# Identification of a Subpopulation of Macrophages in Mammary Tumor–Bearing Mice That Are Neither M1 nor M2 and Are Less Differentiated

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## Abstract

**Systemic and local immune deficiency is associated with cancer, and the role of M2 tumor-associated macrophages in this phenomenon is well recognized. However, the immune status of macrophages from peripheral compartments in tumor hosts is unclear. Peritoneal macrophages (PEM) are derived from circulating monocytes and recruited to the peritoneal cavity where they differentiate into macrophages. We have previously shown that PEMs from mice bearing D1-DMBA-3 mammary tumors (T-PEM) are deficient in inflammatory functions and that this impairment is associated with diminished expression of transcription factors nuclear factor κB and CAAT/enhancer-binding protein. We now provide evidence that T-PEMs display neither M1 nor M2 phenotypes, yet exhibit deficiencies in the expression of several inflammatory cytokines and various proinflammatory signaling pathways. Moreover, due to nuclear factor κB down-regulation, increased apoptosis was observed in T-PEMs. We report for the first time that macrophage depletion is associated with increased macrophage progenitors in bone marrow. Furthermore, T-PEMs have a lower expression of macrophage differentiation markers F4/80, CD68, CD115, and CD11b, whereas Gr-1 is up-regulated. Our results suggest that T-PEMs are less differentiated and represent a newly derived population from blood monocytes. Lastly, we show that transforming growth factor-β and prostaglandin $E_2$, two immunosuppressive tumor-derived factors, may be involved in this phenomenon.** [Cancer Res 2009;69(11):4800–9]

## Introduction

Systemic immune deficiency in cancer (1–3) and the occurrence of a local immune suppression within tumors (4, 5) have been well documented. Indeed, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) and some T-cell subsets colonizing the tumor microenvironment exhibit immunosuppressive properties. However, the contribution of macrophages from extra-tumor sites to the host immune competence has received less attention.

Circulating monocytes differentiate into macrophages upon entry into tissues. Macrophages (6) produce inflammatory cytokines to activate the adaptive immune system and aid in the elimination of microbes and tumor cells. When tissue damage is unresolved, chronic inflammation establishes (7). In this scenario, macrophages continue to exhibit proinflammatory features and generate tissue damage indefinitely.

Two opposite polarization states have been described for macrophages depending on their immune competence: the classically activated M1 and the alternatively activated M2 (5). The M1 macrophage is proinflammatory and is induced by IFN-γ alone or together with microbial stimuli (i.e., LPS). They present antigens efficiently, produce high levels of proinflammatory cytokines such as interleukin (IL)-12, are efficient activators of Th1 T-cell responses, and are cytotoxic against microbes and tumor cells. M1 macrophages are thus considered powerful effectors against the attack of pathogens and tumor cells. M2 macrophages, in contrast, are induced by IL-4 and IL-13, immune complexes, IL-10 or glucocorticoids. They possess less efficient antigen-presenting capacity, have an $IL\text{-}12^{low}IL\text{-}10^{high}$ phenotype, suppress Th1 immunity, and promote wound healing, tissue remodeling, and angiogenesis. Thus, M2 macrophages play a role in the homeostatic mechanisms that terminate inflammatory responses.

Substantial experimental and epidemiologic evidence links chronic inflammation with cancer (7, 8). Macrophages are involved both in initial and later stages of carcinogenesis, paralleling the interplay between inflammation and cancer in their switch from activated M1 in inflamed tissues to M2 tumor-promoting, proangiogenic TAMs (5, 9).

Peritoneal macrophages (PEM) are derived from circulating monocytes and represent peripheral cells exposed to the circulation in the peritoneal cavity. In contrast, TAMs differentiate within the immunosuppressive tumor microenvironment. We have shown that macrophages from mice bearing advanced D1-DMBA-3 mammary tumors (T-PEM) function less efficiently as antigen-presenting cells compared with macrophages from normal animals (N-PEMs; ref. 10) and produce low levels of IL-12 and nitric oxide (11, 12). Moreover, a deficiency in proinflammatory functions is associated with decreased expression of transcription factors nuclear factor κB (NFκB) and CAAT/enhancer-binding protein (C/EBP) in these cells (13). We now provide evidence that T-PEMs are neither M1 nor M2, are more prone to apoptosis, and express lower levels of macrophage differentiation markers. Furthermore, we show for the first time that macrophage depletion is associated with enhanced generation of macrophage precursors in the bone marrow.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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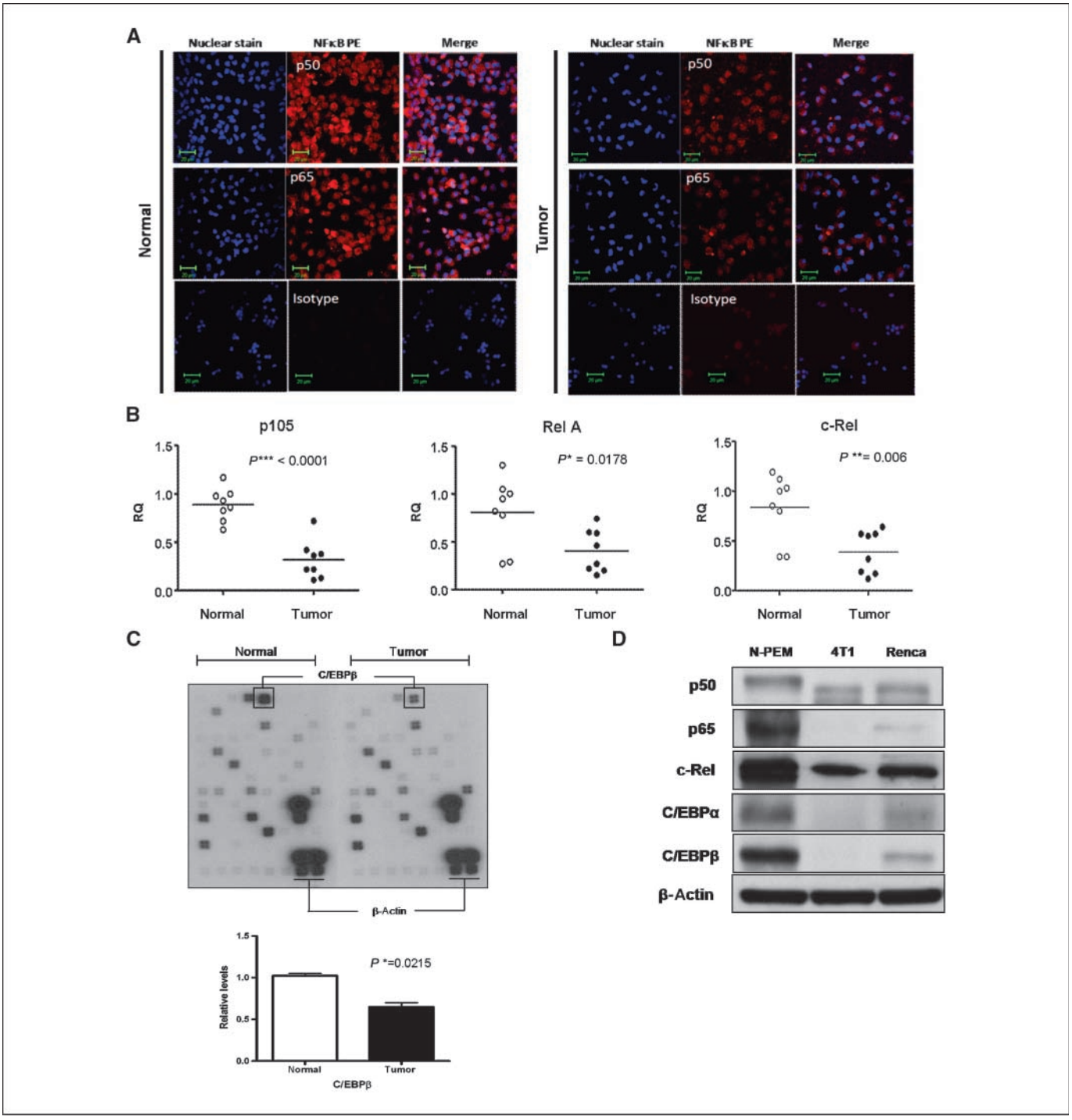


**Figure 1.** NFκB and C/EBP are down-regulated in T-PEMs. *A,* confocal images of N-PEMs and T-PEMs stained with NFκBp50-PE and NFκBp65-PE (*red*) and with DAPI (*blue*). *B,* NFκBp105, RelA, and c-Rel mRNA levels in N-PEMs and T-PEMs were determined by real-time PCR using 18S for normalization. *C,* gene expression array and densitometry shows C/EBPβ mRNA expression in N-PEMs and T-PEMs using β-actin for normalization. *D,* Western blot of NFκB and C/EBP expression in N-PEMs and T-PEMs isolated from Renca and 4T1 tumor-bearing mice.

## Materials and Methods

**Animals, cell lines, and tumors.** BALB/c mice, 10 to 14 weeks of age, were used. D1-DMBA-3 mammary adenocarcinoma and DA-3 mammary tumor cell line, derived from D1-DMBA-3, were maintained and used as previously described (14, 15). Lewis lung carcinoma (LLC), Renca renal cell carcinoma, 4T1 mammary carcinoma, and mouse fibroblast 3T3 cell lines were kindly provided by Eckhard Podack (University of Miami, Miami, FL). The institutional animal care and use committee approved the animal experiments.

**Macrophage collection and culture.** Normal and 4-wk tumor-bearing mice were injected i.p. with 1.5 mL of 3% thioglycolate (Difco Laboratories). On day 4, PEMs were obtained and cultured as described (13).

**Laser confocal microscopy.** Macrophages were adhered to glass slides and Fc receptors blocked using mouse CD16/32 antibody

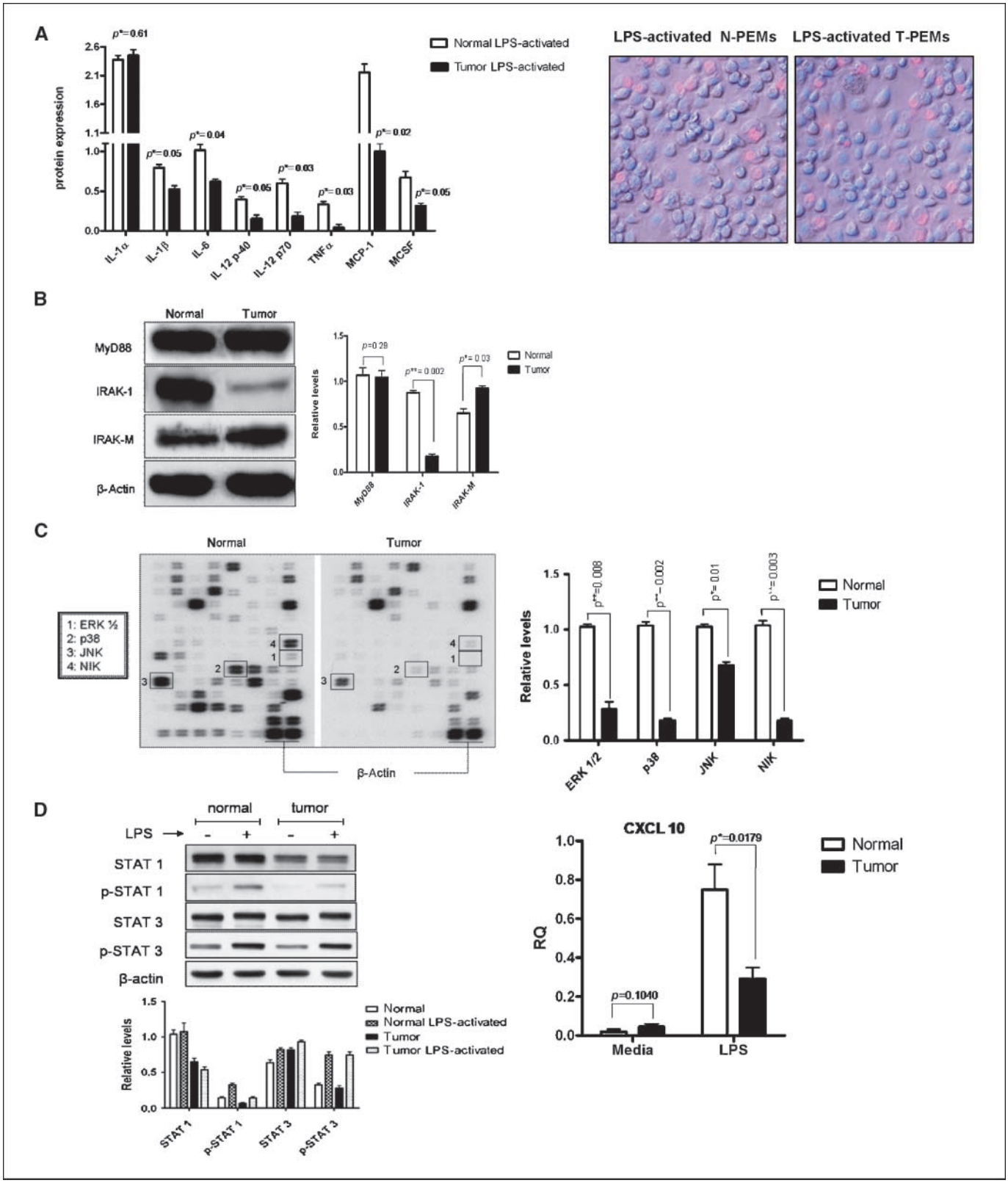


**Figure 2.** Proinflammatory cytokines and various signaling intermediates are down-regulated in T-PEMs. N-PEMs and T-PEMs were isolated and cultured without (*B*) and with 10 μg/mL of LPS (for 20 h in *A*, and for 1 h in *D*) before obtaining whole cell extracts. *A, left,* antibody array histogram showing densitometry after adjustment with a positive control of inflammatory cytokines produced by LPS-stimulated N-PEMs and T-PEMs. *Right,* cell viability assessment using DAPI and PI of N-PEMs and T-PEMs stimulated with LPS for 20 h. *Pink,* dead cells ($PI^+$). *B,* Western blot and densitometry after β-actin adjustment of MyD88, IRAK-1, and IRAK-M expression in resting N-PEMs and T-PEMs. *C,* gene expression array and densitometry from N-PEM and T-PEMs shows ERK1/2, p38, JNK, and NIK mRNA expressions after β-actin normalization. *D, left,* Western blot and densitometry normalized with β-actin of STAT1/pSTA1 and STAT3/pSTA3 expression of resting and LPS-stimulated N-PEMs and T-PEMs. *Right,* CXCL10 mRNA levels in resting and LPS-stimulated N-PEMs and T-PEMs were determined by real-time PCR using 18S for normalization.

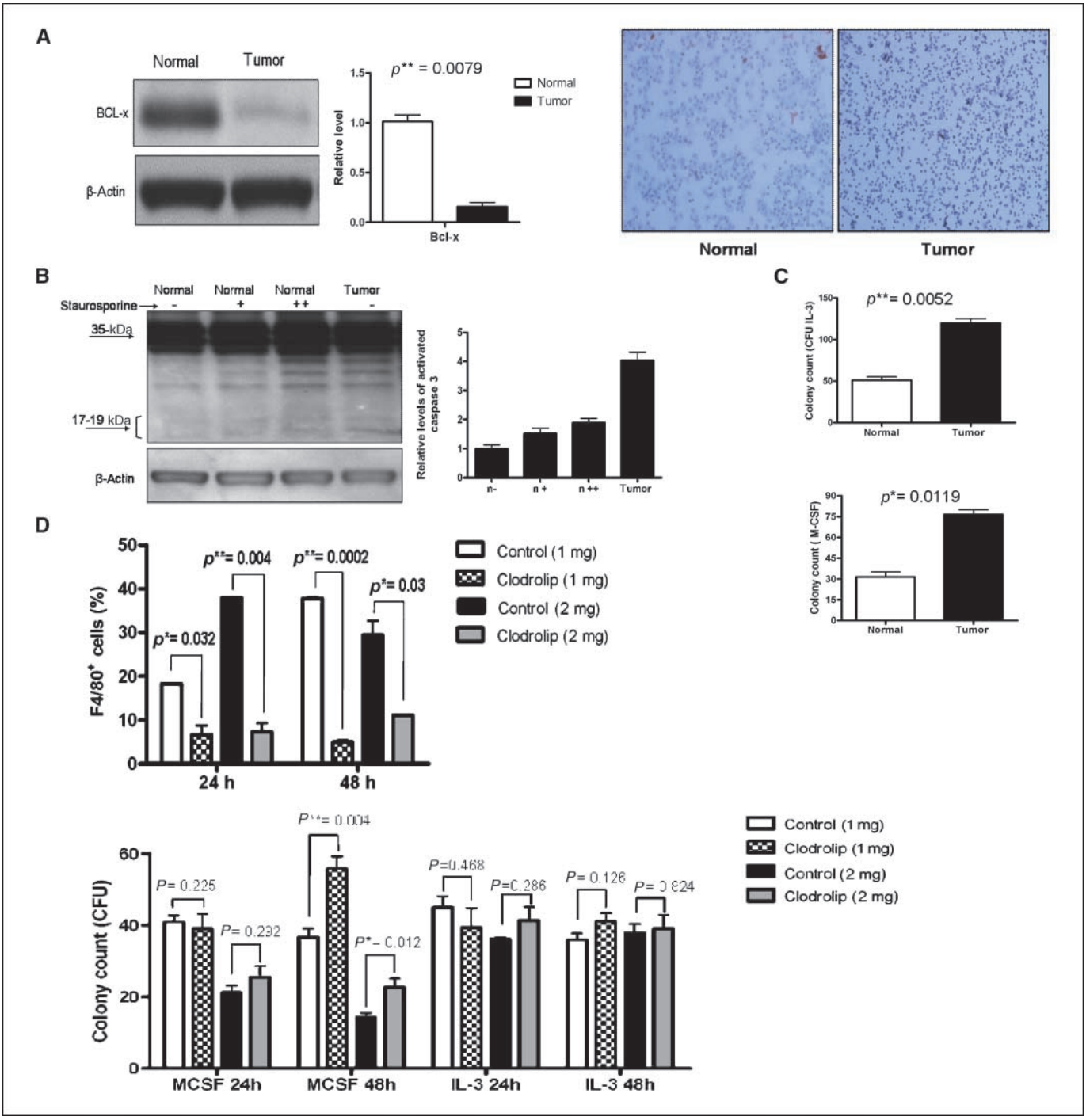


**Figure 3.** Increased apoptosis in T-PEMs and augmentation of committed myeloid progenitors in the marrow of tumor bearers. *A, left,* Western blot and densitometry after normalization with β-actin of Bcl-x expression in N-PEMs and T-PEMs. *Right,* colorimetric TUNEL assay of N-PEMs and T-PEMs. *B,* Western blot analysis and densitometry after normalization with β-actin of activated caspase 3 in N-PEMs and T-PEMs. *Lanes 1* to *3,* N-PEMs cultured with 0, 10, and 100 nmol, respectively, of staurosporine for 2 h; *lane 4,* untreated T-PEMs cultured for the same amount of time. *C,* CFU-IL-3 and CFU-M-CSF assays were performed on marrow cells from normal or tumor-bearing mice. Cells were cultured with rmIL-3 (50 units/mL) and M-CSF (20 ng/mL). *D, top,* F4/80 flow cytometry of resident PEMs from PBS and Clodrolip-treated normal mice. *Bottom,* CFU analysis of IL-3 and M-CSF–induced myeloid progenitors in marrow from control and Clodrolip-treated mice.

(eBioscience). Cells were washed, fixed with 10% formalin, permeabilized with Optimax permeabilization buffer (Sigma-Aldrich) and then incubated with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) to reveal nuclei. For intracellular NFκB staining, fixed and permeabilized macrophages were incubated with NFκBp50-PE and NFκBp65-PE (Santa Cruz Biotechnologies) or with the corresponding isotype control. Fixed cells were then stained with F4/80PE-Cy7 (BioLegend), CD11b-AF488 and Gr-1-AF647 (eBioscience), incubated with DAPI, and then mounted. Slides were analyzed using a Zeiss laser scanning confocal microscope 500 (Zeiss).

**Flow cytometry.** N-PEMs and T-PEMs were gently scraped from tissue culture plates, washed, and counted. Fc receptors were blocked

and cells were stained with CD11b-FITC, CD115-PE, Gr-1-APC antibodies (all from eBioscience), and F4/80-PE Cy7 (BioLegend). Intracellular staining for CD68-AF700 (AbD Serotec) was performed following the instructions of the manufacturer. To assess viability, cells were stained with 7-amino-actinomycin D (7-AAD; BD PharMingen), which was added 15 min prior to analyzing by flow cytometry. Samples were acquired in a LSR II cytometer (BD Biosciences).

**Committed progenitor assay.** Colony-forming unit (CFU) assays using stimulation with IL-3 or M-CSF (Peprotech) were performed (16). Single clusters containing more than 50 cells were counted as a colony. Results are expressed as CFU-IL-3 and CFU-M-CSF per $10^6$ cells plated.

**Real-time PCR.** PEMs ($10^7$ cells) from individual normal and tumor–bearing animals were cultured in complete medium without LPS (NFκB experiments) or with 10 μg/mL of LPS for 2 h (CXCL10 experiments). Total RNA was isolated and cDNA synthesis was performed (1 μg total RNA) using Invitrogen reagents according to the manufacturer's instructions. Real-time or quantitative PCR was done using TaqMan Gene Expression Assays for NFκBp105, relA, c-rel, CXCL10, and eukaryotic 18S RNA on a 7500 Fast Real-time PCR system from Applied Biosystems following the instructions of the manufacturer.

**cDNA arrays.** Relative mRNA expression was analyzed by microarray technology using the mouse JAK/signal transducer and activation of transcription (STAT) signaling pathway (MM-039) and the mouse mitogen-activated protein kinase (MAPK) signaling pathway (MM-017; GE Array Q Series, SuperArray, Inc.), using total RNAs isolated from N-PEMs and T-PEMs as previously described (17).

**Antibody array.** The Mouse Inflammation Antibody Array 1.1 (RayBiotech, Inc.) was used to study the expression of proinflammatory cytokines in whole cell lysates from LPS-activated (Sigma-Aldrich) N-PEM and T-PEMs, following the manufacturer's instructions.

**TUNEL assay.** The DeadEnd Colorimetric Terminal uridine deoxynucleotidyl transferase dUTP nick end-labeling or TUNEL System from Promega was performed according to the manufacturer's instructions, using resting N-PEM and T-PEM.

**Western blot.** N-PEM and T-PEM ($10^7$) were adhered to plastic tissue culture dishes, whole cell extracts were obtained, and Western blot was performed as previously described (13). Rabbit α-mouse polyclonal antibodies (except for IRAK-M, a goat anti-mouse polyclonal antibody) from Santa Cruz Biotechnologies were used as the primary antibody. Anti–Bcl-x was kindly provided by Dr. Larry Boise (Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL; ref. 18), and staurosporine was from EMD Biosciences. Rabbit α-mouse STAT and phosphorylated STAT (STAT1 and STAT3) antibodies and α-caspase 3 antibody were from Cell Signaling Technologies. Rabbit α-mouse actin polyclonal antibody was obtained from Sigma-Aldrich. Goat α-rabbit IgG-HRP, except for the IRAK-M (rabbit α-goat IgG-HRP) was used as the secondary antibody. In some experiments, N-PEMs were pretreated with phosphatidylserine (Avanti Polar Lipids), transforming growth factor-β (TGFβ), vascular endothelial growth factor (VEGF), prostaglandin $E_2$ ($PGE_2$), granulocyte macrophage colony-stimulating factor, and IL-11 (all from Peprotech).

**Macrophage depletion using liposome-encapsulated clodronate (Clodrolip).** Normal mice were injected i.p. with 1 and 2 mg/20 g weight

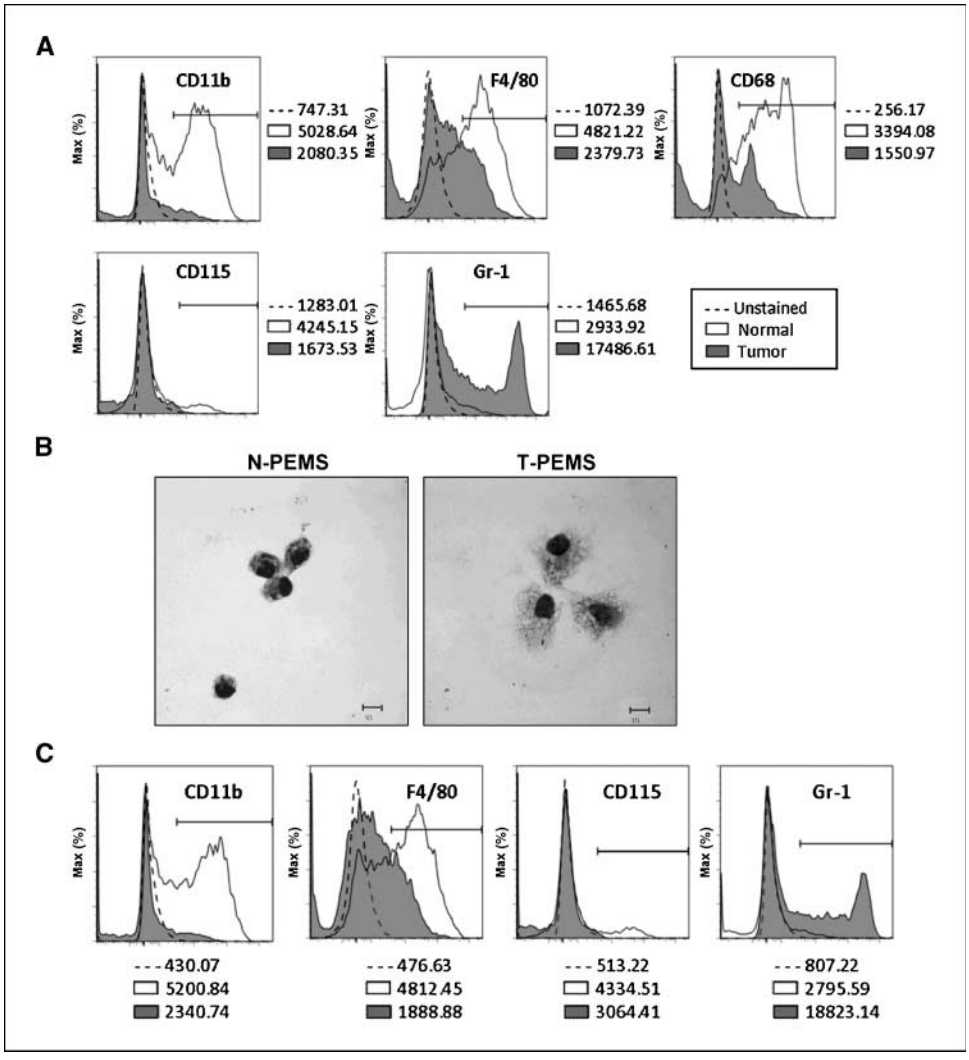


**Figure 4.** T-PEMs express an immature phenotype. *A,* flow cytometric analysis of CD11b, F4/80, CD68, CD115, and Gr-1 in resting N-PEMs and T-PEMs (*right,* MFI values). *B,* Diff-Quick staining reveals the morphology of N-PEMs and T-PEMs. *C,* similar phenotypic analysis as in *A* was performed on 7-AAD$^-$ cells (viable cells).

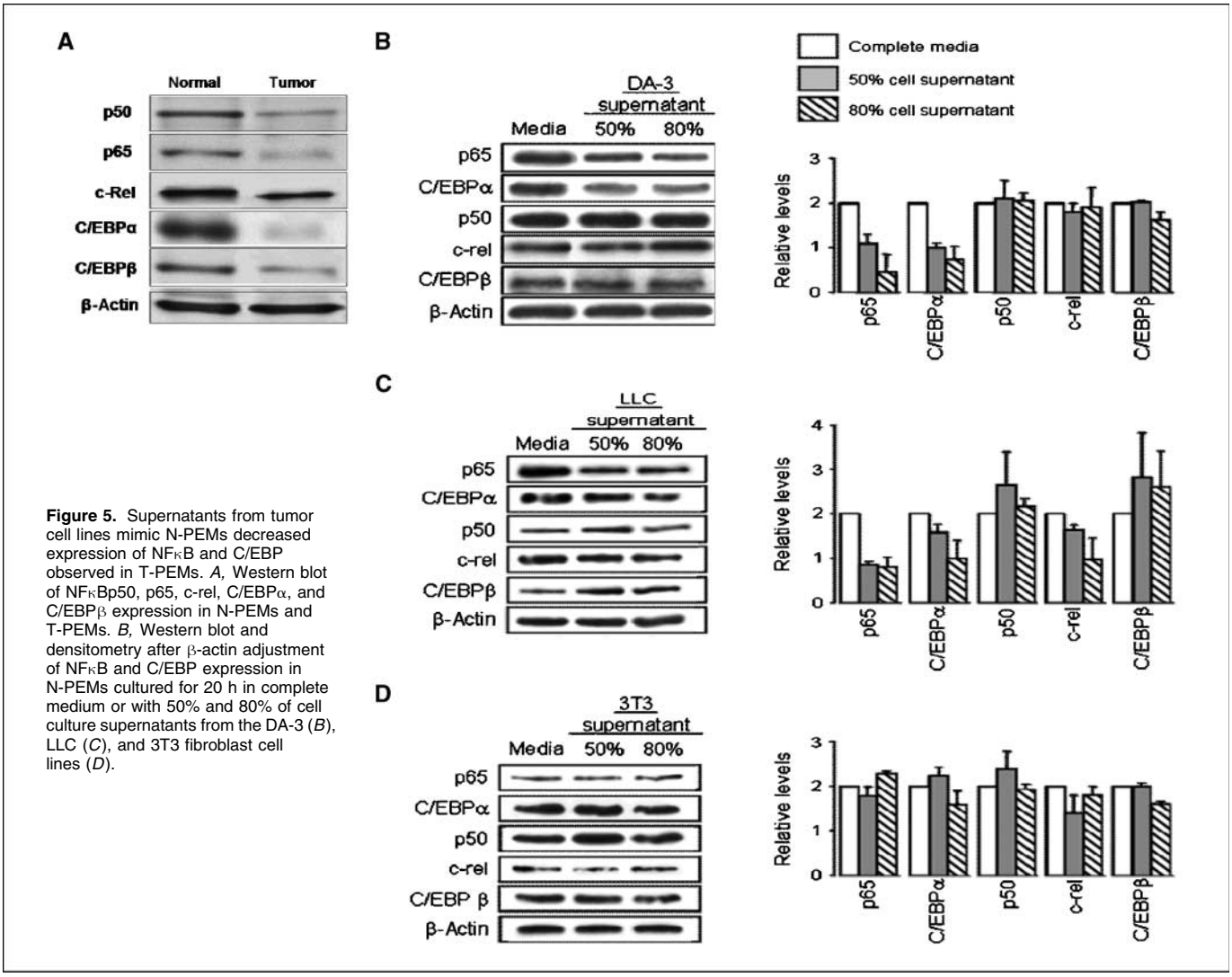


**Figure 5.** Supernatants from tumor cell lines mimic N-PEMs decreased expression of NFκB and C/EBP observed in T-PEMs. *A,* Western blot of NFκBp50, p65, c-rel, C/EBPα, and C/EBPβ expression in N-PEMs and T-PEMs. *B,* Western blot and densitometry after β-actin adjustment of NFκB and C/EBP expression in N-PEMs cultured for 20 h in complete medium or with 50% and 80% of cell culture supernatants from the DA-3 (*B*), LLC (*C*), and 3T3 fibroblast cell lines (*D*).

of Clodrolip (sodium-clodronate tetrahydrate; Farchemia; ref. 19) or equivalent volumes of PBS (three mice/treatment) and sacrificed after 24 and 48 h. PEMs were analyzed for the presence of $F4/80^+$ macrophages by flow cytometry and bone marrow cells were processed for CFU assays.

**Treatment of macrophages with conditioned medium from tumor cell lines and with recombinant tumor factors.** Cell line–conditioned medium from 4-d cultures was centrifuged and supernatants were frozen at −80°C. Recombinant tumor-derived factors were prepared just before use. N-PEMs ($10^7$) were adhered to plastic tissue culture dishes and cultured for 20 h in complete medium containing 50% and 80% of tumor cell line supernatants or recombinant tumor-derived factors. Cells were lysed and Western blot analysis was performed.

**Statistical analysis.** Paired *t* test was used to analyze statistical significance, and error bars represent SEM.

## Results

**T-PEMs express diminished levels of NFκB and C/EBP at mRNA and protein levels.** Our previous studies using Western blot and EMSA revealed that resting and LPS-activated T-PEMs exhibit decreased levels and binding activities of transcription factor proteins NFκBp50, p65, c-rel, and C/EBPα and C/EBPβ (13). Down-regulation of NFκBp50 and p65 proteins in these cells was confirmed using intracellular staining and laser confocal microscopy (Fig. 1*A*). We examined whether NFκB down-regulation was also reflected at the mRNA levels in T-PEMs. Real-time PCR confirmed that the mRNAs encoding for NFκBp50, p65, and c-rel proteins are down-regulated in T-PEMs (Fig. 1*B*). Moreover, gene microarray experiments (Fig. 1*C*; C/EBPα was not assessed in the microarray) corroborated the decreased levels of C/EBPβ transcription factor protein in T-PEMs, which we have previously described (13). These results suggest that the factors responsible for NFκB and C/EBP diminished expression in T-PEMs may act through transcriptional or posttranscriptional mechanisms. To analyze whether this might be a more general mechanism existing in T-PEMs, we studied the protein expression of NFκB and C/EBP in T-PEMs from other mouse tumor models. Our results (Fig. 1*D*), using T-PEMs from mice bearing Renca renal cell carcinomas and 4T1 mammary tumors, confirm the decreased protein expression of NFκB and C/EBP in these models.

**T-PEMs are defective in the production of several proinflammatory cytokines.** A major factor contributing to proin-

flammatory impairment in T-PEMS is their diminished expression of NFκB and C/EBP. We thus hypothesized that in addition to IL-12 and nitric oxide synthase (iNOS), other inflammatory molecules transcriptionally regulated by NFκB may also be down-regulated in T-PEMs. Protein microarray analysis (Fig. 2*A*) using cell lysates of LPS-activated N-PEMs and T-PEMs revealed that T-PEMs, in addition to IL-12, are deficient in the production of IL-1β, IL-6, tumor necrosis factor-α, MCP-1, and M-CSF. Interestingly, LPS-activated T-PEMs did not up-regulate expression of the anti-inflammatory cytokine IL-10 compared with N-PEMs (13). Cell viability of cultured N-PEMs and T-PEMs as determined by DAPI and PI showed no significant lethality ($PI^+$ cells) in either cell type upon cell culture (Fig. 2*A*).

**IRAK-1, NIK, and critical MAPK are reduced, whereas IRAK-M is increased in T-PEMs.** Because different signaling pathways are involved in the control of cytokine expression, we analyzed other proinflammatory signaling pathways in T-PEMs. Several crucial intermediates operate upstream of NFκB in the Toll-like receptor/NFκB signaling pathway. Although MyD88 and IRAK-1 are important in the signaling cascade triggered by activation of Toll-like receptors on the cell surface (20), IRAK-M is an inhibitor of this pathway (21). Analysis of the protein expression of these intermediates in T-PEMs showed that, although there are no significant changes in the expression of MyD88 in T-PEMs, kinase IRAK-1 is dramatically diminished whereas the inhibitor kinase IRAK-M is up-regulated in T-PEMs (Fig. 2*B*). Because MAPKs also play an important role in the regulation of proinflammatory cytokine gene expression (22), their levels were analyzed in T-PEMs. Gene microarray analysis using total RNA obtained from N-PEMs and T-PEMs showed a general decrease in the mRNA expression of most of the MAPKs examined (Fig. 2*C*). Furthermore, a substantial reduction of NIK (NFκB-inducing kinase), JNK, p38, and ERK1/2 gene expression was observed. These data indicate major dysfunctional signaling in several pathways of proinflammatory cytokines in T-PEMs.

**STAT1, but not STAT3, is down-regulated in T-PEMs.** STATs are transcription factors that regulate many aspects of cell growth, survival, and differentiation. STAT1 is involved in up-regulating genes upon stimulation by IFNs, and is associated with the activation of a Th1-type of antitumor immune response (23). In contrast, STAT3 is overexpressed in tumor cells and is an antiapoptotic factor that leads to tumor tolerance in the host (24). Up-regulation of the STAT3 pathway leads to increased angiogenesis, tumor survival, and immunosuppression (25). We investigated whether T-PEMs exhibit altered expression of STAT1 and STAT3. Because these transcription factors are activated by phosphorylation, the expression of both their resting and activated (phosphorylated) forms was compared in N-PEMs and T-PEMs

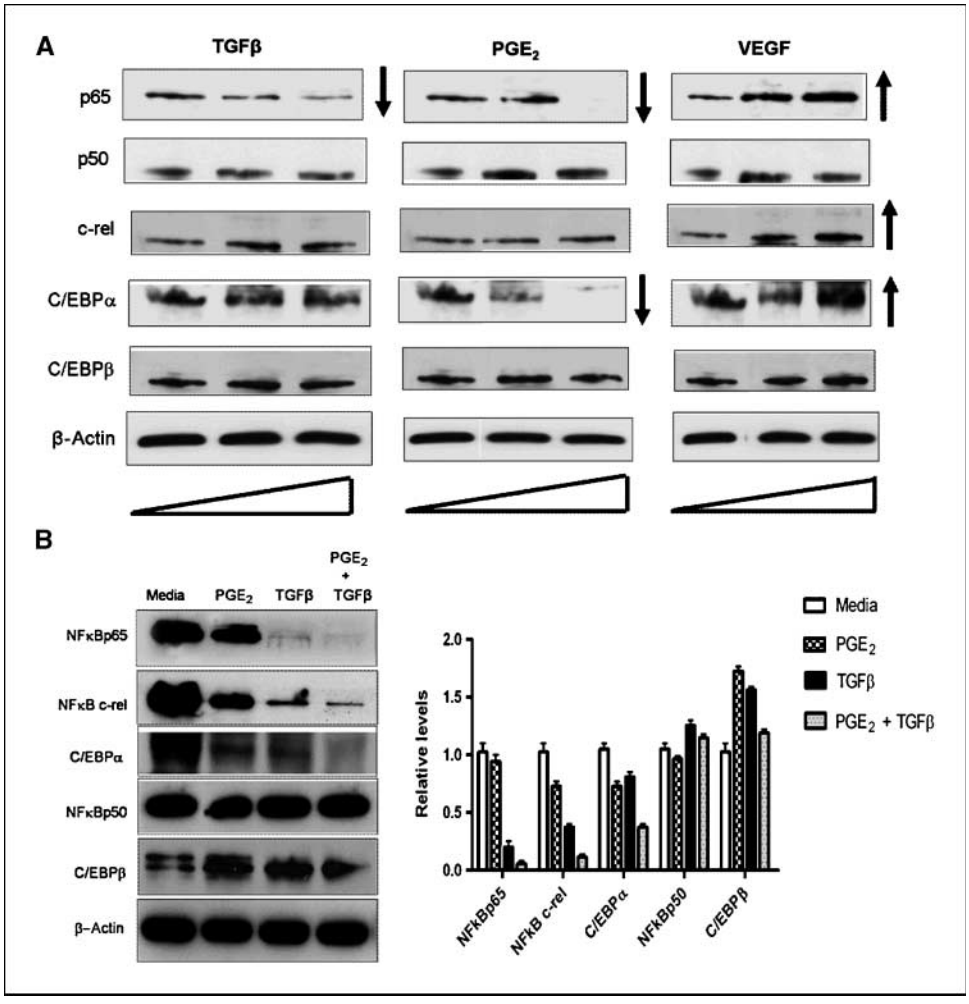


**Figure 6.** DA-3–derived TGFβ and $PGE_2$ induce decreased expression of NFκB and C/EBP in N-PEMs. Western blot analysis of NFκB and C/EBP expression: *A,* N-PEMs treated for 20 h with increasing concentrations of TGFβ (0, 50, and $10^5$ pg/mL), $PGE_2$ (0, 1, and 360 ng/mL), and VEGF (0, 1,700, and $10^5$ pg/mL); *B,* $PGE_2$ and TGFβ individually or in combination. Histograms in *B* show densitometry after β-actin adjustment.

using Western blot analysis. As seen in Fig. 2*D*, STAT1 is down-regulated in resting as well as LPS-activated T-PEMs. Accordingly, these cells also exhibit reduced STAT1 phosphorylation upon LPS activation compared with their normal counterparts. Interestingly, the levels of STAT3 or pSTAT3 were similar in the two cell types. We examined whether STAT1-regulated genes were also affected. Thus, the expression of the chemokine CXCL10 in LPS-stimulated N-PEMs and T-PEMs was determined. Real-time PCR analysis showed decreased CXCL10 mRNA expression in LPS-stimulated T-PEMs (Fig. 2*D*).

**T-PEMs express low levels of Bcl-x and high levels of activated caspase 3 and are more apoptotic.** NFκB, in addition to promoting the described pathways, also induces the expression of several antiapoptotic genes such as Bcl-x. Using Western blots (Fig. 3*A*), a substantial down-regulation in Bcl-x protein expression was observed in resting T-PEMs. Furthermore, TUNEL assays confirmed higher levels of apoptosis in T-PEMs, and apoptotic nuclei in these cells were pyknotic and fragmented (Fig. 3*A*). Activation of caspase 3 is crucial for apoptosis. Thus, we determined whether the increased apoptosis was associated with higher levels of activated caspase 3 in T-PEMs compared with N-PEMs. Treatment with increasing concentrations of staurosporine, an apoptosis-inducing agent, was included as a positive control of caspase 3 activation in N-PEMs. Results showed an increase in the basal expression of the 17 to 19 kDa bands (activated caspase 3) in T-PEMs, even at higher levels than those observed in staurosporine-treated N-PEMs, indicating caspase 3 activation and thus apoptosis execution in T-PEMs (Fig. 3*B*). Consequently, T-PEMs are not only dysfunctional but may be less frequent because of their greater susceptibility to apoptosis.

**Macrophage apoptosis in the periphery is associated with increased myeloid progenitors in the bone marrow of tumor-bearing mice.** Because our results suggest that the tumor induces decreased NFκB expression and enhanced apoptosis in T-PEMs, a compensatory increase in the generation of myeloid progenitors may be occurring in the bone marrow of tumor bearers. Thus, functional CFU assays for marrow-derived myeloid progenitors from normal and tumor-bearing mice were carried out using IL-3 and M-CSF. Our results confirm that the numbers of different myeloid progenitors are significantly increased in tumor hosts when compared with normal mice (Fig. 3*C*). To determine whether decreased numbers of peripheral macrophages contribute to the induction of marrow myelopoiesis, normal mice were treated with i.p. injections of Clodrolip, a macrophage-depleting agent (19). Flow cytometry analyses showed that treatment of normal mice with 1 or 2 mg of Clodrolip (for 24 and 48 hours) significantly depleted $F4/80^+$ resident PEMs, compared with PBS-treated animals at all dose combinations (Fig. 3*D*). Importantly, marrow cells from these macrophage-depleted animals exhibited significantly enhanced production of CFU-M-CSF but not CFU-IL-3–induced myeloid progenitors after treatment with Clodrolip for 48 hours. Thus, 48 hours of macrophage depletion was sufficient to induce myelopoiesis in the bone marrow (Fig. 3*D*). These results suggest that macrophage depletion is associated with a specific increase in marrow progenitors committed to macrophage but not other myeloid lineages.

**T-PEMs down-regulate expression of F4/80, CD68, CD115, and CD11b and up-regulate Gr-1.** Macrophages normally express the myeloid markers F4/80, CD68, CD115 (M-CSF receptor), and CD11b, and the elevated expression of these molecules is considered indicative of macrophage differentiation (26, 27). T-PEMs express significantly lower levels of F4/80, CD68, CD115, and CD11b than N-PEMs (Fig. 4*A*), consistent with the phenotype of newly recruited blood monocytes ($F4/80^{low}$ $CD68^{low}$C-$D115^{low}CD11b^{low}$). These results suggest that T-PEMs are less-differentiated than N-PEMs. Interestingly, T-PEMs also exhibit up-regulation of the granulocytic myeloid marker Gr-1. Yet, they appear as a homogeneous cell population of macrophages of larger size and increased vacuolation (Fig. 4*B*). As T-PEMs are prone to apoptosis, 7-AAD viability marker was included in our flow cytometric experiments. Similar to Fig. 4*A*, expression of macrophage markers was decreased whereas Gr-1 expression was increased on viable ($7\text{-}AAD^-$) T-PEMs (Fig. 4*C*).

***In vitro* pretreatment of N-PEMs with supernatants from tumor cell lines mimics the reduced expression of NFκB and C/EBP observed in T-PEMs.** Because the presence of advanced mammary tumors results in a profound decrease in expression of NFκB and C/EBP at the gene and protein levels in T-PEMs, we investigated whether *ex vivo* pretreatment of N-PEMs with supernatants collected from 4-day-old cultures of the DA-3 cell line produced similar effects. Figure 5*A* shows diminished expression of NFκB and C/EBP proteins in T-PEMs as a positive control to compare with the effect of the *in vitro* treatment of N-PEMs with tumor supernatants. Culturing N-PEMs with increasing concentrations of DA-3 cell supernatant induced reductions in the amounts of some NFκB and C/EBP proteins in these macrophages (Fig. 5*B*). To assess whether NFκB and C/EBP down-regulation is associated with tumor-secreted factors, N-PEMs were pretreated with supernatants from different cell lines. Pretreatment of N-PEMs with supernatants from the tumorigenic LLC cell line down-regulated the expression of NFκB and C/EBP (Fig. 5*C*), whereas N-PEMs pretreated with supernatants from the nontumorigenic 3T3 cell line did not (Fig. 5*D*), implicating factors secreted by tumor cell lines in NFκB and C/EBP down-regulation. Cell viability of N-PEMs cultured with 80% of DA-3 and LLC cell supernatants was assessed by PI staining. No significant toxicity (increased numbers of $PI^+$ cells) was observed in these treated cells (Supplemental Fig. S1). Furthermore, expression of F4/80, CD11b, and Gr-1 did not change significantly when N-PEMs were cultured with LLC supernatants compared with untreated N-PEMs (Supplemental Fig. S2).

**TGFβ and $PGE_2$ tumor factors are associated with the diminished expression of NFκB and C/EBP observed in T-PEMs.** The DA-3 tumor cell line secretes several factors (1, 15, 28, 29) which are also produced by other tumors (30, 31). Pretreatment of N-PEMs with tumor-derived factors results in diminished expression of IL-12 and of iNOS (11, 32). To examine whether some of these tumor-derived factors might be associated with the impairment of NFκB and C/EBP expression observed in T-PEMs, resting N-PEMs were pretreated with increasing concentrations of these tumor-derived products. Pretreatment of N-PEMs with increasing concentrations of TGFβ and $PGE_2$ resulted in a substantial decrease in NFκB and C/EBP expression in these cells (Fig. 6*A*). However, the addition of phosphatidylserine, GM-CSF, or IL-11 did not alter their expression (data not shown). Interestingly, pretreatment of N-PEMs with VEGF resulted in enhanced expression of NFκB and C/EBP. To study the possible interactions between TGFβ and $PGE_2$ that might occur within macrophages *in vivo*, we pretreated N-PEMs with these tumor factors simultaneously. Our results show that these two tumor factors operate in a synergistic fashion to decrease the expression levels of NFκB and C/EBP (Fig. 6*B*).

## Discussion

Macrophages are plastic in that their phenotype changes to adapt to their environment (33). Our unpublished data confirm that TAMs from mammary D1-DMBA-3 tumor bearers are classic M2 macrophages, with IL-12$^{low}$ and IL-10$^{high}$. The immune competence of T-PEMs was examined and there was evidence that these cells, which reside in a nontumor location, are not M2 suppressors, they are IL-12$^{low}$, but are not IL-10$^{high}$. No enhanced expression of other suppressor markers shared by M2 macrophages was detected (34, 35), such as higher expression of VEGF, matrix metalloproteinase-9, or arginase (data not shown). We have previously shown that T-PEMs do produce elevated levels of the suppressor cytokine TGFβ (29). We now provide evidence that these cells display a mixed phenotype (neither M1 nor M2), with down-regulation of several proinflammatory signaling pathways and cytokine production. Potent levels of circulating tumor factors may interact with T-PEMs, leading to such a phenotype. Immune modulation may occur in the circulation when they are still monocytes, or perhaps during their differentiation to macrophages in the peritoneal cavity. The fact that both NFκB and C/EBP are constitutively decreased in these cells results in their deficiency to produce inflammatory cytokines and chemokines, such as IL-12, IL-6, tumor necrosis factor-α, IL-1β, M-CSF, and MCP-1 (36, 37). Furthermore, the down-regulation of NIK mRNA in resting T-PEMs and the involvement of this kinase in both the classic (38) and the alternative (39) NFκB activation pathways, together with the deficiency of T-PEMs in p38, JNK, and ERK, indicate critical impairments in NFκB and related signaling pathways in these cells. STAT1, another crucial signaling mediator of Th1 immunity, is also impaired in T-PEMs. Resting or LPS-activated T-PEMs exhibit lower levels of STAT1 and pSTAT1 expression compared with N-PEMs. Moreover, the STAT1-induced gene CXCL10 was also down-regulated at the mRNA level. In contrast with our results in T-PEMs, increased phosphorylation of STAT1 has been observed in TAMs (40).[5] Kusmartsev and Gabrilovich have also described elevated STAT1 in TAMs, which they associate with an up-regulation of iNOS and arginase activity in these cells (41). In this respect, we have reported that T-PEMs and TAMs in our tumor model have very low iNOS expression (12), and in the present study, no differences in arginase production between N-PEMs and T-PEMs were observed (data not shown). This suggests a mixed phenotype displayed by T-PEMs, presenting some characteristics of M1 macrophages and only some of the M2-suppressive traits. Furthermore, the patterns of cytokine expression displayed by T-PEMs provide functional evidence that these cells are different from M1 and M2 macrophages. Remarkably, T-PEMs are not fixed in their mixed M1/M2 phenotype, and can become high producers of IL-12 or of IL-10. T-PEMs can be experimentally switched to express high amounts of IL-12p70 by culturing in the presence of LPS and IFN-γ. Moreover, they can be induced to produce elevated amounts of IL-10 by culturing them with peptidoglycan, a Toll-like receptor 2 ligand (Supplemental Fig. S3).

We sought to examine the possibility of enhanced apoptosis in T-PEMs, as a consequence of their decreased NFκB. Results indicate that resting T-PEMs express dramatically diminished levels of NFκB-induced antiapoptotic Bcl-x, enhanced activated caspase-3, and increased apoptosis. Importantly, apoptotic cells such as T-PEMs may still be PI$^-$ in early apoptotic stages and thus detected as "viable" macrophages due to intact membranes. To analyze whether apoptosis could induce the marrow of tumor-bearing mice to compensate for the loss of macrophages in the periphery by generating higher numbers of myeloid progenitor cells, bone marrow from normal and tumor-bearing mice were compared using CFU assays specific for committed myeloid progenitors. Our data show that the frequency of committed myeloid progenitors for either a broad spectrum of myeloid cell types (IL-3) or specific for the monocyte's/macrophage's lineage (M-CSF) are both significantly augmented in tumor-bearing mice. Increased myelopoiesis in the marrow of tumor–bearing mice has been associated with tumor factors (42, 43). However, we show for the first time that a decrease in macrophage numbers, in the absence of a tumor, can also induce marrow myelopoiesis. Furthermore, macrophage-depleted mice show myelopoiesis which specifically replenishes cells of the macrophage lineage. Compensatory homeostasis due to increased macrophage apoptosis could be an additional contributing factor to the increased myelopoiesis observed in tumor bearers.

The migration of newly produced monocytes from bone marrow to the circulation could result in macrophages with an immature phenotype. Thus, we compared the phenotype of T-PEMs with that of N-PEMs. Flow cytometry analysis revealed significantly decreased expression of F4/80, CD68, CD115, and CD11b in T-PEMs, all markers of macrophage differentiation. Interestingly, these cells express the granulocyte marker Gr-1 at higher levels than N-PEMs. Gr-1 is considered a marker of neutrophils and of immunosuppressive MDSC, which are CD11b$^+$/Gr-1$^+$ (44). CD68$^+$/Gr-1$^+$ macrophages have been described in the peritoneal cavity of mice infected with low doses of *Toxoplasma gondii*; these macrophages also express CD11b and F4/80 but at lower levels than PEMs from uninfected mice (45). Gr-1 is also expressed by immature TAMs thought to function in immunosuppression (46). Our T-PEMs do not express characteristic markers of MDSC, such as arginase and/or nitric oxide (15). In contrast to our T-PEMs, splenic MDSCs isolated from DA-3-tumor–bearing mice do not express F4/80 (47). Our data suggest that T-PEMs are a less differentiated cell population and thus represent recently recruited monocytes from the blood that may become apoptotic during differentiation into macrophages. The fact that T-PEMs are less differentiated may also contribute to their highly plastic behavior.

We sought to determine whether tumor-derived factors were associated with the changes observed in T-PEMs. Our results show that factors secreted by DA-3 and LLC decreased NFκB and C/EBP expression in N-PEMs. Indeed, TGFβ and $PGE_2$, individually and additively induced down-regulation of NFκB and C/EBP family members. Interestingly, pretreatment with VEGF induced elevated levels of NFκB and C/EBP in N-PEMs, in sharp contrast to our data with T-PEMs. VEGF levels that circulate in tumor bearers may not be sufficient to increase NFκB and C/EBP in T-PEMs, or alternatively, VEGF might interact with other factors *in vivo* which block the induction of NFκB and C/EBP.

Tumors produce immunomodulatory factors in excess, which may thus alter immune function both locally at the tumor site and systemically. Reversing tumor-induced immune function in cancer patients may be carried out by targeting impaired immune cells.

[5] Our unpublished results.

One possibility is to re-educate M2 TAMs or mixed phenotype M1/M2 T-PEMs to become antitumor M1s. Augmenting NFκB expression may be one approach, as peripheral macrophages as well as TAMs from various tumor models exhibit down-regulated NFκB (48).[6]

[6] Our unpublished results.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## References

1. Fu YX, Watson G, Jimenez JJ, Wang Y, Lopez DM. Expansion of immunoregulatory macrophages by granulocyte-macrophage colony-stimulating factor derived from a murine mammary tumor. Cancer Res 1990;50:227–34.
2. Kirchner H, Chused TM, Herberman RB, Holden HT, Lavrin DH. Evidence of suppressor cell activity in spleens of mice bearing primary tumors induced by Moloney sarcoma virus. J Exp Med 1974;139:1473–87.
3. North RJ. Down-regulation of the antitumor immune response. Adv Cancer Res 1985;45:1–43.
4. Li H, Fan X, Houghton J. Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem 2007;101:805–15.
5. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002;23:549–55.
6. Ross JA, Auger MJ. The biology of macrophages. In: Burke B, Lewis CE, editors. The macrophage. 2nd ed. Oxford: Oxford University Press; 2002.
7. Balkwill F, Charles KA, Mantovani A. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell 2005;7:211–7.
8. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet 2001;357:539–45.
9. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. Nat Rev Cancer 2004;4:71–8.
10. Watson GA, Lopez DM. Aberrant antigen presentation by macrophages from tumor-bearing mice is involved in the down-regulation of their T cell responses. J Immunol 1995;155:3124–34.
11. Handel-Fernandez ME, Cheng X, Herbert LM, Lopez DM. Down-regulation of IL-12, not a shift from a T helper-1 to a T helper-2 phenotype, is responsible for impaired IFN-γ production in mammary tumor-bearing mice. J Immunol 1997;158:280–6.
12. DiNapoli MR, Calderon CL, Lopez DM. The altered tumoricidal capacity of macrophages isolated from tumor-bearing mice is related to reduce expression of the inducible nitric oxide synthase gene. J Exp Med 1996;183:1323–9.
13. Torroella-Kouri M, Ma X, Perry G, et al. Diminished expression of transcription factors nuclear factor κB and CCAAT/enhancer binding protein underlies a novel tumor evasion mechanism affecting macrophages of mammary tumor-bearing mice. Cancer Res 2005;65:10578–84.
14. Medina D, DeOme KB. Response of hyperplastic alveolar nodule outgrowth-line D1 to mammary tumor virus, nodule-inducing virus, and prolonged hormonal stimulation acting singly and in combination. J Natl Cancer Inst 1969;42:303–10.
15. Calderon C, Huang ZH, Gage DA, Sotomayor EM, Lopez DM. Isolation of a nitric oxide inhibitor from mammary tumor cells and its characterization as phosphatidyl serine. J Exp Med 1994;180:945–58.
16. Jones M, Komatsu M, Levy RB. Cytotoxically impaired transplant recipients can efficiently resist major histocompatibility complex-matched bone marrow allografts. Biol Blood Marrow Transplant 2000;6:456–64.
17. Torroella-Kouri M, Herbert L, Perry G, Lopez DM. Altered IL-12 signaling pathways contribute to the deficient IFN-γ production by T splenocytes from tumor-bearing mice. Cancer Genomics Proteomics 2004;1:345–54.
18. Boise LH, Minn AJ, Noel PJ, et al. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. Immunity 1995;3:87–98.
19. Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, Schwendener RA. Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Br J Cancer 2006;95:272–81.
20. Muroi M, Tanamoto K. TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-κB. J Leukoc Biol 2008;83:702–7.
21. Kobayashi K, Hernandez LD, Galan JE, Janeway CA, Jr., Medzhitov R, Flavell RA. IRAK-M is a negative regulator of Toll-like receptor signaling. Cell 2002;110:191–202.
22. Raingeaud J, Gupta S, Rogers JS, et al. Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 1995;270:7420–6.
23. Robinson DS, O'Garra A. Further checkpoints in Th1 development. Immunity 2002;16:755–8.
24. Cheng F, Wang HW, Cuenca A, et al. A critical role for Stat3 signaling in immune tolerance. Immunity 2003;19:425–36.
25. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol 2007;7:41–51.
26. Hickstein DD, Baker DM, Gollahon KA, Back AL. Identification of the promoter of the myelomonocytic leukocyte integrin CD11b. Proc Natl Acad Sci U S A 1992;89:2105–9.
27. Sweet MJ, Hume DA. CSF-1 as a regulator of macrophage activation and immune responses. Arch Immunol Ther Exp (Warsz) 2003;51:169–77.
28. Owen JL, Iragavarapu-Charyulu V, Gunja-Smith Z, Herbert LM, Grosso JF, Lopez DM. Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. J Immunol 2003;171:4340–51.
29. Torroella-Kouri M, Lopez DM. Mammary tumor derived TGFβ-1 impairs crucial innate immune response in tumor hosts. J Immunol Immunopathol 2003;5:31–8.
30. Dohadwala M, Batra RK, Luo J, et al. Autocrine/paracrine prostaglandin E2 production by non-small cell lung cancer cells regulates matrix metalloproteinase-2 and CD44 in cyclooxygenase-2-dependent invasion. J Biol Chem 2002;277:50828–33.
31. Kalkhoven E, Kwakkenbos-Isbrucker L, Mummery CL, et al. The role of TGF-β production in growth inhibition of breast-tumor cells by progestins. Int J Cancer 1995;61:80–6.
32. DiNapoli MR, Calderon CL, Lopez DM. Phosphatidyl serine is involved in the reduced rate of transcription of the inducible nitric oxide synthase gene in macrophages from tumor-bearing mice. J Immunol 1997;158:1810–7.
33. Biswas SK, Sica A, Lewis CE. Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. J Immunol 2008;180:2011–7.
34. Lin EY, Li JF, Gnatovskiy L, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 2006;66:11238–46.
35. Rodriguez PC, Quiceno DG, Zabaleta J, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. Cancer Res 2004;64:5839–49.
36. Murphy TL, Cleveland MG, Kulesza P, Magram J, Murphy KM. Regulation of interleukin 12 p40 expression through an NF-κB half-site. Mol Cell Biol 1995;15:5258–67.
37. Plevy SE, Gemberling JH, Hsu S, Dorner AJ, Smale ST. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. Mol Cell Biol 1997;17:4572–88.
38. Hu WH, Mo XM, Walters WM, Brambilla R, Bethea JR. TNAP, a novel repressor of NF-κB-inducing kinase, suppresses NF-κB activation. J Biol Chem 2004;279:35975–83.
39. Mordmuller B, Krappmann D, Esen M, Wegener E, Scheidereit C. Lymphotoxin and lipopolysaccharide induce NF-κB-p52 generation by a co-translational mechanism. EMBO Rep 2003;4:82–7.
40. Biswas SK, Gangi L, Paul S, et al. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-κB and enhanced IRF-3/STAT1 activation). Blood 2006;107:2112–22.
41. Kusmartsev S, Gabrilovich DI. STAT1 signaling regulates tumor-associated macrophage-mediated T cell deletion. J Immunol 2005;174:4880–91.
42. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. Nat Rev Immunol 2004;4:941–52.
43. Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 2007;117:1155–66.
44. Bronte V, Apolloni E, Cabrelle A, et al. Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood 2000;96:3838–46.
45. Mordue DG, Sibley LD. A novel population of Gr-1+-activated macrophages induced during acute toxoplasmosis. J Leukoc Biol 2003;74:1015–25.
46. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 2001;166:5398–406.
47. Ilkovitch D, Lopez DM. Urokinase-mediated recruitment of myeloid-derived suppressor cells and their suppressive mechanisms are blocked by MUC1/sec. Blood. Epub 2009 Feb 4.
48. Saccani A, Schioppa T, Porta C, et al. p50 nuclear factor-κB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. Cancer Res 2006;66:11432–40.