Tumor Cell Apoptosis Polarizes Macrophages—Role of Sphingosine-1-Phosphate

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Macrophage polarization contributes to a number of human pathologies. This is exemplified for tumor-associated macrophages (TAMs), which display a polarized M2 phenotype, closely associated with promotion of angiogenesis and suppression of innate immune responses. We present evidence that induction of apoptosis in tumor cells and subsequent recognition of apoptotic debris by macrophages participates in the macrophage phenotype shift. During coculture of human primary macrophages with human breast cancer carcinoma cells (MCF-7) the latter ones were killed, while macrophages acquired an alternatively activated phenotype. This was characterized by decreased tumor necrosis factor (TNF)- α and interleukin (IL) 12-p70 production, but increased formation of IL-8 and -10. Alternative macrophage activation required tumor cell death because a coculture with apoptosis-resistant colon carcinoma cells (RKO) or Bcl-2-overexpressing MCF-7 cells failed to induce phenotype alterations. Interestingly, phenotype alterations were achieved with conditioned media from apoptotic tumor cells, arguing for a soluble factor. Knockdown of sphingosine kinase (Sphk) 2, but not Sphk1, to attenuate S1P formation in MCF-7 cells, restored classical macrophage responses during coculture. Furthermore, macrophage polarization achieved by tumor cell apoptosis or substitution of authentic S1P suppressed nuclear factor (NF)- κ B signaling. These findings suggest that tumor cell apoptosis-derived S1P contributes to macrophage polarization.

INTRODUCTION

Macrophages participate in a number of (patho)physiological settings, due to high plasticity of their functional responses. Classical activation, achieved by microbial cell wall components or interferon (IFN)- γ triggers proinflammatory macrophage activation, characterized among other mediators, by the production of nitric oxide (NO), superoxide (O_2^-), tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and IL-6, thus resembling the M1 macrophage phenotype (Gordon, 2003). In contrast, activation toward the M2 phenotype can be elicited by stimuli such as glucocorticoids, IL-4, -13, or -10 (Mantovani *et al.*, 2002; Gordon, 2003).

In 1863 Virchow observed the presence of leukocytes in human tumors. Later on a link between cancer and chronic inflammation was suggested, and nowadays it seems accepted that macrophages are a major cell component infiltrating certain tumors (Mantovani *et al.*, 2002). High numbers of tumor-associated macrophages (TAMs) often predict a poor survival prognosis for patients with solid human tumors, such as breast, prostate, ovarian, and cervical cancers (Bingle *et al.*, 2002; Lewis and Pollard, 2006). Moreover, the presence of TAMs is often correlated with tumor cell survival. Tumor growth-promoting activities of TAMs are connected to alternative activation, because TAMs display a polarized M2 phenotype (Mantovani *et al.*, 2002, 2004a). In

contrast to M1-activated macrophages, TAMs show a reduced capacity to produce, e.g., TNF- α or NO. TAMs not only support tumor survival and growth, but also contribute to metastasis, tumor angiogenesis, and immune evasion (Mantovani *et al.*, 2004a; Pollard, 2004; Lewis and Pollard, 2006). Therefore, it is desirable to understand mechanisms of macrophage polarization toward the TAM phenotype because maneuvers to reprogram a M2 macrophage toward a M1 type may be beneficial (Sica *et al.*, 2006). TAM polarization seems to be affected by the tumor microenvironment, with the likely contribution of tumor-derived molecules such as IL-4, IL-10, transforming growth factor (TGF)- β , prostaglandin E_2 (PGE_2), and chemokines, as well as tumor hypoxia (Mantovani *et al.*, 2002, 2004b; Lewis and Pollard, 2006).

Some years ago, another concept of tumor-induced macrophage polarization was introduced. Administration of apoptotic tumor cells reduced macrophage cytotoxicity against vital tumor cells (Reiter *et al.*, 1999) and disrupting recognition of ACs by macrophages or dendritic cells in vivo induced tumor regression (Bondanza *et al.*, 2004). Interactions of macrophages with apoptotic cells (ACs) provokes alternative activation profiles, which might be critical for termination of inflammation and/or repair of tissue during wound healing (Savill *et al.*, 2002; Gregory and Devitt, 2004). ACs trigger the formation of IL-10, TGF- β , or PGE_2 from macrophages (Gregory and Devitt, 2004), but also release immunosuppressive molecules such as TGF- β or IL-10 themselves (Tomimori *et al.*, 2000; Chen *et al.*, 2001). Recently, we noticed the release of sphingosine-1-phosphate (S1P) from ACs (Weigert *et al.*, 2006), a sphingolipid known to be involved in tumor progression by promoting angiogenesis (LaMontagne *et al.*, 2006).

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Here we provide evidence for the release of S1P from apoptotic tumor cells as a modulator of macrophage polarization. In coculture, human monocyte-derived macrophages induced cell death of MCF-7 breast carcinoma cells, but not of apoptosis-deficient Bcl-2-overexpressing MCF-7 cells. Only apoptotic tumor cells decreased levels of TNF- α and elevated those of IL-8 in macrophages after lipopolysaccharide (LPS) administration. The impact of apoptotic tumor cells on the macrophage phenotype was dependent on S1P production in apoptotic MCF-7 cells because knockdown of Sphk2 abrogated the phenotype shift as well as S1P production in cocultures. Interestingly, apoptotic MCF-7 cells and authentic S1P reduced nuclear factor (NF)- κ B activation in macrophages in response to LPS. These findings suggest a role for tumor cell apoptosis-derived S1P in macrophage polarization toward a TAM-like phenotype.

MATERIALS AND METHODS

Cell Culture and Reagents

MCF-7 breast carcinoma cells, Colo 201 human colon adenocarcinoma cells, and Hep-G2 hepatocellular carcinoma cells were maintained in RPMI 1640. RKO colon carcinoma cells were kept in DMEM high glucose, A549 lung carcinoma cells in DMEM/Ham's F12, and Caco-2 colon adenocarcinoma in EMEM, each supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% heat-inactivated fetal calf serum (FCS). LPS was from Sigma-Aldrich (St. Louis, MO) and human recombinant IFN- γ was obtained from Roche (Indianapolis, IN).

Human Monocyte Isolation and Culture

Human monocytes were isolated as described (Von Knethen and Brune, 2001). In brief, monocytes were isolated from buffy coats (DRK-Blutspendendienst Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunhämatologie, Frankfurt am Main, Frankfurt, Germany) using Ficoll-Hypaque gradients (PAA Laboratories, Karlsruhe, Germany). Peripheral blood mononuclear cells were washed twice with phosphate-buffered saline (PBS) and were allowed to adhere to culture dishes (Primaria 3072, Becton Dickinson, Lincoln Park, NJ) for 1 h at 37°C. Nonadherent cells were removed. Monocytes were then differentiated into macrophages with RPMI 1640 containing 10% AB-positive human serum (PAA Laboratories) for 7 d.

Coculture Experiments

Primary human monocyte-derived macrophages were cultured at a density of 2×10^5 cells/ml. After differentiation, tumor cells were added at the same density, and cocultures were maintained for 5 d. Subsequently, residual tumor cells were removed from the plates by incubations with accutase (PAA Laboratories; Albee *et al.*, 2007) for 5 min, which left adherence of macrophages unaltered. Remaining macrophages were treated with 1 μ g/ml LPS and 100 U/ml IFN- γ or were exposed a second time to equal amounts of tumor cells. In some experiments higher ratios of tumor cells compared with macrophages were used, as indicated.

Quantification of Cell Death

Cocultures of human macrophages and tumor cells were harvested after 12, 24, or 48 h by incubation with accutase for 30 min, which removed both macrophages and tumor cells. Cell death was quantified by fluorescence-activated cell sorting (FACS) after incubations with a monoclonal human CD44-PE antibody (Ancell, Bayport, MN) to discriminate between macrophages and tumor cells, followed by the annexin V-fluorescein isothiocyanate (FITC) method (ImmunoTools, Friesoythe, Germany). In detail, quantification of macrophage versus tumor cell contents in cocultures was performed with FACS analysis using α -CD44-PE. The macrophage population was identified by staining control macrophages, thereby gating this population, which was used to identify macrophages in cocultures. All remaining cell counts were attributed to tumor cells.

Production of Conditioned Media from Apoptotic Tumor Cells

Conditioned media from apoptotic MCF-7 or RKO cells were produced as described previously (Weigert *et al.*, 2006). Briefly, MCF-7 cells, MCF-7 cells transfected with siRNA targeting SphK2, or RKO cells were exposed to 0.5 μ g/ml staurosporine (Sigma) for 4 h in case of MCF-7 cells and 5 h in case of RKO cells. Subsequently, cells were washed twice with PBS, followed by an 2-h incubation in full medium. Thereafter, these conditioned media were harvested by centrifugation ($13,000 \times g$, 5 min) and filtration through 0.2- μ m pore filters, to remove apoptotic bodies.

Quantification of Cytokines

FACS analysis using BD Cytometric Bead Array Flex Sets following the instructions provided by the manufacturer allowed quantification of cytokines (TNF- α , IL-10, IL-8, IL-12-p70) in the supernatant of cocultures. The samples were acquired with the FACSCanto (BD Biosciences, San Jose, CA) flow cytometer and analyzed with BD Biosciences' FCAP software. Performance matched the specifications of the manufacturer. Minimal detection limits were 1.9 pg/ml for TNF- α , 3.8 pg/ml for IL-10, 4.9 pg/ml for IL-8, and 4.8 pg/ml for IL-12-p70. Routinely, medium from coculture setups were changed every 24 h. Thus, cytokine production reflects a sampling period corresponding to the last 24 h of the coculture, only. If not stated otherwise, cocultures lasted for 5 d with medium changes every 24 h.

Western Blot Analysis

Western blot analysis was performed as described (von Knethen *et al.*, 2005). A mAb directed toward Bcl-2 (BD Transduction Laboratories, Lexington, KY) and polyclonal antibodies against Sphk1 or Sphk2 (Exalpha Biologicals, Woburn, MA) were used.

Electrophoretic Mobility Shift Assays

Nuclear extracts were prepared as described (Von Knethen and Brune, 2001). An established electrophoretic mobility shift assay (EMSA) method, with slight modifications, was used (Camandola *et al.*, 1996). Nuclear protein (20 μ g) was incubated for 30 min at room temperature with 2 μ g poly(dI-dC) from Amersham Biosciences (Freiburg, Germany), 2 μ l buffer D (20 mM HEPES/KOH, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, 0.5 mM PMSF, pH 7.9), 4 μ l buffer F (20% Ficoll-400, 100 mM HEPES/KOH, 300 mM KCl, 10 mM DTT, 0.5 mM PMSF, pH 7.9), and 250 fmol 5'-IRD700-labeled oligonucleotide (Metabion, Planegg-Martinsried, Germany) in a final volume of 20 μ l. Specific p65 and p50 supershift antibodies (2 μ g) were added as indicated. DNA-protein complexes were resolved at 200 V for 2 h using native 4% polyacrylamide gels and visualized with the Odyssey infrared imaging system (Li-Cor, Lincoln, Nebraska). Oligonucleotides with the consensus NF- κ B site (boldface letters) were used (Peng *et al.*, 1995): 5'-GCC AGT TGA GGG GAC TTT CCC AGG C-3'; 3'-C GGT CAA CTC CCC TGA AAG GGT CCG-5'. Supershift analysis was performed with α -p65 and α -p50 from Santa Cruz Biotechnology (Heidelberg, Germany). The latter antibody is known to attenuate the protein-DNA interaction rather than causing a classical supershift (He *et al.*, 2004).

Transfections

To overexpress Bcl-2, MCF-7 cells were transfected with the pRc/CMVbcl2 plasmid (Messmer *et al.*, 1996) using Nucleofector technology (Amara, Köln, Germany), according to the manufacturer's instructions. For knockdown of Sphk isoforms, Sphk1-specific small interfering RNA (siRNA) or Sphk2 pre-designed Hs_SPHK2_3 siRNA (Qiagen, Chatsworth, CA) were nucleofected into MCF-7 cells. Nucleofection efficiency was about 70%, as verified by flow cytometry after nucleofection of MCF-7 cells with pmxGFP (Amara; data not shown). Overexpression of Bcl-2 and Sphk1 or Sphk2 knockdown was controlled by Western blot analysis 48 h subsequent to nucleofection. Knockdown of Sphk isoforms was controlled by siCONTROL nontargeting Duplex 1 (Dharmacon Research, Boulder, CO). After knockdown of Sphk isoforms, MCF-7 cells were cultured for another 24 h and subsequently were added to macrophage cultures.

S1P Quantification in Cell Culture Supernatants

Quantification of S1P from cell culture supernatants with liquid chromatography tandem mass-spectrometry (LC-MS/MS) was performed as described previously (Schmidt *et al.*, 2006; Weigert *et al.*, 2006). After 24 h, medium in cocultures of human macrophages and MCF-7 cells was replaced by medium free of FCS. Cocultures were maintained for another 24 h, and supernatants were harvested and analyzed. The lower limit of quantification was found to be 0.5 ng/ml. Variations in accuracy and intraday and interday precision ($n = 6$ for each concentration) were $<10\%$.

Statistical Analysis

p values were calculated using the paired Student's *t* test combined with Bonferroni correction, as well as ANOVA. Differences were considered significant at $p < 0.05$, unless indicated otherwise.

RESULTS

MCF-7 Cells Induced an Anti-inflammatory Cytokine Profile in Cocultured Macrophages

In a first experimental approach, we asked whether or not a coculture of breast carcinoma cells (MCF-7) with human monocyte-derived macrophages affected the cytokine profile in the latter ones. Therefore, we incubated macrophages

Figure 1. Coculture of human macrophages with MCF-7 cells induced a cytokine shift. Supernatants of human monocyte-derived macrophages or MCF-7 cells or from cocultures of macrophages with MCF-7 cells were assayed at times indicated for TNF- α (A), IL-10 (B), or IL-8 (C). Quantification was by FACS with BD Cytometric Bead Array Flex Sets as described in *Material and Methods*. Data are presented as the mean $\pm$ SEM from four independent experiments. Differences between supernatants from control macrophages and cocultures marked with an asterisk are statistically significant ($p < 0.05$).

with an equal amount of MCF-7 cells for 5 d and quantified the contents of TNF- α , IL-10, and IL-8 in the coculture supernatants at different time points (Figure 1). The coculture provoked induction of TNF- α and IL-10 at 24 h, followed by a decrease from 48 to 120 h, which was markedly stronger in case of TNF- α (Figure 1A) compared with IL-10 (Figure 1B). In contrast, IL-8 production did not peak after 24 h, but increased steadily up to 120 h (Figure 1C). Cytokine production in either control macrophages or MCF-7 cells, cultured for 24 h was low. Interestingly, adding fresh MCF-7 cells for 24 h to the culture systems that already lasted for 120 h (second coculture) failed to induce TNF- α (Figure 1A), but elicited an increase in IL-10 (Figure 1B), whereas IL-8 production remained high (Figure 1C). We concluded that MCF-7 cells, cocultured with macrophages elicited a transient proinflammatory response followed by an early production of TNF- α , a more persistent formation of the anti-inflammatory cytokine IL-10 and a strong induction of IL-8.

To prove that a prolonged coculture setup provoked an alternative activation profile in macrophages, we stimulated macrophages with LPS/IFN- γ at the end of the 5-d coculture with MCF-7 cells compared with control macrophages (Figure 2A). Residual MCF-7 cells were removed by incubations with accutase for 5 min, which left adherence of macrophages unaltered, thus proving that this setup exclusively determines cytokine production from macrophages (Figure 2B). Activation of naive macrophages with LPS/IFN- γ stim-

Figure 2. Coculture with MCF-7 cells induced an alternative activation profile in human macrophages. (A and C) Human primary macrophage remained as controls or were incubated with MCF-7 cells for 5 d. Subsequently residual MCF-7 cells were removed from cocultures, and 1 μ g/ml LPS and 100 U IFN- γ were added to macrophages from cocultures or control macrophages for 6 h. TNF- α (A and C), IL-10 (A), IL-8 (A and C), and IL-12-p70 (A) contents in supernatants were quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from at least six independent experiments. Differences between supernatants from LPS/IFN- γ -treated macrophages and macrophages from cocultures marked with an asterisk are statistically significant ($p < 0.05$). (B) Macrophages and MCF-7 cells were cocultured for 12 h. Cocultures were harvested after incubation with accutase for 30 min, with or without accutase pretreatment for 5 min, followed by washing, subsequent staining with α -CD44-PE, and analysis by FACS. (D) Cocultures of human primary macrophages with MCF-7 cells were maintained for the times indicated. Cocultures, control macrophages or MCF-7 cells were incubated for 30 min with accutase, harvested, stained with α -CD44-PE as a discrimination marker between macrophages and MCF-7 cells, and analyzed by FACS or fluorescence microscopy. Top panels display phase-contrast and α -CD44-PE staining of macrophage/MCF-7 cocultures after 12 h. White arrows mark α -CD44 positive macrophages; black arrows mark MCF-7 cell colonies. FACS traces are representative for three independent experiments. The bottom graph shows quantification of FACS data. Statistically significant ($p < 0.05$) reduced cell numbers of MCF-7 cells in cocultures are marked with asterisks.

ulated the production of TNF- α and IL-10 but not of IL-8 compared with resting cells. Addition of LPS/IFN- γ to macrophages from the coculture setup produced significantly less TNF- α and revealed markedly increased levels of IL-8,

whereas IL-10 remained unchanged. In the past, decreased production of proinflammatory IL-12-p70 was connected to alternative macrophage activation and was attributed to increased IL-10 (Sica *et al.*, 2000). Because IL-10 levels in macrophages from cocultures were enhanced compared with naive macrophages even without LPS/IFN- γ stimulation (Figure 1B), we investigated the amount of IL-12-p70 in our system. Administration of LPS/IFN- γ strongly induced IL-12-p70 in control macrophages, but remained significantly lower when stimulating macrophages from the coculture setup (Figure 2A). These results suggest that MCF-7 cells provoked a macrophage phenotype shift toward an alternative activation profile. Activation of macrophages from cocultures with LPS/IFN- γ substantiated this phenotype switch, when following the production of classical pro-versus anti-inflammatory mediators. Although principal observations pointing to alternative macrophage activation during coculture with MCF-7 cells were obvious (Figure 1), stimulation of naive versus coculture-primed macrophages with LPS/IFN- γ made the phenotype shift more definite. Therefore, we used LPS/IFN- γ stimulation in further experiments to demonstrate macrophage phenotype alterations. Infiltrating macrophages may comprise up to 50% of the tumor cell mass (Murdoch *et al.*, 2004). Nevertheless, we asked whether or not higher cell numbers of tumor cells may change the phenotypic alterations that we observed. When we used ratios of 5:1 or 10:1 in cocultures, the decrease of TNF- α became more pronounced, with more tumor cells added to macrophages, whereas the increase in IL-8 was unaffected (Figure 2C).

Viability Changes of Cancer Cells during Coculture with Macrophages

To follow the ratio of macrophages to MCF-7 cells, cocultures were stained with anti-CD44. CD44 proved to be a valid discrimination marker between highly CD44-positive macrophages and weakly CD44-expressing tumor cells (Draffin *et al.*, 2004) in our system (Figure 2D). We noticed a continuous reduction of MCF-7 cells starting at 24 h after initiating the coculture system that resulted in a complete loss of tumor cells at 72 h (Figure 2D). To ensure that MCF-7 did not simply up-regulate CD44, we also monitored the morphology of cells in cocultures. Based on morphological criteria, MCF-7 cells were completely absent after 72 h (data not shown). Therefore, we suspected macrophage-induced AC death of cancer cells. To prove this assumption, cells from cocultures were harvested at 12, 24, and 48 h and stained with anti-CD44 to distinguish between macrophages and MCF-7 cells. In addition, annexin V staining quantified phosphatidylserine exposure as an early marker of cell death. Annexin V positive MCF-7 cells were noticed 24 h after coculture with macrophages. The amount of apoptotic MCF-7 cells increased to ~50% after 48 h, whereas no significant changes in annexin V binding were observed in macrophages from cocultures or control macrophages, i.e., cultured in the absence of MCF-7 cells (Figure 3A). Our working hypothesis predicted that only apoptotic tumor cells evoked phenotype changes in macrophages. In further experiments, we therefore used two cell lines that were resistant to cell death by cocultured macrophages. First, we used MCF-7 cells that overexpress Bcl-2 (Figure 3, B and D), and second, we used RKO cells, which turned out to be naturally resistant (Figure 3C). The reason for RKO cell survival in cocultures remained unknown. However, they expressed higher amounts of Bcl-2 than naive MCF-7 cells, which may account for apoptosis resistance (data not shown). A 48-h coculture of MCF-7-Bcl-2 or RKO cells with

Figure 3. Impact of human primary macrophages on the viability of different human cancer cell lines. Human primary macrophages remained as controls or were incubated with MCF-7 cells (A), Bcl-2-overexpressing MCF-7 cells (B), or RKO cells (C) for the times indicated. Cocultures and control macrophages were incubated for 30 min with accutase, harvested, stained with α -CD44-PE as a discrimination marker between macrophages and tumor cells, followed by annexin V-FITC staining as a marker for cell death, and analyzed by FACS. Data are presented as the mean $\pm$ SEM from three independent experiments. Differences between annexin V binding of macrophages and MCF-7 cells from coculture (cc) marked with an asterisk are statistically significant ($p < 0.05$). (D) Western analysis shows Bcl-2 expression of control MCF-7 cells and MCF-7 cells transfected with a plasmid encoding human Bcl-2 (MCF-7-Bcl-2). One of two representative experiments is displayed.

human macrophages showed no signs of cell death, either in tumor cells or in macrophages. Under these conditions, tumor cells and macrophages coexist, without initiation of cell death parameters. This is in contrast to the coculture of apoptosis sensitive MCF-7 cells exposed to macrophages.

Alternative Activation of Macrophages Demands Tumor Cell Apoptosis

The notion that Bcl-2-overexpressing MCF-7 cells (MCF-7-Bcl-2) and RKO do not enter the route of programmed cell death upon coculture with macrophages qualified these cells, compared with sensitive MCF-7 cells, to study LPS/IFN- γ -evoked cytokine responses after 5 d of coculture (Figure 4). Cytokine production of TNF- α , IL-10, and IL-8 in naive macrophages after LPS/IFN- γ addition was significantly increased compared with unstimulated controls. Activation of macrophages coming from cocultures with MCF-7-Bcl-2 or RKO cells revealed a cytokine profile resembling that of naive macrophages after classic stimulation with LPS/IFN- γ . In contrast, cytokine formation of macrophages

Figure 4. Activation profile of human macrophages after coculture with different human tumor cell lines. Human primary macrophages remained as controls or were incubated with MCF-7 cells, Bcl-2-overexpressing MCF-7 cells (MCF-7-Bcl-2) or RKO cells for 5 d. Residual tumor cells were removed from cocultures with 5-min accutase treatment. Afterward, 1 μ g/ml LPS and 100 U IFN- γ were added to macrophages from cocultures and to control macrophages for 6 h. TNF- α , IL-10, and IL-8 contents in supernatants were quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from five independent experiments. Statistically significant differences ($p < 0.05$) are marked with an asterisk.

derived from cocultures with apoptotic sensitive MCF-7 cells was different. TNF- α was lower, IL-8 was higher, and IL-10 remained unaltered. Apparently, MCF-7-Bcl-2 as well as RKO cells did not change the ability of macrophages to respond to LPS/IFN- γ with the production of these cytokines compared with naive cells. Only the coculture with MCF-7 cells, which underwent AC death upon coculture with macrophages, altered cytokine production after LPS/IFN- γ stimulation. To corroborate that tumor cell death indeed was essential for alternative macrophage polarization, we induced apoptosis in RKO and MCF-7 cells with staurosporine and collected conditioned medium from these apoptotic tumor cells, which then was added to macrophages for 24 h. After changing the medium, macrophages were stimulated with LPS/IFN- γ . Supernatants from apoptotic tumor cells alone induced significant changes in the macrophage cytokine profile, independent of direct tumor cell-macrophage contacts. Secretion of IL-10 and IL-8 was enhanced, whereas IL-12-p70 production was decreased compared with naive macrophages (Figure 5A). Supernatants of apoptotic RKO or MCF-7 cells per se did not contain

Figure 5. Activation profile of human macrophages induced by conditioned medium of apoptotic tumor cells and different tumor cell lines. (A) Human primary macrophages remained as controls or were incubated with conditioned medium from apoptotic MCF-7 (CM-MCF-7) or RKO (CM-RKO) cells for 24 h. Thereafter, supernatants were removed, cells were washed with PBS, fresh medium was added, and macrophages were stimulated with 1 μ g/ml LPS and 100 U IFN- γ for 6 h. Production of TNF- α , IL-10, IL-8, and IL-12-p70 was quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from five independent experiments. Statistically significant differences ($p < 0.05$) are marked with an asterisk. (B) Human macrophages remained as controls or were incubated with different tumor cell lines as indicated. Tumor cell death in cocultures is marked with a plus (+). Residual tumor cells were removed from cocultures with 5-min accutase treatment. Afterward, 1 μ g/ml LPS and 100 U IFN- γ were added to macrophages from cocultures and to control macrophages for 6 h. TNF- α and IL-8 contents in supernatants were quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from six independent experiments. Statistically significant differences compared with LPS/IFN- γ -stimulated control macrophages ($p < 0.05$) are marked with an asterisk.

detectable amounts of cytokines (data not shown), except for IL-8 (about 200 pg/ml), which was nevertheless extremely low compared with the amounts that were produced by macrophages. Stimulation of macrophages exposed to tumor cell conditioned medium with LPS/IFN- γ elicited those

phenotypic alterations, which we observed in direct cocultures, where tumor cell death occurred. TNF- α and IL-12-p70 were decreased and IL-8 was enhanced, whereas IL-10 was high, but not significantly different from control macrophages (Figure 5A). We concluded that the macrophage phenotype shift was dependent on tumor cell apoptosis, rather than representing intrinsic features of different tumor cell lines. Moreover, because supernatants of apoptotic tumor cells evoked macrophage phenotype alterations, macrophage polarization demanded soluble, AC-derived factors. To further validate our data to be independent of a specific tumor cell line, we used four additional well-characterized tumor cell lines, such as the colon carcinoma cell lines Caco-2 and Colo201, the lung cancer cell line A549, and the hepatocellular carcinoma cell line Hep-G2 in our coculture system. After 5 d of coculture, Caco-2, Colo201, and A549 cells were absent from cocultures, whereas Hep-G2 cells were growing normally (data not shown). After removal of residual tumor cells and stimulation with LPS/IFN- γ , macrophages that had killed tumor cells displayed a cytokine profile comparable to, but even more pronounced than those from MCF-7 cocultures (Figure 5B). TNF- α was significantly lower compared with control macrophages stimulated with LPS/IFN- γ , whereas IL-8 was significantly increased. Macrophages from cocultures with Hep-G2 cells displayed an activation profile similar to control macrophages, stimulated with LPS/IFN- γ (Figure 5B).

S1P Production in Apoptotic MCF-7 Cells Accounts for Macrophage Polarization

Recently, we obtained evidence that ACs release S1P into the culture medium, which was sphingosine kinase (Sphk) 2-dependent (Weigert *et al.*, 2006). Considering existing evidence that associates S1P production with tumor vascularization and growth (LaMontagne *et al.*, 2006; Visentin *et al.*, 2006), we wanted to know whether S1P production in apoptotic MCF-7 cells contributed to macrophage polarization. This question was approached by using siRNA technology to knock down Sphk1 versus Sphk2 in MCF-7 cells. Knockdown of either Sphk isoforms in MCF-7 cells was successful (Figure 6D). siRNA supplied was isoform specific, and a control siRNA further proved specificity. As an additional control, we ruled out an interference of Sphk1 or Sphk2 knockdown on induction of MCF-7 cell death upon coculture with macrophages (Figure 6E). We then used naive macrophages, macrophages derived from a coculture with MCF-7 cells, or cells from a coculture of MCF-7 cells with either Sphk1 or Sphk2 being knocked down (Figure 6). After 5 d of coculture, tumor cells were removed, and macrophages were stimulated with LPS/IFN- γ to follow cytokine production of TNF- α (Figure 6A), IL-10 (Figure 6B), or IL-8 (Figure 6C). As seen in previous experiments, macrophages from cocultures with MCF-7 cells displayed reduced TNF- α and increased IL-8 but unaltered IL-10 production compared with naive macrophages exposed to LPS/IFN- γ . Knockdown of Sphk1 did not alter macrophage cytokine production compared with changes provoked by apoptotic MCF-7 cells. However, knockdown of Sphk2 affected the ability of MCF-7 cells to alter cytokine production in macrophages upon LPS/IFN- γ stimulation. With Sphk2 being suppressed in MCF-7 cells during the coculture, the macrophage response upon LPS/IFN- γ addition was similar to naive cells, producing high TNF- α and low IL-8, with no changes in IL-10. A similar response in macrophages was noticed when exposed to conditioned media from apoptotic, i.e., staurosporine-treated MCF-7 cells, with SphK2 being knocked down by siRNA. Compared with conditioned media from

apoptotic and SphK2-expressing MCF-7 cells, the release of IL-8 was attenuated and the production of IL-10 was lower in unstimulated macrophages (Figure 6F). Along that line, after stimulation with LPS/IFN- γ , repression of TNF- α seen with conditioned medium from apoptotic MCF-7 cells was reversed when exposed to conditioned medium derived from SphK2-knockdown cells (Figure 6F). To substantiate that tumor cell apoptosis and SphK2 knockdown in tumor cells affect the release of S1P, we used LC-MS/MS to quantify the lipid mediator, released into supernatants of cocultures of macrophages with MCF-7 cells or into the supernatant of MCF-7 cells treated with staurosporine (Table 1). Supernatants from cocultures of macrophages with MCF-7 cells, MCF-7 cells overexpressing Bcl-2, or MCF-7 cells with Sphk2 being knocked down, were collected at a time when apoptosis was high in MCF-7 cells, i.e., the 24–48-h period of coculture. Although S1P amounts in supernatants of control macrophages, cocultures of macrophages with Bcl-2-overexpressing- or Sphk2 knockdown MCF-7 cells were below the reliable limit of quantification (0.5 ng/ml), S1P levels in cocultures of macrophages with naive MCF-7 cells were significantly elevated (Table 1; $p \leq 0.01$). We obtained similar results when we quantified S1P in supernatants of viable or apoptotic, i.e., staurosporine-treated MCF-7 cells with or without SphK2 knockdown. Supernatants of viable MCF-7 lack S1P, whereas S1P was released from apoptotic MCF-7 cells upon staurosporine treatment. Knockdown of SphK2 abrogated this response significantly (Table 1; $p \leq 0.01$). These results imply that S1P is generated by Sphk2 and is derived from dying MCF-7 cells.

Cocultured MCF-7 Cells or Authentic S1P Impaired NF- κ B Activation in Macrophages

M2 polarization of TAMs has previously been attributed to diminished p65 nuclear translocation (Biswas *et al.*, 2006). To validate our model with respect to this TAM marker, we looked for NF- κ B activation in macrophages exposed to apoptosis-sensitive versus -resistant MCF-7 cells. EMSA analysis showed NF- κ B activation in response to LPS/IFN- γ stimulation in macrophages from cocultures with MCF-7-Bcl-2 or RKO cells (Figure 7A, lanes 4–9). Supershift analysis was evident with an α -p65 antibody, whereas α -p50 reduced NF- κ B-DNA binding without shifting the complex to higher molecular mass. When macrophages had been cocultured with MCF-7 cells before stimulation with LPS/IFN- γ , activation of NF- κ B was impaired (Figure 7A, lanes 1–3). Negligible activation of NF- κ B was also seen when macrophages were pre-exposed for 2 d to authentic S1P (Figure 7B). Statistical quantification of relevant changes is shown in Figure 6C. Collectively, these data imply that S1P as well as apoptotic tumor cells suppress activation of NF- κ B in macrophages, whereas apoptotic resistant tumor cells do not share this behavior. Macrophage polarization by apoptotic tumor cells is evident at the level of cytokine production as well as NF- κ B activation.

DISCUSSION

Increasing evidence suggests TAMs as important players in tumor progression. In some human cancers they even emerge as prognostic markers (Bingle *et al.*, 2002; Lewis and Pollard, 2006). Their protumor activities are associated with polarization toward an M2 phenotype. This alternative activation program is driven by the tumor microenvironment, which includes tumor-derived immunosuppressants as well as tumor hypoxia (Pollard, 2004). Our study points to tumor cell apoptosis as an additional process in affecting the tumor

Figure 6. Knockdown of Sphk2, but not Sphk1 in MCF-7 cells restored a classical activation profile in human macrophages upon coculture. Human primary macrophages remained as controls or were incubated for 5 d with MCF-7 cells or MCF-7 cells with Sphk1 or Sphk2 being knocked down with specific siRNA. Residual tumor cells were removed from cocultures with 5-min accutase treatment, and 1 μ g/ml LPS and 100 U IFN- γ were added to macrophages from cocultures and to control macrophages for 6 h. TNF- α (A), IL-10 (B), and IL-8 (C) contents in supernatants were quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from five independent experiments. Statistically significant differences ($p < 0.05$) are marked with an asterisk. (D) Western analysis shows Sphk1 and Sphk2 expression in MCF-7 cells transfected either with control siRNA (siControl), siRNA against Sphk1 (siSphk1), or Sphk2 (siSphk2). One of three representative experiments is displayed. Western analysis was performed 48 h after nucleofection. (E) Human primary macrophages remained as controls or were incubated with naive MCF-7 cells or MCF-7 cells, with Sphk1 or Sphk2 being knocked down with specific siRNA for the times indicated. Cocultures and control macrophages were incubated for 30 min with accutase, harvested, stained with α -CD44-PE as a discrimination marker between macrophages and tumor cells and annexin V-FITC as a marker for cell death, and analyzed by FACS. Data are presented as the mean $\pm$ SEM from three independent experiments. (F) Human macrophages remained as controls or were incubated with conditioned medium from apoptotic MCF-7 (CM-MCF-7) or MCF-7 cells transfected with siRNA to target Sphk2 (CM-MCF-7 siSphk2) cells for 24 h. Thereafter, supernatants were removed, cells were washed with PBS, fresh medium was added, and macrophages were stimulated with 1 μ g/ml LPS and 100 U IFN- γ for 6 h. Production of TNF- α , IL-10, and IL-8 was quantified by FACS with BD Cytometric Bead Array Flex Sets. Data are presented as the mean $\pm$ SEM from five independent experiments. Statistically significant differences ($p < 0.05$) are marked with asterisks.

microenvironment and thus macrophage polarization (Kim *et al.*, 2006). It has been shown previously that pretreatment of macrophages with apoptotic but not necrotic tumor cells reduced their cytotoxic behavior (Reiter *et al.*, 1999). Considering the production of TNF- α as a potentially harmful agent our work supports the notion that TNF- α is produced in a coculture of macrophages with MCF-7 cells, with the latter ones being killed. However, once ACs had been generated in

the coculture setup, the production of TNF- α declined, and cocultures produced substantially smaller amounts of the cytotoxic agent upon LPS/IFN- γ stimulation compared with naive cell activation. Moreover, the second administration of fresh tumor cells to macrophages primed by apoptotic tumor cells did not elicit a new peak of TNF- α formation and did not result in tumor cell killing (data not shown). Along that line, MCF-7 cells overexpressing Bcl-2 were not killed

Table 1. Release of S1P from MCF-7 cells during coculture or staurosporine treatment

Sample	S1P (ng/ml)
MCF-7 cells	
Control	n.d.
Staurosporine (0.5 ng/ml)	4.02 ± 1.51
siSphK2 + Staurosporine (0.5 ng/ml)	0.65 ± 0.12
Cocultures	
Macrophages	n.d.
Macrophages + MCF-7	5.65 ± 2.79
Macrophages + MCF-7 siSphK2	n.d.
Macrophages + MCF-7-Bcl-2	n.d.

S1P contents in supernatants were measured with LC-MS/MS as described in *Materials and Methods*. Values are means ± SD; n ≥ 5. The lower limit of quantification was 0.5 ng/ml. S1P concentrations in supernatants below this limit are marked with n.d. MCF-7 cells were treated with staurosporine for 4 h, washed and suspended in full medium for 2 h followed by S1P determinations. Coculture conditions were as described in *Materials and Methods*.

by macrophages, because Bcl-2-overexpressing cells, especially Bcl-2-expressing MCF-7 cells, are resistant to TNF- α -induced apoptosis (Burow *et al.*, 1998). Thus, the presence of ACs attenuates TNF- α formation in macrophages, thereby affecting their cytotoxic potential toward tumor cells. Presumably the interaction with apoptotic tumor cells affects other mechanisms of macrophage cytotoxicity, which were investigated previously (Reiter *et al.*, 1999). Nevertheless, tumor cell killing by macrophages that were primed with dying tumor cells beforehand was disabled. Apoptotic cells, besides reducing macrophage cytotoxicity, may further affect the tumor microenvironment because they release immune modulators such as TGF- β and IL-10 (Tomimori *et al.*, 2000; Chen *et al.*, 2001). We did not observe the release of IL-10 from dying tumor cells in our system (data not shown). Nevertheless, conditioned medium from apoptotic tumor cells induced phenotypical alterations in macrophages.

As in cocultures with MCF-7 cells, IL-10 production in macrophages was enhanced with conditioned medium from dying tumor cells. Although mechanistically unexplained, an increase in IL-10 was correlated to defective NF- κ B binding in TAMs (Sica *et al.*, 2000), and the inability to activate NF- κ B was also observed upon recognition of ACs by macrophages (Cvetanovic and Ucker, 2004). A short-term binding of ACs to macrophages did not affect NF- κ B DNA binding, but depleted p300 to impair transcriptional activation of NF- κ B (Cvetanovic and Ucker, 2004). Importantly, attenuated NF- κ B activity appeared critical in shaping the TAM phenotype (Biswas *et al.*, 2006). Our experiments favored S1P, a soluble factor, in blocking NF- κ B, after long-term exposure of ACs to macrophages. This was unexpected, because NF- κ B activation rather than inhibition was shown for S1P in other cell systems (Siehler *et al.*, 2001). Despite S1P might induce low NF- κ B activation, it completely abrogated TNF- α -mediated stimulation of NF- κ B in THP-1 human monocytes (Kimura *et al.*, 2006). Although mechanistically unclear at present, the impact of S1P on NF- κ B may help to understand macrophage polarization by apoptotic tumor cells. Activation versus inhibition may depend on the S1P receptor profile and/or duration toward S1P exposure, as well as the presence or absence of costimuli such as LPS/IFN- γ .

Figure 7. Macrophages display reduced NF- κ B activation after coculture with MCF-7 cells or addition of S1P. Human primary macrophage, remained as controls, were incubated with MCF-7 cells, Bcl-2-overexpressing MCF-7 cells (MCF-7-Bcl-2), RKO cells for 5 d, or 10 μ M S1P for 2 d. Residual tumor cells were removed, and 1 μ g/ml LPS and 100 U IFN- γ were added to macrophages from cocultures and to control macrophages for 4 h. Activation of NF- κ B was analyzed by EMSA using a specific 5'-IRD700-labeled oligonucleotide as described in *Materials and Methods*. Supershift analysis was performed with p65 and p50 antibodies (Santa Cruz Biotechnology) as indicated. (A) EMSA for NF- κ B DNA-binding activity in macrophages from cocultures. One of three representative experiments is displayed. (B) EMSA for NF- κ B DNA-binding activity in control and S1P-exposed macrophages after LPS/IFN- γ stimulation. One of three representative experiments is displayed. Cells were pre-exposed for 48 h with 10 μ M S1P before stimulation with 1 μ g/ml LPS and 100 U IFN- γ . (C) The histogram shows quantification of EMSA data. Data are presented as the mean ± SEM from five independent experiments. Differences between LPS/IFN- γ -stimulated macrophages and macrophages from cocultures or S1P-treated macrophages marked by asterisks are statistically significant ($p < 0.05$).

S1P from apoptotic MCF-7 cells changed the LPS/IFN- γ activation profile in macrophages. It suppressed TNF- α formation but increased IL-8 production in macrophages upon LPS/IFN- γ activation and most likely provoked IL-10 liberation during phagocytosis of dying cells. Reduced TNF- α

production may result from a diminished NF- κ B activity, whereas alterations in interleukin production may be associated with activation of STAT1 and/or PI3K-signaling, because these pathways characterize M2 cells (Rauh *et al.*, 2005; Biswas *et al.*, 2006). Production of the immunosuppressive cytokine IL-10 is characteristic for TAMs. Although underlying molecular pathways remain unclear, it is known that S1P provoked secretion of IL-10 from T-cells and dendritic cells, which was correlated to a diminished proinflammatory activity in these cells (Idzko *et al.*, 2002; Wang *et al.*, 2005). Moreover, IL-10 formation may require PI3K signaling, a pathway which can be activated by S1P receptors (Taha *et al.*, 2004). Furthermore, PI3K may negatively regulate TLR signaling during inflammation and has been proposed as a potential target to circumvent immunosuppression in cancer (Fukao and Koyasu, 2003).

As stated, IL-10 was previously related to defective IL-12 production in TAM, which was furthermore explained by defective NF- κ B signaling (Sica *et al.*, 2000). IL-10 was produced in cocultures with MCF-7 cells, even after tumor cells were killed. Therefore, we expected that coculture-primed macrophages should produce less IL-12-p70, which indeed was the case. Because IL-12-p70 and TNF- α production in macrophages from cocultures was equally affected, we did not follow the release of IL-12-p70 in further experiments. However, we showed that S1P reduced NF- κ B DNA-binding, which is likely to attenuate generation of IL-12-p70 as well.

Enhanced production of IL-8 in macrophages derived from MCF-7 cocultures is not immediately apparent considering the attenuated activity of NF- κ B, a known inducer of IL-8 (Hoffmann *et al.*, 2002). Under conditions of S1P release, other transcriptional activators of IL-8 such as C/EBP β , AP-1, or STAT1 may substitute for NF- κ B, and it will be interesting in the future to define how S1P enhances IL-8 production.

Despite uncertainties in IL-8 regulation, its production may explain how S1P contributes to tumor angiogenesis, because IL-8 expression was related to TAM-dependent angiogenesis and poor prognosis in uterine cancer (Fujimoto *et al.*, 2002). Recent *in vivo* studies refer to a role of S1P in tumor vascularization (LaMontagne *et al.*, 2006; Visentin *et al.*, 2006). Forced S1P receptor desensitization with FTY720 abrogated S1P- and VEGF-induced angiogenesis and attenuated metastatic tumor growth (LaMontagne *et al.*, 2006). In addition, the use of a monoclonal S1P antibody reduced tumor growth via inhibition of tumor angiogenesis and survival (Visentin *et al.*, 2006). These observations support a direct connection between S1P production and tumorigenesis, although the source of S1P in human tumors is undefined.

Considering that high levels of Sphk1 in glioblastoma correlated with low patient survival (Van Brocklyn *et al.*, 2005), it seems attractive to assume gain of expression regulation as an underlying mechanism. In our study, we identified Sphk2 activity in close association with tumor cell apoptosis by macrophages as another possible S1P origin. As shown by LC-MS/MS, the release of S1P from apoptotic, but not viable tumor cells induced macrophage polarization toward an alternatively activated phenotype, as characterized by a change in cytokine production, reduced tumor cytotoxicity and an attenuated NF- κ B response.

Previously, other factors such as TGF- β or phosphatidylserine were considered to provoke phenotype alterations in macrophages after the interaction with ACs (Chen *et al.*, 2001; Savill *et al.*, 2002). Because suppression of S1P release via SphK2 knockdown did not restore TNF- α completely,

these mediators might contribute in shaping the macrophage phenotype. However, S1P was shown to mimic TGF- β responses by cross-activating the TGF- β II receptor (Xin *et al.*, 2004), which might support a prominent role of S1P. Taken together, our results suggest that induction of tumor cell apoptosis by invading macrophages, at least during early stages of tumor formation, may help to understand formation and action of TAMs.

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