F). Once again, the induction by apoptotic cells or mAb217 was blocked in the presence of the truncated TGF-β receptor. On the other hand, TGF-β did not appear to play a significant role in the induction of 15-LO or 15-HETE by LPS. Products of 15-LO have been suggested to participate in LXA₄ production and activation of PPARγ (33, 34), which may also have anti-inflammatory effects in macrophages (35–37). Accordingly, we also examined the effect of apoptotic cells and mAb217 to induce LXA₄ and alter the levels of PPARγ. As shown in Fig. 7, A and B, both stimuli initiated LXA₄ production and increased the amounts of PPARγ protein in the cells, as did direct addition of TGF-β. This supports a possible additional anti-inflammatory effect of apoptotic cells via LXA₄ and/or activation of PPARγ.

**DISCUSSION**

Apoptotic cells are known to induce an anti-inflammatory and anti-immunogenic response, mediated in part by their induction of active TGF-β in responding cells. Here we show that the effect of the apoptotic cells is to drive a complex coordinated inhibition of potentially inflammatory mediators along with induction of potentially anti-inflammatory molecules in macrophages that are orchestrated by the production of TGF-β. By studying the responses induced by apoptotic cells in macrophages that are unresponsive to TGF-β by virtue of their transfection with a dominant negative TGF-β receptor, the role of this important mediator on a wide variety of eicosanoids, nitric oxide, and related molecules was delineated. Thus, the earlier demonstrations that apoptotic cells induced the production of PGE₂, but suppressed thromboxane (6, 20) were both shown to be due to the effects of TGF-β, and these observations extended to other potentially pro- and anti-inflammatory arachidonate metabolites. It should be noted that the prostanoids PGE₂, PGI₂, and PGD₂ are known to exhibit both pro- and anti-inflammatory actions, in part for PGE₂, depending upon the receptors that are engaged (38). Similar pleomorphic effects should be noted for NO. In our earlier study (6), we showed that PGE₂ suppressed LPS-induced inflammatory mediators from macrophages (6), and in the data reported here, prostanoid pro-
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FIGURE 6. Apoptotic cell or mAb217 stimulation decreases LPS-induced 5-lipoxygenase and leukotrienes but increases 15-lipoxygenase and 15-HETE through TGF-β. A, RAW 264 macrophages were pre-incubated with apoJ or mAb217 for 30 min and then stimulated with or without LPS for 18 h. Leukotriene production in the conditioned medium was analyzed by ELISA. *, significantly different from LPS alone. B, time course of leukotriene production. C, RAW-V or RAW-TJRIII cells were incubated with mAb217, isotype IgM, apoJ, or ViableJ for 30 min and then with LPS for 18 h. 5-LO levels in the total cell lysate were analyzed by Western blot. D–F, RAW-V or RAW-TJRIII cells were incubated with the stimuli or TGF-β (C, 10 ng/ml) for 18 h. D, 15-LO production in the conditioned medium was analyzed by ELISA. *, significantly different from RAW-V cells.

FIGURE 7. Apoptotic cells or mAb217 increase LXA₄ production and PPARγ. A, RAW-V or RAW-TJRIII cells were incubated with stimuli or TGF-β for 18 h, and PPARγ levels in the total cell lysate were analyzed by Western blot. B, RAW 264 macrophages were incubated with the stimuli or TGF-β for 18 h. LXA₄ concentration was measured in supernatant by ELISA. *, significantly different from RAW-V.

production also showed a feedback enhancement of TGF-β production. Thus, in this limited context, we see these prostanoids as potentially anti-inflammatory, and their induction as contributing to the overall anti-inflammatory consequences of apoptotic cell recognition.

The observations required the demonstration of TGF-β production in response to the apoptotic cells, shown earlier by numerous investigators and confirmed herein. A number of ligands have been demonstrated on apoptotic cells that can interact with a number of "receptors" on responding cells, in this case macrophages. Additionally there are a large group of "bridge" molecules (39) that can link the apoptotic cell ligands to the receptors. We have suggested that two important ligands are phosphatidylserine and calreticulin. The latter, as well as the collectin family of bridge molecules (40), has been suggested to interact with LRP as a receptor and, in isolation, seems to induce a more pro-inflammatory response (5, 13). On the other hand, PS and its receptors and possibly some or all of its bridge molecules appear to induce the anti-inflammatory effects and, in most cases, to act in a dominant fashion in the normal response to apoptotic cells. Necrotic cells are usually thought to be pro-inflammatory (9, 15) and may have reversed this PS-driven dominance. Other studies that have suggested that apoptotic cells can in some circumstances act in a pro-inflammatory fashion may also reflect variations in balance between pro-inflammatory (e.g. LRP) versus anti-inflammatory (e.g. PS-driven) responses.

In the studies reported here, we show that the TGF-β induction by apoptotic cells was dependent on exposed PS by blockade with the PS-binding protein factor Va, which has the advantage over the more usually employed annexin V by binding in physiologic concentrations of calcium. Although direct stimulation of TGF-β by PS-containing liposomes was weak, these agents readily enhanced TGF-β production to other stimuli. The amounts of TGF-β measured by ELISA only represents that in the supernatant and is therefore relatively insensitive for a molecule that binds to surfaces and cell membranes. Early in vivo demonstrations of PS liposome effects were also relatively weak in comparison with apoptotic cells (8) and, we suspect, may reflect issues of presentation and avidity.

The other approach employed here was to examine the effect of mAb217, an IgM-activating antibody that is suspected of binding to an as yet unidentified receptor for PS on responding cells. Its ability to bind all cells that respond to PS-exposing apoptotic cells, to be blocked itself by pretreatment of the cells with l- but not d-phosphatidylserine, and its close mimicking of the activation induced by PS-exposing apoptotic cells supports this contention. In all cases in this study, mAb217 and apoptotic cells behaved identically. The implication is that recognition of PS drives the production of TGF-β and the downstream anti-inflammatory responses reported herein.

The eicosanoids are derived from metabolism of arachidonic acid through a number of pathways. In this study we focused on prostanoid synthesis through PGH synthases (COX) and downstream prostaglandin and thromboxane synthases as well
as through lipoygenases and their downstream products of leukotrienes and HETES. Apoptotic cell and TGF-β induction of PGE₂ and PGI₂ were shown. The effect appeared to be the up-regulation of the respective synthases PGES and PGIS whose protein levels were increased in response to the apoptotic cells and blocked in the presence of the dominant negative TGF-β receptor. Likewise, PGD synthase protein levels were up-regulated by the apoptotic cells and mAb217 but not in the absence of TGF-β effects. By contrast, the pro-inflammatory eicosanoid thromboxane was suppressed. In this case the resting macrophages or those stimulated with apoptotic cells alone did not produce thromboxane or exhibit significant levels of its synthase. When stimulated to induce thromboxane synthase by LPS or LPS and IFN-γ, however, the apoptotic cells or mAb217 suppressed the up-regulation and the mediator production, again in a TGF-β-dependent fashion.

These effects on prostanoid balance implied an up-regulation of both COX and of sources of substrate, namely arachidonic acid, and both increases in COX2 (but not COX1) and released arachidonate were demonstrated in response to apoptotic cells. The effect on COX2 was also shown to be dependent on TGF-β (41, 42) and at least part of the arachidonate release. TGF-β itself also induced liberation of arachidonate. Although not explored directly herein, it seems reasonable to assume that the apoptotic cells and/or TGF-β induce activation of existing phospholipases 2 and that the TGF-β may also initiate some up-regulation of these that contributes to greater and/or more prolonged release of the arachidonate.

Our earlier study had suggested that apoptotic cells also suppressed the production of potentially pro-inflammatory sulfidopeptide leukotrienes (6). Here we show that levels of the upstream enzyme 5-lipoxygenase, as well as of the leukotrienes themselves, were also suppressed by the apoptotic cells and that this too was reversed in the absence of TGF-β signaling. The decrease in 5-lipoxygenase levels noted here are at odds with studies showing enhanced production in response to TGF-β during macrophage maturation under the influence of vitamin D₃ (43). We therefore suspect a discordant effect of TGF-β on maturing versus mature macrophages. Intriguingly, and in keeping with the coordinated anti-inflammatory effects, 15-lipoxygenase was up-regulated, and its stable product 15-HETE was increased by the apoptotic cells or mAb217, again because of TGF-β. Products of 15-HPETE are generally thought to be anti-inflammatory (44–47), and some may possibly achieve some up-regulation of these that contributes to greater and/or more prolonged release of the arachidonate.

The other pathway and mediator addressed in this study was the potentially immunoregulatory and broad spectrum signaling molecule NO. Apoptotic cells and mAb217 suppressed the generation of NO and the up-regulation of iNOS. TGF-β is well known to inhibit production of iNOS (48–50); therefore, the TGF-β dependence of this was expected and shown by the dominant receptor approach. Intriguingly, arginase, which reduces the substrate for NOS enzymes, was reciprocally up-regulated by the apoptotic cells, again via TGF-β. This would provide an additional brake on the NO generation by macrophages responding to apoptotic cells.

The results indicate a complex effect of apoptotic cells acting through release of TGF-β to up-regulate generally anti-inflammatory mediators and inhibit the production of pro-inflammatory molecules. The systems employed here support an effect on synthesis of the various enzymes involved in generating these mediators, i.e. acting on transcription, translation, or both. However, we cannot exclude additional effects on metabolism or secretion of the mediators acting in the shorter term. In response to apoptotic cells, macrophages appear to release preformed TGF-β almost immediately (30–60 min (8, 16)) followed by new synthesis and more prolonged generation of the active molecule. Combined, this would achieve a fairly rapid and then prolonged effect on the inflammatory state of the cell. However, it should be noted that the immediate generation of free arachidonate in response to apoptotic cells might lead to generation of potentially pro-inflammatory eicosanoids by enzymatic pathways constitutively present in the cell (e.g. 5-LO and leukotriene synthases) before the slower effects of TGF-β had a chance to kick in (see for example Refs. 51–54). Given the pluripotentiality and plasticity of this cell type, we are generally skeptical of the concept of stable macrophage “phenotypes” (3). Nevertheless, it should also be noted that some of the effects of apoptotic cell exposure mimic those seen in the so-called alternatively activated macrophage (55, 56), thus raising intriguing questions regarding how long these effects persist following the interaction with apoptotic cells and/or the consequences of prolonged exposure as might be experienced in resolving inflammatory response in vivo.

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