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Tumor-Derived GM-CSF Promotes Granulocyte Immunosuppression in Mesothelioma Patients

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Abstract

Purpose: The cross-talk between tumor cells, myeloid cells, and T cells can play a critical role in tumor pathogenesis and response to immunotherapies. Although the etiology of mesothelioma is well understood, the impact of mesothelioma tumor cells on the surrounding immune microenvironment is less well studied. In this study, the effect of the mesothelioma tumor microenvironment on circulating and infiltrating granulocytes and T cells is investigated.

Experimental Design: Tumor tissues and peripheral blood from mesothelioma patients were evaluated for presence of granulocytes, which were then tested for their T-cell suppression potential. Different cocultures of granulocytes and/or mesothelioma tumor cells and/or T cells were set up to identify the mechanism of T-cell inhibition.

Results: Analysis of human tumors showed that the mesothelioma microenvironment is enriched in infiltrating granulocytes, which inhibit T-cell proliferation and activation. Characterization of the whole blood at diagnosis identified similar, circulating, immunosuppressive CD11b$^+$CD15$^+$HLADR$^-$ granulocytes at increased frequency compared with healthy controls. Culture of healthy-donor granulocytes with human mesothelioma cells showed that GM-CSF upregulates NOX2 expression and the release of reactive oxygen species (ROS) from granulocytes, resulting in T-cell suppression. Immunohistochemistry and transcrip-tomic analysis revealed that a majority of mesothelioma tumors express GM-CSF and that higher GM-CSF expression correlated with clinical progression. Blockade of GM-CSF with neutralizing antibody, or ROS inhibition, restored T-cell proliferation, suggesting that targeting of GM-CSF could be of therapeutic benefit in these patients.

Conclusions: Our study presents the mechanism behind the cross-talk between mesothelioma tumors and the immune microenvironment and indicates that targeting GM-CSF could be a novel treatment strategy to augment immunotherapy in patients with mesothelioma.

Introduction

Malignant mesothelioma is an aggressive cancer arising from the mesothelial cells lining the pleura, peritoneum, and pericardium (1). The majority of patients present with advanced-stage disease and are not candidates for surgery. Although chemotherapy improves outcome for these patients, the median overall survival is less than 24 months (2). Immunotherapy approaches relying on T-cell anticancer activity, such as peptide vaccines and CAR T cells, have shown only limited efficacy, suggesting that the underlying immune microenvironment may play a role in muting the immune response (3, 4).

Myeloid cells play an important role in the balance of pro- and anticancer T-cell responses. Murine models of mesothelioma have shown that monocytes, macrophages, and dendritic cells may be modulated by the tumor microenvironment (5–7). However, the functional role of granulocytes and their mechanism of action in human mesothelioma are not well understood. Studies in mesothelioma have suggested the ratio between peripheral blood or intratumoral neutrophils and lymphocytes correlates with prognosis, indicating a key interaction between these cells in tumor pathogenesis (8). In other cancers, secreted factors within the tumor microenvironment control the differentiation of granulocytes. In turn, this may promote inflammation within the tumor microenvironment or lead to changes in the interaction with the adaptive immune response. Here, we investigate the mechanisms underlying the cross-talk between mesothelioma tumor cells, granulocytes, and T cells.

Materials and Methods

Patients and sample collection

Heparinized blood samples were obtained from patients with malignant mesothelioma ($n = 47$) who were enrolled in IRB-approved protocols at the National Cancer Institute, Bethesda, and the University of Birmingham, UK, before treatment (Table S1). Written informed consent was obtained from all the patients and the study was conducted in accordance with recognized ethical guidelines. Blood from healthy donors was obtained from the NIH Blood Bank ($n = 30$) and at the University of Birmingham, UK ($n = 18$) in heparin tubes. Patients with both histologically confirmed pleural ($n = 24$) and peritoneal ($n = 9$) mesothelioma were included in this study and at the time of enrolment had clinical and/or radiological evidence of disease. A number of patients had received prior treatments including surgery and systemic chemo- or immunotherapy (Table S1). The transcriptomes of 87 mesothelioma tumors diagnosed between 1999 and 2013, held within the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl) were analyzed for CSF2 expression. Patients were aged from 28 to 81 years of age at diagnosis. Fifty-six patients had a history of asbestos exposure, 14 had no history, and 17 were not known. Of the 87 patients' samples histologies were distributed as follows: 23 biphasic, 5 diffuse, 57 epithelioid, and 2 sarcomatoid.

Cell lines

Human mesothelioma cell lines [ED (MSTO211)-H, AC-Meso Y9-Meso, MPM15, MPM26, MPM30, MPM34, and MPM43] purchased from the Aichi Cancer Research Centre Institute and Mesobank UK were cultured in RPMI-1640 (Invitrogen) with 10% heat-inactivated fetal bovine serum, glutamine ($1\times$), sodium pyruvate ($1\times$), and penicillin-streptomycin (RPMI 10% = R10%). The cell lines were cultured in a humidified atmosphere at 37°C with 5% CO_2. All cell lines were verified by Northgene DNA short-tandem repeat analysis within the last 6 months. All cell lines were tested of mycoplasma and were negative. Cell lines were used for up to 5 passages.

Flow cytometric analysis of whole blood and tumors

Whole blood and fresh tumor samples from diagnostic surgery were processed within 12 hours of collection. Ten samples from patients with benign pleural pathologies of infectious and inflammatory nature were also included as a comparison. Whole blood was either lysed using ammonium chloride solution according to the manufacturer's instructions (Qiagen) or using a hypertonic ammonium chloride solution (150 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1mmol/L EDTA) for 10 minutes at room temperature (maintained at 21°C –23°C) at a ratio of 1:9 (volume of sample: volume of lysing solution) prior to antibody staining. Where indicated peripheral blood was separated using a Lymphoprep density gradient. Tissue samples were digested using type II collagenase (Worthington) for 3 hours at 37°C. Immune populations were identified by staining with anti-CD11b, anti-HLA-DR, anti-CD13, anti-CD14, anti-CD15, anti-CD66b, and anti CD45 antibodies (BD Biosciences) on ice or at room temperature for 30 minutes. Cells were acquired using FACS-Canto II (BD Biosciences) and Cyan (Beckman Coulter) and analyzed either by FCS Express 4 software (DeNovo Software) or FlowJo (TreeStar).

Isolation of granulocytes, T cells, and mesothelioma cells for functional assays

The whole blood from healthy donors and patients were processed as described above. We isolated the low-density granulocytes from the peripheral blood mononuclear layer and high-density granulocytes from the layer of white cells on the red cell pellet following Lymphoprep centrifugation by magnetic bead isolation using anti-CD15 microbeads (BD Pharmingen) and MACS LS separation columns (Miltenyi Biotech) according to the manufacturer's instructions. Cell purity was >98% as confirmed by flow cytometry. Cell populations were similarly isolated from collagenase digested tumors using MACS beads (anti-CD15 for granulocytes and anti-CD14 for monocytes), followed by flow cytometric confirmation of purity. The dose of collagenase selected has previously been established to not affect cell surface marker expression or cell viability.

For isolation of autologous T cells and myeloid cells from the whole blood, the target populations were enriched first using positive selection with CD45 magnetic beads (Miltenyi Biotech) followed by staining with myeloid antibodies (above) and anti-CD3 antibody (BioLegend). Cells were sorted on Astrios (Beckman Coulter) using a 100 μm nozzle. DAPI was used as a viability marker to gate out the dead cells.

Granulocyte polarization

To generate tumor-conditioned media (TCM), cell lines or sorted patients' tumor cells were plated (1.5×10^6 cells) and cultured for 72 hours. The conditioned media were removed and filtered prior to use. Following Lymphoprep isolation high-density granulocytes were enriched by CD15 magnetic bead isolation as above, healthy donor granulocytes were plated in R10% in 24-well plates, at concentrations of 1×10^6 per well. TCM was added as 25% of the total volume as indicated. Granulocytes were harvested following 24 hours of culture and washed twice prior to use in suppression assays. Granulocyte viability was confirmed to be >90% in all cases, by flow cytometry, before further experimentation.

Autologous T-cell proliferation assays

Sorted $CD3^+$ T cells were labeled with 10 μmol/L carboxy-fluorescein diacetate succinimidyl ester (CFSE; Life Technologies) and cultured with sorted granulocytes at ratios of 1:0, 1:0.5, 1:1 in complete media at 37°C, 5% CO_2 for 4 days in the presence of 1:1 ratio of anti-CD3/ anti-CD28 dynabeads (Invitrogen). Cells were stained with V450 anti-CD4 (Clone-RPA-T4; BD Biosciences) and APC-Cy7 anti-CD8 (Clone-RPA-T8; BioLegend) and proliferation was determined by CFSE dilution. Unstimulated T cells were used as a negative control. The effect of the addition of L-NMMA (0.5 mmol/L, NG-Methyl-L-arginine acetate), nor-NOHA (0.5 mmol/L, N-Omega-hydroxy-nor-L-arginine) and iNAC (10 mmol/L; all from Sigma- Aldrich) was similarly tested. The percentage of cells that diluted CFSE (divided cells) was determined.

Peripheral blood lymphocyte cell proliferation assay

Peripheral blood lymphocytes (PBL; 2×10^5) were cultured in 96-well flat bottom plates with coated anti-CD3 antibody (3 μg/mL) and anti-CD28 antibody (2 μg/mL), in 200 μL R10%. Cells were incubated at 37°C, 5% CO_2 for 4 days and then 1 μCi/well ^{3}H-thymidine (Perkin Elmer Life Sciences) was added for 12 to 16 hours. ^{3}H-thymidine incorporation was

measured using a TopCount reader (Perkin Elmer). The suppressive ability of autologous or conditioned granulocytes was assessed by coculturing-purified cells together with the PBLs. nor-NOHA (0.5 mmol/L), L-NMMA (0.5 mmol/L), iNAC (10 mmol/L; Sigma-Aldrich) was added to cells in culture. HEPES (25 mmol/L) was added to the medium to maintain the pH after iNAC addition. Data are expressed as a percentage of PBL proliferation driven by antibody costimulation in the presence of MDSC compared with PBL proliferation in the absence of suppressive cells (100%).

Reactive oxygen species (ROS) assay

Sorted granulocytes were stained with $2'$, $7'$-dichlorofluorescein diacetate (DCFDA) using DCFDA cellular ROS detection assay kit (Abcam) for 30 minutes at 37°C. The stained cells were analyzed on a BD FACS Calibur and Cyan (Beckman Coulter). Cells stained with Tert-butyl hydrogen peroxide (TBHP), TCM polarized granulocytes were also incubated with Phorbol 12-myristate 13-acetate (PMA; concentration need to be added) during the staining with DCFDA, this was used as a positive control.

Quantification of H_2O_2 production was measured using the Amplex Red Hydrogen Peroxidase assay kit (Invitrogen). Following culture in mesothelioma conditioned media for 24 hours, sorted granulocytes were washed twice in R10%, counted and plated in Krebs–Ringer phosphate buffer according to the manufacturer's guidelines. Detection of H_2O_2 was carried out following 30 minutes of incubation at 37°C using a microplate reader at 560 nm.

ELISA

The concentrations of cytokines within conditioned media following culture with T cells, mesothelioma cell lines (1×10^6/mL) or sorted tumor cells were quantified using a competitive enzyme-linked immunoassay according to the manufacturers' instructions. The following molecules were tested GM-CSF (Biolegend), IL13 (BD Biosciences), IL8 (BioLegend), IL6 (BioLegend), G-CSF (R&D Systems), VEGF (R&D Systems), Mesothelin (BioLegend). The concentration of IFNγ in coculture supernatants was determined by Ready Set Go ELISA kit (eBioscience).

RT-Q-PCR analysis

RT-Q-PCR was used to detect NOX2 expression in cell line supernatant-conditioned granulocytes (0, 4, 8, 12, 24 hours' time points). RNA was extracted using an RNeasy Mini kit (Qiagen). cDNA was prepared using SuperScriptTM III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions. RT-Q-PCR was done in duplicate using FAST SYBR Green Master Mix (Applied Biosystems) and the Applied Biosystems 7500 Fast Real-Time PCR system. Analysis of gene expression was calculated according to the $2^{-\Delta T}$ method and plotted as arbitrary units of mRNA relative to GAPDH. Gene specific primer sequences were NOX2 (CAAGATGCGTGGAAACTA, F; TCCCTGCTCCCACTAACA, R) and GAPDH (CCAGCCGAGCCACATCGCTC, F; ATGAGCCCCAGCCTTCTC, R; Eurofins).

Immunohistochemistry

Mesothelioma sections from diagnostic tumor biopsies ($n = 38$) were deparaffinized in Histoclear (National diagnostics) and ethanol and rehydrated in 0.3% hydrogen peroxide for 15 minutes. Antigen retrieval was performed in 10 mmol/L sodium citrate buffer (pH 6.0) for 20 minutes in amicrowave oven. Slides were cooled and washed prior to blocking in $5 \times$ Caesin (Thermo Fisher Scientific) for 30 minutes at room temperature. Sections were then incubated over night with primary antibody, rabbit anti–GM-CSF (Novus Biologicals), diluted in PBS. Sections were washed and secondary antibody (Universal ImmPRESS antibody, Vector Laboratories) was added at room temperature for 30 minutes followed by further washing and addition of DAB substrate (ImmPACT DAB, Vector Laboratories) for 5 minutes. After coun-terstaining with Harris hematoxylin (Sigma-Aldrich), slides were dehydrated using ethanol and Histoclear and mounted using Omnimount (National Diagnostics). Slides were examined and photographed using a Nikon Eclipse 400 microscope.

Statistical analysis

Continuous parameter values were compared between two groups using an exact form of a Wilcoxon rank sum test. Paired comparisons were performed using a Wilcoxon signed rank test. Spearman correlation analysis was used to determine the correlation between age and MDSC parameters. The correlations are interpreted as follow: strong if $|r| > 0.70$; moderately strong if $0.50 < |r| < 0.70$; weak to moderately strong if $0.30 < |r| < 0.50$; weak if $|r| < 0.30$. All P values are two-tailed and presented without adjustment for multiple comparisons because all tests performed were considered to be exploratory.

Results

Mesothelioma tumors modulate infiltrating myeloid cells to suppress T-cell responses

The immune microenvironment in mesothelioma has been shown to have strong prognostic implications with infiltration by CD8+ lymphocytes conferring a favorable prognosis (9) and the association of peripheral blood granulocyte-to-lymphocyte ratio with poorer prognosis (10–12). However, our understanding of the biological cross-talk between mesothelioma cells, granulocytes, and T cells in human patients is limited.

Interrogation of the transcriptomic profile of 87 mesothelioma tumors, held within the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl), suggested a significant infiltration of immune cells in the tumor microenvironment (Fig. 1A). Flow cytometric analysis of 18 digested human mesothelioma tumors confirmed this data at the cellular level within the tumor microenvironment (mean: CD15 = 8.6%, CD14 = 4.8%, CD3 = 6.7%; Fig. 1B). Immuno-histochemical staining of a further 38 mesothelioma tumors supported the findings, with identification of granulocytes in all samples at diagnosis (Fig. 1C). characterization of tumor-associated granulocytes by flow cytometry showed that they expressed CD11b, CD15, with low/absent CD33 expression, and low/absent HLA-DR expression (Fig. 1D). Murine model of mesothelioma recently identified that the immune-regulatory tran-scriptome of granulocytes may be altered within the tumor microenvironment with potential effects on surrounding T cells and tumor cells (13). To

investigate the effects of granulocytes on T cells, $CD15^+$ granulocytes were sorted from mesothelioma tumors at the time of resection and cocultured with T cells from healthy donors. Tumor-derived granulocytes suppressed T-cell proliferation to a greater extent compared with those from healthy-donor blood or those extracted from pleural tissue with benign pathologies (Fig. 1E). Similarly, $CD14^+$ monocytes from mesothelioma tumors were sorted and also found to suppress T-cell proliferation ($P = 0.0002$; Supplementary Fig. S1A). Therefore, the tumor microenvironment is able to locally modulate infiltrating myeloid cells to inhibit T-cell proliferation.

Mesothelioma creates a systemic immunosuppressive environment through circulating granulocytes

The effects of mesothelioma tumors on the immune system may be limited to the local tissue microenvironment or could also lead to systemic alteration. To test this, we compared T cells from the blood of patients at diagnosis to those from healthy donors and observed that mesothelioma patients' T cells have a reduced proliferation capacity compared with those in healthy donors (Fig. 2A). We have previously identified that tumor metabolism of arginine can create a systemic environment inhibitory to T-cell responses. As mesotheliomas are known to be arginine auxotrophs, we measured the arginase activity of mesothelioma cell lines (14). No significant arginase activity was identified suggesting an alternative mechanism must be responsible (Supplementary Fig. S1B). As we identified immunosuppressive granulocytes infiltrating mesothelioma tumors, we hypothesized that the T-cell suppression may be due to the presence of these circulating immunosuppressive myeloid cells.

To investigate the hypothesis, the frequency of granulocytic and monocytic cells was characterized in the whole blood of healthy donors and mesothelioma patients ($n = 33$) at diagnosis (Table S2). There were significant increases in the percentage of HLA-DR-granulocytes ($CD14^-CD15^+CD11b^+HLADR^-$) in the whole blood compared with healthy controls ($P = 0.013$; Fig. 2B). Subpopulation analysis revealed only a marginal difference in HLA-DR- monocytes ($CD14^+CD15^-CD11b^+HLADR^-$) compared with healthy donors ($P = 0.05$; Fig. 2C) and no difference in the frequency of HLA-DR$^+$ monocytes ($CD14^+CD11b^+HLADR^+$; median 7.0% vs. 8.9%; $P = 0.14$) or HLA-DR$^+$ granulocytes ($CD15^+CD14^-CD11b^+HLADR^+$) cells (median 0.83% vs. 1.46%; $P = 0.29$) between healthy donors and mesothelioma patients. Consistent with reports of increased granulocyte:lymphocyte ratios in mesothelioma patients, granulocytes were the most predominant population overall (median frequency: $CD14^+$ monocytes: 0.198% vs. $CD15^+$ granulocytes: 66.8%). Immunophenotyping revealed significant differences in the relative expressions of granulocyte markers CD11b and CD66b compared with those from healthy donors (Fig. 2D; Supplementary Fig. S1C).

As $CD15^+$ granulocytes are the major population of circulating myeloid cells in mesothelioma patients and shared the same immunophenotype as tumor-infiltrating granulocytes described above, their functional effects on T cells was examined further. Coculture of sorted whole blood granulocytes from patients decreased both autologous

CD4$^+$ and CD8$^+$ T-cell proliferation at ratios of 1:1 and 1:0.5 (T cells:granulocytes; Fig. 2E; Supplementary Fig. S1D) and activation ($P = 0.0078$, Fig. 2F).

Immunosuppressive granulocytes may be methodologically identified in the PBMC layer (classical G-MDSC; low-density) and on the red cell pellet (high density) following density gradient centrifugation of whole blood. Analysis of separated whole blood identified a significant increase in the frequency of granulocytes within the PBMC layer; however, the frequency is extremely low (median <10%) with 90% of the granulocytes lying on the red cell pellet (Fig. 3A). This small population of granulocytes had only a weak ability to suppress T-cell proliferation (Fig. 3B). Only the high-density granulocytes, which had increased frequency in the patients, had suppressive activity (Fig. 3B). Granulocytes from healthy donors had minimal effects on T-cell proliferation (Fig. 1E; Supplementary Fig. S2A) or IFNγ release (Supplementary Fig. S2B). Therefore, granulocytes within the blood and tumors of mesothelioma patients share the same immunophenotype and functional capacity to suppress T-cell proliferation and activation, thus extending the immunosuppressive microenvironment.

Mesothelioma-conditioned granulocytes suppress T-cell proliferation by generation of ROS

Granulocytes can impair T-cell proliferation through a number of mechanisms including arginine depletion, nitric oxide species or ROS production, and release of immunosuppressive cytokines (15–19). We examined iNOS and arginase I expression in patients' granulocytes and those from healthy donors identifying no significant differences in expression (Fig. 3C). The addition of the arginase or iNOS inhibitors, nor-NOHA or L-NMMA respectively, to sorted patients' granulocytes did not rescue T-cell proliferation thus excluding these mechanisms (Fig. 3D). No evidence for immunosuppressive cytokine release from these cells was identified in patient plasma by ELISA (IL10, IL1β, IL4, IL13; Supplementary Fig. 2C). In a mesothelioma murine model, ROS have been demonstrated to suppress T-cell responses (20). Gating on granulocytes identified that mesothelioma patients upregulate ROS compared with healthy controls ($P = 0.03$; Fig. 3E; Supplementary Fig. S2D). Addition of the ROS inhibitor iNAC to sorted patients' granulocytes restored both autologous CD4$^+$ and CD8$^+$ T-cell proliferation (Fig. 3F; Supplementary Fig. S2E) and IFNγ release (Fig. 3G). We confirmed that ROS production was reduced by the addition of the inhibitor iNAC ($P = 0.031$; Fig. 3H). PDL1 is another mechanism that myeloid cells may use to modulate T cells. There was no significant difference in the frequency of CD15$^+$PDL1$^+$ cells in the blood or tumors of patients compared with those from healthy controls (Supplementary Fig. S2F). Correlating the frequency of PDL1$^+$CD15$^+$ cells with CD3$^+$ frequency revealed no significant correlation in the blood ($P = 0.4976$, $r = -0.3214$; Supplementary Fig. S2F), but there was a significant correlation in the tumor ($P = 0.0583$, $r = 0.8286$; Supplementary Fig S2G and S2H). The findings suggest that in the tumor, granulocyte PDL1 may be a secondary mechanism of modulating T-cell numbers inside the tumor microenvironment, but not peripherally.

GM-CSF from mesothelioma tumor cells drives granulocyte ROS production

Although it is recognized that mesothelioma tumors release G-CSF, which may contribute to granulocyte expansion and recruitment, the mechanism by which granulocytes are polarized

to upregulate ROS production and suppress T cells is unknown (21, 22). Granulocytes produce ROS through the activity of NADPH oxidase enzyme (NOX2) expression. Consistent with this, we demonstrated that NOX2 is expressed in patients' granulocytes and healthy donors (Fig. 4A). To examine the effect of the mesothelioma microenvironment on granulocytes, healthy-donor-derived granulocytes were cultured in the conditioned supernatants of mesothelioma cell lines or primary tumors. Conditioned supernatants led to an upregulation of NOX2 expression over time (Fig. 4B), with accompanying increase in the production (Fig. 4C; Supplementary Fig. S3A and S3B) and release of ROS (Fig. 4D). PMA was used as positive control for ROS induction in granulocytes upregulation, confirming the mesothelioma specific mechanism (Supplementary Fig. S3C). The mesothelioma-conditioned granulocytes showed a strong ability to suppress T-cell proliferation (Fig. 4E), which could be rescued by the addition of iNAC (Fig. 4F). LOX-1 has been reported to be a marker for some granulocytic MDSCs (23). Conditioned media led to no change in LOX-1 on healthy-donor granulocytes (Supplementary Fig. 3D). Therefore, mesothelioma cells signal to granulocytes to modulate their function.

To identify the nature of the mesothelioma-granulocyte cross-talk, mesothelioma-conditioned supernatant was first boiled to denature all proteins. Boiled supernatant lost the ability to polarize granulocytes to suppress T-cell proliferation (Supplementary Fig. S3E) consistent with the release of a soluble molecule from the mesothelioma cells. Arginine depletion, a potential mechanism of polarization due to mesothelioma arginine auxotrophism, similarly did not polarize healthy-donor granulocytes to produce ROS consistent with this finding (Supplementary Fig. 3F).

ELISAs for cytokines involved in granulocyte signaling were performed of supernatants from mesothelioma cell lines and mesothelioma human primary tumor cells identified a number of key molecules were highly expressed—G-CSF, GM-CSF, IL13, IL6, IL8, VEGF, PGE2, mesothelin (Fig. 5a). In particular mesothelioma cells release IL8 (neutrophil chemotactic protein; mean concentration 981 pg/mL) and G-CSF (mean concentration 283 pg/mL), which are known to attract granulocytes into the tumor microenvironment. No evidence of serum amyloid-A release from mesothelioma cells was found. Interrogation of the R2 database confirmed a similar cytokine expression profile from 87 human tumors (Fig. 5B). Culture of healthy-donor granulocytes with individual recombinant cytokines identified above showed that GM-CSF led to the highest upregulation of ROS production (Fig. 5C) with associated upregulation in NOX2 expression (Supplementary Fig. S3G). Granulocytes treated with recombinant GM-CSF inhibited T-cell proliferation (Fig. 5D and E), and T-cell proliferation was rescued by the inhibitor (iNAC) or removal of ROS with catalase (Fig. 5E). No increases in plasma GM-CSF concentrations are identified in patients at diagnosis, suggesting that the intratumoral release of GM-CSF drives the ROS upregulation in granulocytes (Supplementary Fig. S4A). Addition of anti-GM-CSF–neutralizing antibody to mesothelioma cell line cocultures inhibited ROS upregulation (Fig. 6A and B) and the release of peroxide species in conditioned granulocyte supernatants (Supplementary Fig. S4B) confirming mesothelioma-released GM-CSF drives granulocyte ROS production and T-cell–suppressive activity. The addition of anti-GM-CSF–neutralizing antibody rescued T-cell proliferation, confirming the mechanism of mesothelioma polarization of granulocytes (Fig. 6C).

ROS production from conditioned healthy granulocytes correlated with the concentration of GM-CSF in tumor-conditioned media (r = 0.438, P = 0.0118; Fig. 6D). Immunohistochemistry of mesothelioma tumors confirmed that GM-CSF is expressed within the tumor microenvironment of patients (Fig. 6E; Supplementary Fig. S4C) and transcriptomic analysis of 87 primary tumor samples within the R2: database demonstrated that GM-CSF is expressed in over 50% of the samples and does not correlate with histological subtype (Fig. 6F).

In summary, mesothelioma creates an immunosuppressive microenvironment locally and systemically through the release of GM-CSF from tumor cells, which induces granulocyte ROS production to inhibit T-cell function.

Discussion

Although the role of monocytes and macrophages in mesotheliomas has previously been well documented in human tissue and murine models, granulocytes have received little attention. In this study, we focused on human mesotheliomas, identifying the mechanism by which tumor cells modulate granulocyte function to suppress T-cell responses. A previous immune-histochemical study identified that high CD4 T-cell counts or low neutrophil counts within mesothelioma tumors are linked to better patient outcomes (24). To evaluate the seemingly reciprocal relationship between granulocytes and T lymphocytes, we first confirmed that granulocytes make up a significant proportion of infiltrating immune cells with relatively fewer T cells. To date, granulocyte function in mesotheliomas has almost exclusively been studied in murine cell line xenografts. Murine granulocytes may be alternatively activated in mesotheliomas (N1 vs. N2) or characterized as granulocytic MDSCs (G-MDSC; refs. 13, 20, 25). In all of these murine cases, the granulocytic cells express ROS—a well-established mechanism of T-cell suppression (26). We carefully considered whether our tumor-infiltrating and -circulating granulocytes could be G-MDSC according to recent guidelines for nomenclature which define MDSCs based on immunophenotype, density, and suppressive activity (27). In our mesothelioma patients, both circulating and tumor-associated granulocytes were CD11b^{+}CD14^{−}CD15^{+}/CD66b^{+} fitting with the G-MDSC phenotype. However, blood G-MDSCs are classically described as being low-density cells following separation with density centrifugation. We showed that in mesothelioma patients' blood, low-density granulocytes are a minority population within the PBMC layer and have minimal T-cell suppressive activity—thus the two populations are distinct. In addition, no evidence of altered LOX-1 expression, a marker recently identified on low-density G-MDSCs, was found after mesothelioma conditioning of healthy-donor granulocytes (23). The most suppressive granulocytes are those of high density and act through ROS release. The intratumoral granulocytes we studied share the same immunophenotype and suppressive mechanism. As discussed in the consensus recommendations based on current technology, there is no unique marker to distinguish suppressive granulocytes from G-MDSC, particularly for intra-tumoral cells. Notably, the need to use cell density on separation as a method to define immune cell subsets is extremely limited, and alternative methodologies will be developed for the future characterization of these cells. Our findings highlight the plasticity of granulocytes in humans and their place in regulating the tumor-associated immune microenvironment (13).

Similar examples of human cancer–associated, immuno-suppressive granulocytes, as opposed to G-MDSC, have been identified in melanoma and non–small lung cancer, driven through the release of tumor-derived factors (15, 28).

Our analysis of the supernatants of mesothelioma cell lines and primary tumor tissue revealed a cytokine profile consistent with granulocyte attraction and modulation within the tumor microenvironment. A number of factors have been reported to modulate granulocyte function in murine models of mesothelioma. In a murine model of mesothelioma, prostaglandin inhibition reduced the number of granulocytic MDSCs (20). TGFβ within murine mesothelioma tumors also drives the expression of the chemokines CCL3, CCL5, and CCL2 in pro-tumoral granulocytes (29). For humans, no direct mechanism of mesothelioma modulation of granulocytes has been shown although the mesothelioma-inducing mineral erionite can directly stimulate ROS production in healthy-donor–derived neutrophils (30). IL8 (CXCL8) is a potent proinflammatory cytokine and is primarily known for its chemotactic and activating action on neutrophils, along with inhibition of normal neutrophil apoptosis (31–33). Our finding of moderate levels of IL8 released from mesothelioma cells may contribute to the enhanced granulocytes infiltration of mesothelioma tumors. Targeting of IL8 in models of tumors such as fibrosarcoma and prostate carcinoma prevents the influx of host neutrophils (34). IL8 is also an autocrine growth factor in a number of cancer types (35–37) including mesothelioma (38).

We identified that meostheliomas can also release G-CSF, a second well-established cytokine that induces granulocyte infiltration. Notably, G-CSF production by mesothelioma is reported to confer a more aggressive phenotype (39–41). Although we confirmed mesothelioma tumors release IL8 and G-CSF or prostaglandins, these factors had no impact in generating suppressive granulocytes. Instead, we demonstrated that GM-CSF is expressed by mesothelioma tumor cells and can polarize granulocytes to upregulate ROS production. No differences in the effect of granulocyte-derived ROS was found on CD4+ versus CD8+ T cells. Establishment of cell lines from primary mesotheliomas has reported significant production of GM-CSF (42) and this cytokine can drive suppressive granulocyte activity in murine models for a number of solid tumors (43–45). Although we identified GM-CSF was widely expressed in our samples studied, the effects of prior therapies in our patient population on GM-CSF expression is unknown.

Clinically, GM-CSF has been used as an alternative to G-CSF to support myeloid cell recovery postchemotherapy in mesothelioma patients (46, 47). No differences in outcome were reported for the two growth factors, although the effects on immune parameters are not available. Recombinant GM-CSF has also been administered alongside a tumor vaccine in this patient group (48–50) and used alongside immunotherapy approaches in neuroblastoma. In the mesothelioma studies, GM-CSF was administered to patients in all study arms, regardless of whether they received the investigational tumor/peptide vaccines or not. Although responses are noted, it is not possible to understand whether the cytokine had any effect on outcomes both within the trial populations or compared with historical controls. It is possible that administration of GM-CSF may inhibit antitumor T-cell responses, through the induction of G-MDSC, contributing to the lack of clinically relevant T-cell responses seen in these patients. In two trials where GM-CSF was administered intralesionally to

mesothelioma, neutrophil infiltration and maturation were enhanced; however, this was not associated with tumor responses in the majority of patients (51, 52). Indeed, a phase II clinical trial in neuroblastoma demonstrated difference in prognosis if GM-CSF is administered intravenously versus subcutaneously, which could affect the dose-dependent effects of this cytokine on granulocyte phenotype (53, 54).

Preclinical studies that block GM-CSF have resulted in reversal of T-cell inhibition by MDSCs in the setting of pancreatic tumors and improvements in phenotype in inflammatory disease models (55, 56). Our data suggest that targeting the GM-CSF pathway may be of benefit in mesothelioma. Clinically relevant approaches to target GM-CSF have been focused on inflammatory diseases (57). Mavrilimumab (CAM-3001) is a human anti–GM-CSF receptor—an antibody that has completed phase I and II clinical trials in the setting of rheumatoid arthritis (58). Our findings suggest that anti–GM-CSF or anti–GM-CSF receptor antibodies could play a critical role in mesothelioma treatment, particularly alongside T-cell immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

The functional role of granulocytes and their cross-talk with tumor cells and T cells in human mesothelioma is not well understood. We demonstrated that GM-CSF is expressed by mesothelioma tumor cells and can polarize granulocytes to upregulate reactive oxygen species production, which in turn suppresses the T-cell proliferation and function. As GM-CSF plays a role in driving an immunosuppressive granulocyte phenotype in mesothelioma, targeting GM-CSF could represent an alternative therapeutic approach for these patients.
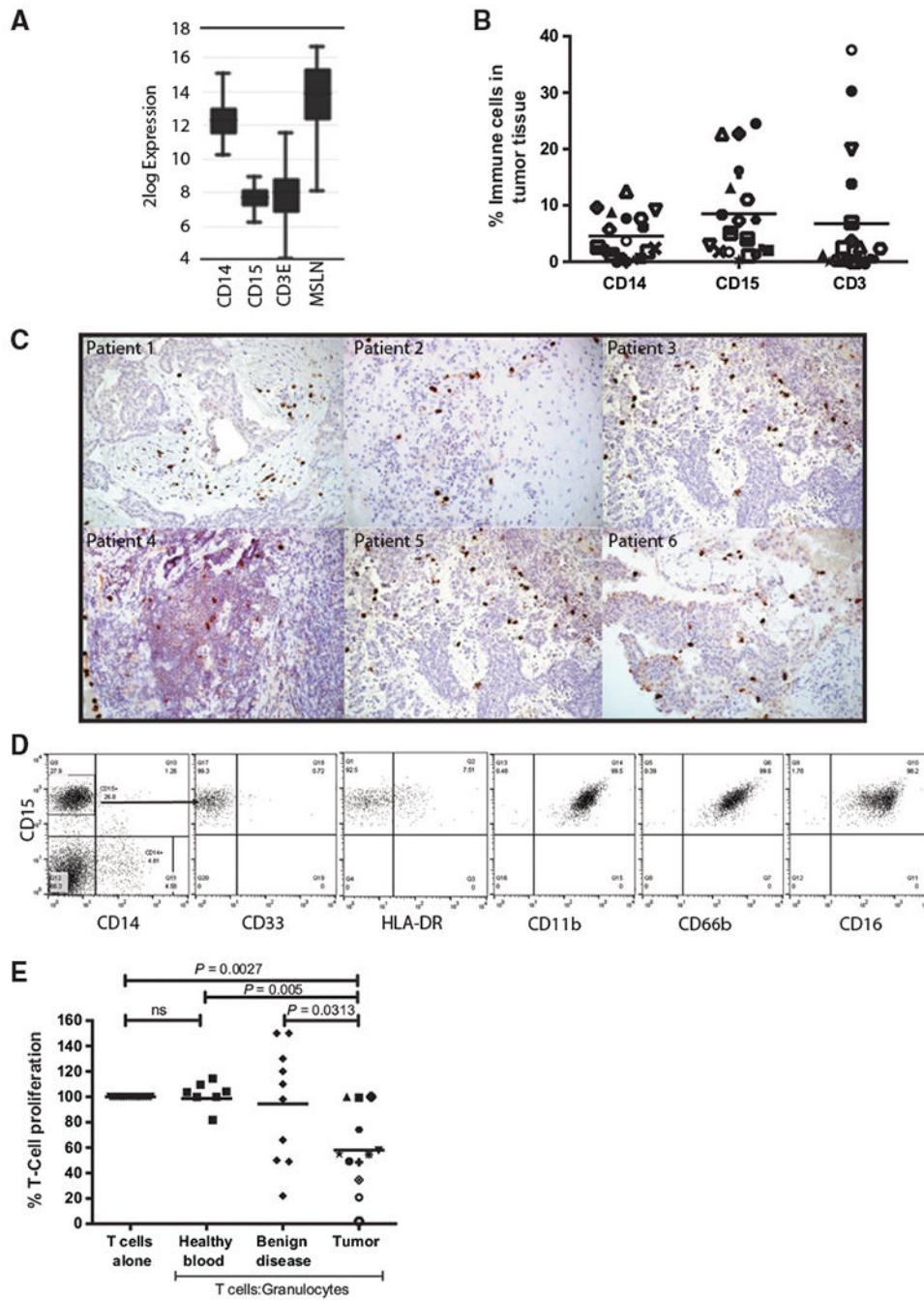
Figure 1.
Granulocytes in tumor tissue suppress T-cell proliferation. **A,** Transcriptomic expression of CD14, CD15, CD3E, and Mesothelin in 87 mesothelioma tumors from the R2: Genomics Analysis and Visualisation Platform (http://r2.amc.nl). **B,** Percentages of CD15+, CD14+, and CD3+ cells detected by flow cytometry in the digested tumor tissue of 18 patients with confirmed mesothelioma. **C,** Representative tumor sections from 6 patients (total stained = 38 tumors) demonstrating infiltration of CD15 expressing cells within mesothelioma tumors. Images were taken at 20× magnification. **D,** Immunophenotype of tumor-associated

granulocytes by flow cytometry identified that they expressed CD11b, CD15, with low/absent CD33 expression, and low/absent HLA-DR expression. **E,** T-cell proliferation from healthy donors is suppressed following culture with CD15$^+$ granulocytes (representative 1:0.5 ratio) sorted from mesothelioma tumors, compared with those cultured in complete media alone, with CD15$^+$ granulocytes from the blood of healthy donors, or from pleural tissue with benign pathologies.
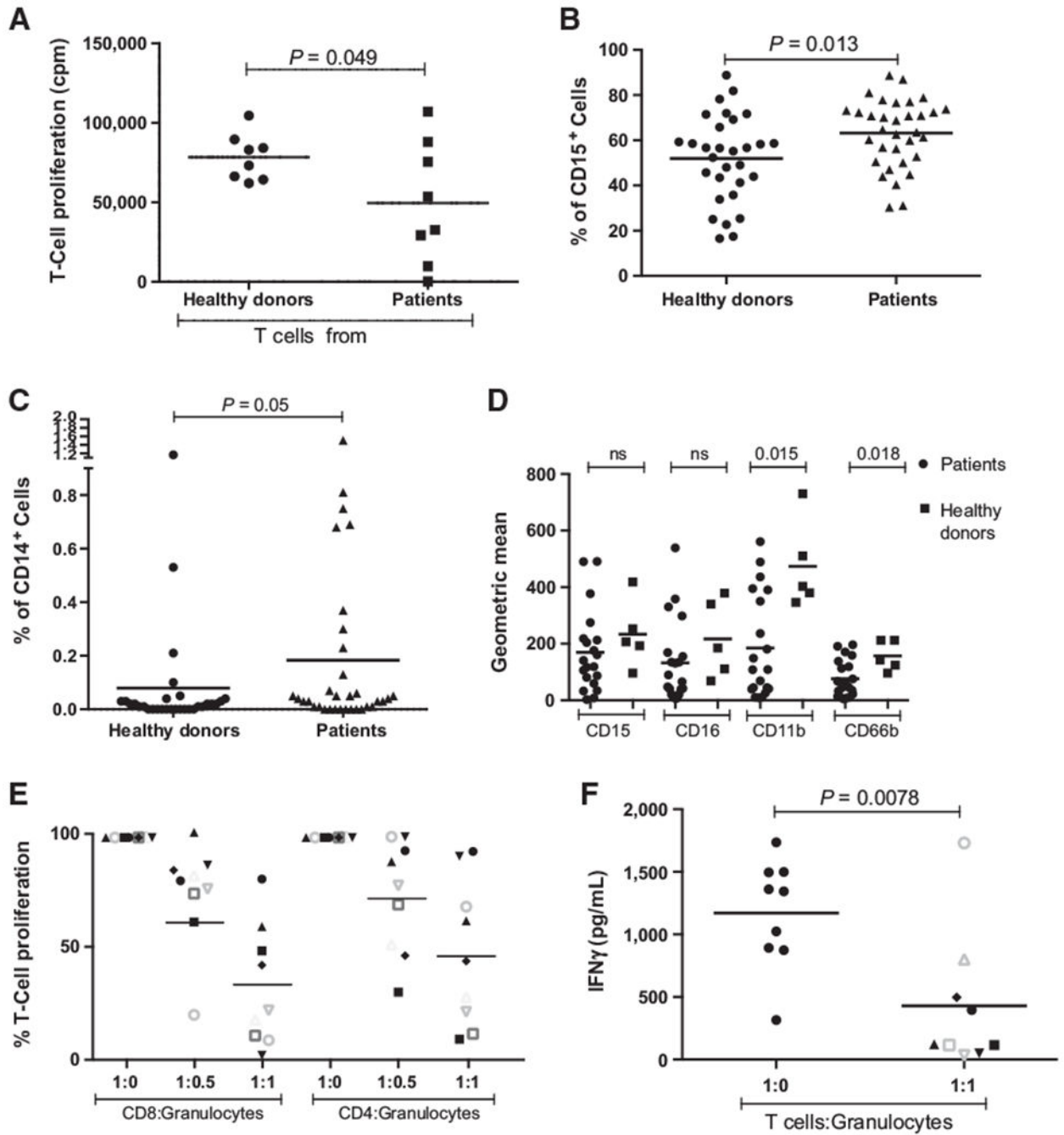
Figure 2.
Granulocytes are elevated in peripheral blood of mesothelioma patients and suppress T-cell proliferation and activation. **A,** Sorted CD3$^+$ T cells from the blood of mesothelioma patients (6 untreated, 2 with prior therapy) have reduced proliferative capacity compared with those sorted from the blood of healthy donors. **B,** Increased frequency of CD15$^+$ granulocytes in the peripheral blood of mesothelioma patients ($n = 33$) and healthy donors ($n = 30$) at diagnosis. **C,** Marginal increased frequency of CD14$^+$HLADR$^-$ monocytes in the peripheral blood of mesothelioma patients ($n = 33$) and healthy donors ($n = 30$). **D,**

Expression of CD15, CD16, CD11b, and CD66b markers on granulocytes of healthy donors and mesothelioma patients, as detected by flow cytometry. **E,** Autologous CD4$^+$ and CD8$^+$ T-cell proliferation is suppressed following culture with CD15$^+$ granulocytes sorted from the blood of patients at diagnosis. T cells and granulocytes were cocultured at ratios of 1:0.5 and 1:1, respectively, and compared with T cells alone (1:0). **F,** T-cell-derived IFNγ release in culture supernatants is significantly impaired following coculture with CD15$^+$ granulocytes sorted from the blood of patients at diagnosis.
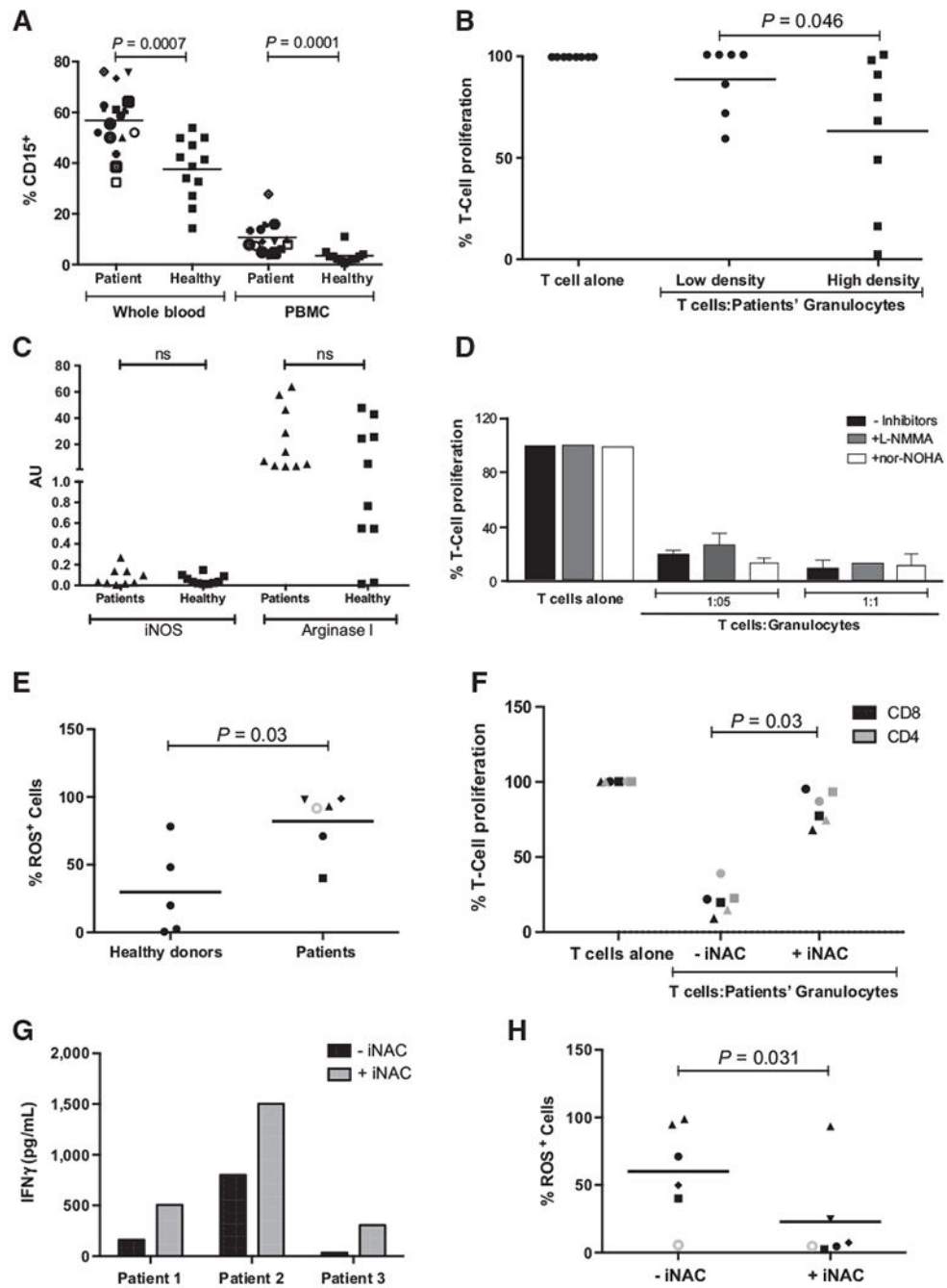
Figure 3.
Granulocytes from mesothelioma patients suppress T-cell proliferation through ROS. **A,** The frequency of CD11b+CD15+ cells was compared in the whole blood and PBMC layer following Lymphoprep separation for 18 mesothelioma patients and 12 healthy donors. The majority of CD15+ granulocytes lie on the red cell pellet following Lymphoprep separation. **B,** Healthy donor T-cell proliferation is most suppressed following culture in the presence of blood CD15+ granulocytes from mesothelioma patients which have been collected from the red cell pellet (high density) after Lymphoprep separation. Low-density granulocytes

isolated in the PBMC layer of the same blood samples were comparatively less suppressive to T-cell proliferation. **C,** QPCR analysis of the expression of iNOS and arginase in granulocytes sorted from healthy donors or patients. **D,** T-cell proliferation is not restored by the addition of L-NMMA or nor-NOHA to the cultures in the presence of CD15$^+$ granulocytes from patients. Two representative patients are shown. **E,** Increased frequency of ROS$^+$ CD15$^+$ granulocytes in the blood of patients from mesothelioma patients compared with healthy donors. **F,** Inhibition of NOX2 activity with iNAC reversed the suppressive effect of granulocytes on CD4 and CD8 T-cell proliferation. **G,** Inhibition of NOX2 activity with iNAC restored T-cell activation, as measured by IFNγ release into cell supernatants. **H,** Culture of patients' granulocytes with iNAC reduced the intracellular production of ROS confirming the known specificity of drug action.
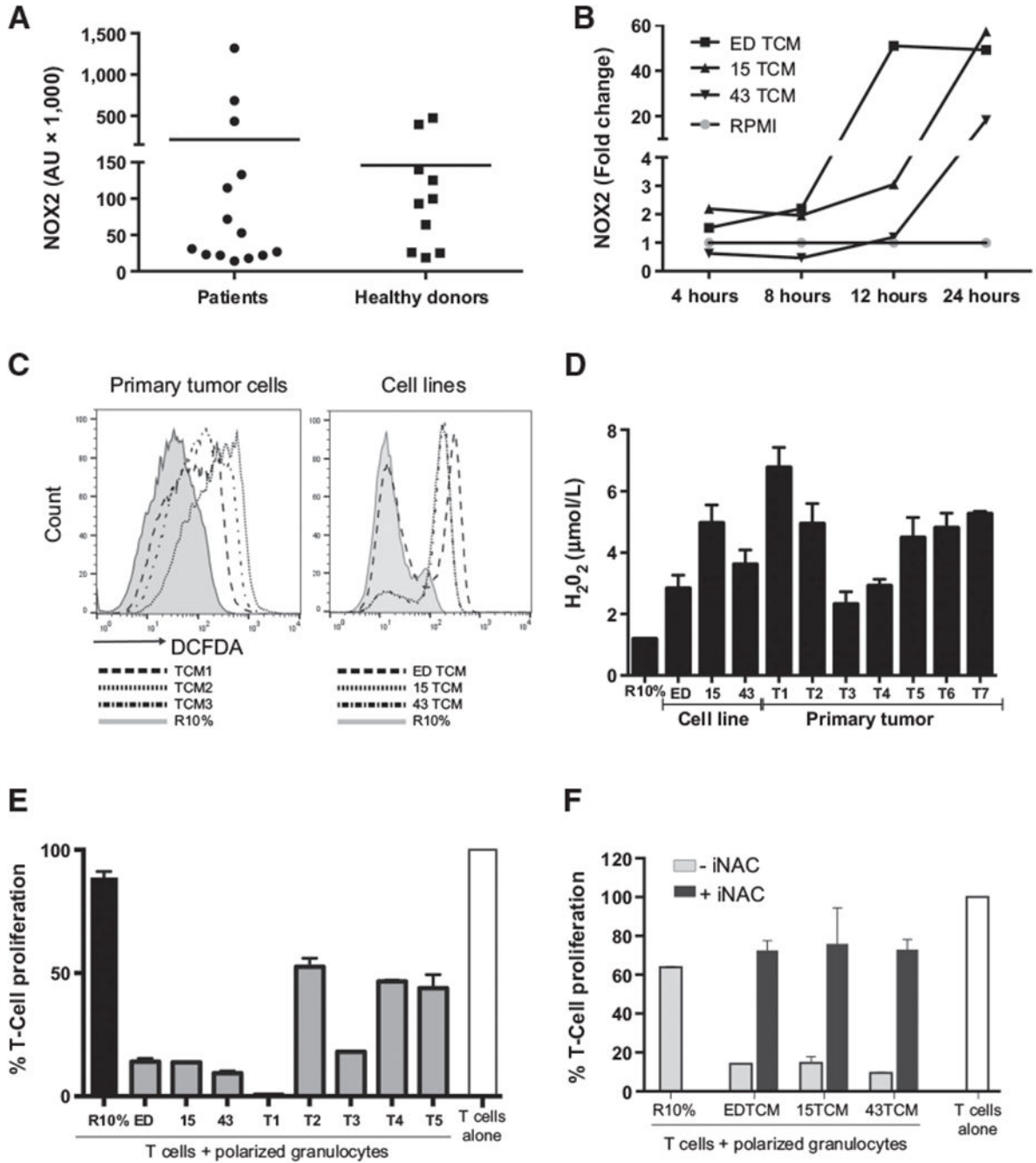
Figure 4.
ROS generation by granulocytes is upregulated by the mesothelioma microenvironment. **A,** Expression of NOX2 by qRT-PCR in CD15^{+} cells from the blood of healthy donors and mesothelioma patients. **B,** NOX2 expression in CD15^{+} granulocytes is upregulated over time following coculture with mesothelioma cell lines, as assessed by qRT-PCR. **C,** ROS production is upregulated in healthy-donor-derived granulocytes following culture in conditioned media from sorted mesothelioma malignant cells or mesothelioma cell lines, compared with complete RPMI. ROS species are detected by DCFDA staining and flow

cytometry. **D,** Release of ROS from CD15$^+$ granulocytes is upregulated after culture with mesothelioma cell lines or sorted mesothelioma malignant cells as detected by hydrogen peroxide species, using a colorimetric assay. **E,** T-cell proliferation is significantly inhibited following culture with cell line conditioned- or tumor-conditioned granulocytes. Comparison made with T cells cultured with granulocytes conditioned by completed media alone. **F,** Treatment of mesothelioma cell line conditioned granulocytes with iNAC prevents suppression of T-cell proliferation.
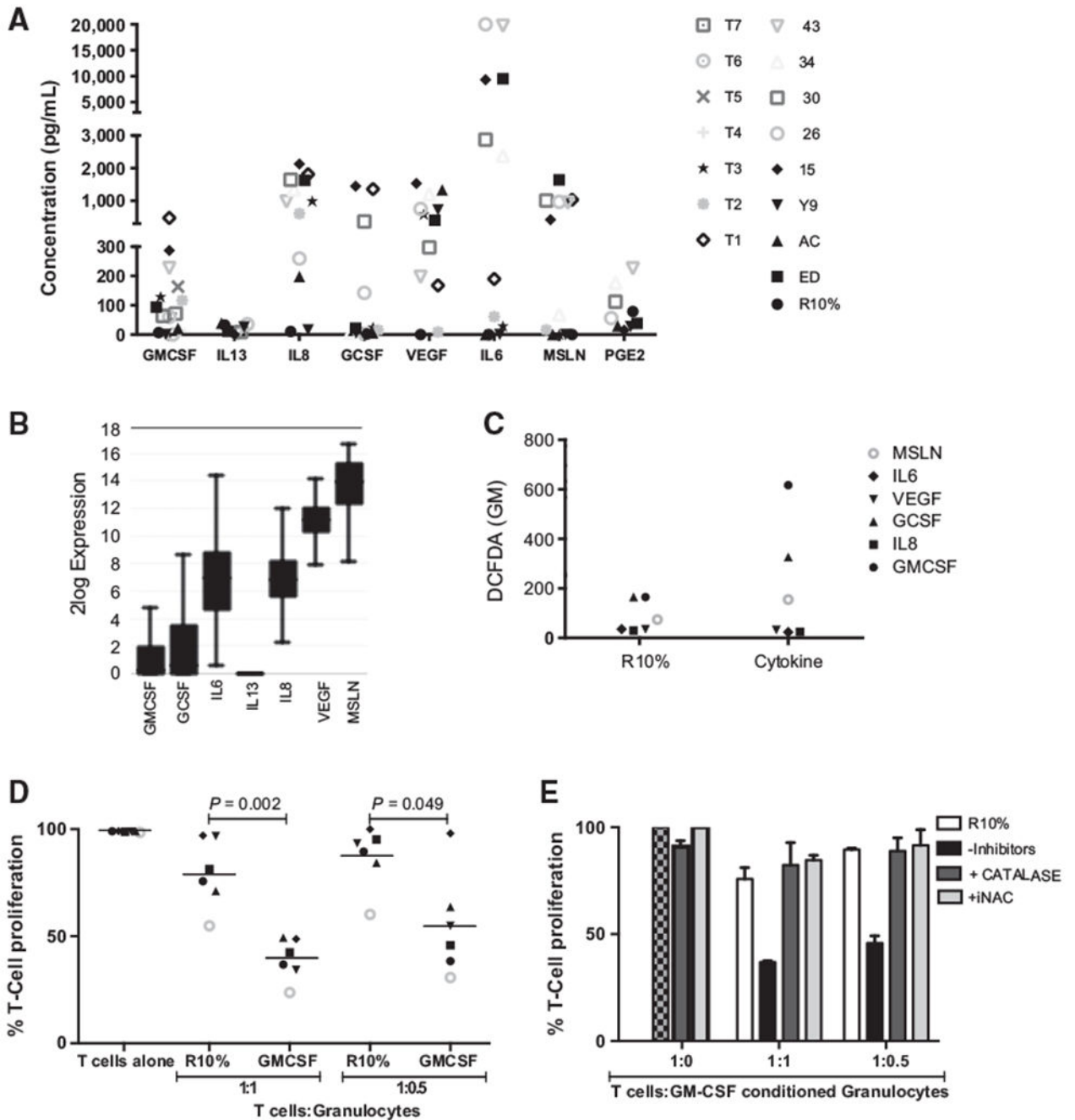
Figure 5.

Mesothelioma cells release GM-CSF to upregulate granulocyte ROS and suppressive activity. **A,** Cytokine multiplex assay determined the cytokine profile of tumor cell supernatants and cell line supernatants. Increased concentrations of GM-CSF, IL8, GCSF, VEGF, IL6, and mesothelin are found. Low concentrations of prostaglandin E2 and IL13 were detected. **B,** Transcriptomic expression of GMCSF, GCSF, IL6, IL13, IL8, VEGF, and mesothelin in 87 mesothelioma tumors from the R2: Genomics and Visualisation Platform. **C,** ROS production (DCFDA staining) by healthy donor CD15+ cells treated with detected

cytokines to determine which were capable of enhancing ROS production. GM-CSF increased ROS production most prominently. **D,** T-cell proliferation was significantly suppressed by granulocytes conditioned with recombinant GM-CSF, compared with control granulocytes. Ratios of 1:1 and 1:0.5 T cells:granulocytes shown. **E,** Inhibition of granulocyte ROS production (iNAC) or accumulation (catalase) after healthy-donor granulocytes were conditioned with GM-CSF restores T-cell proliferation compared with controls.
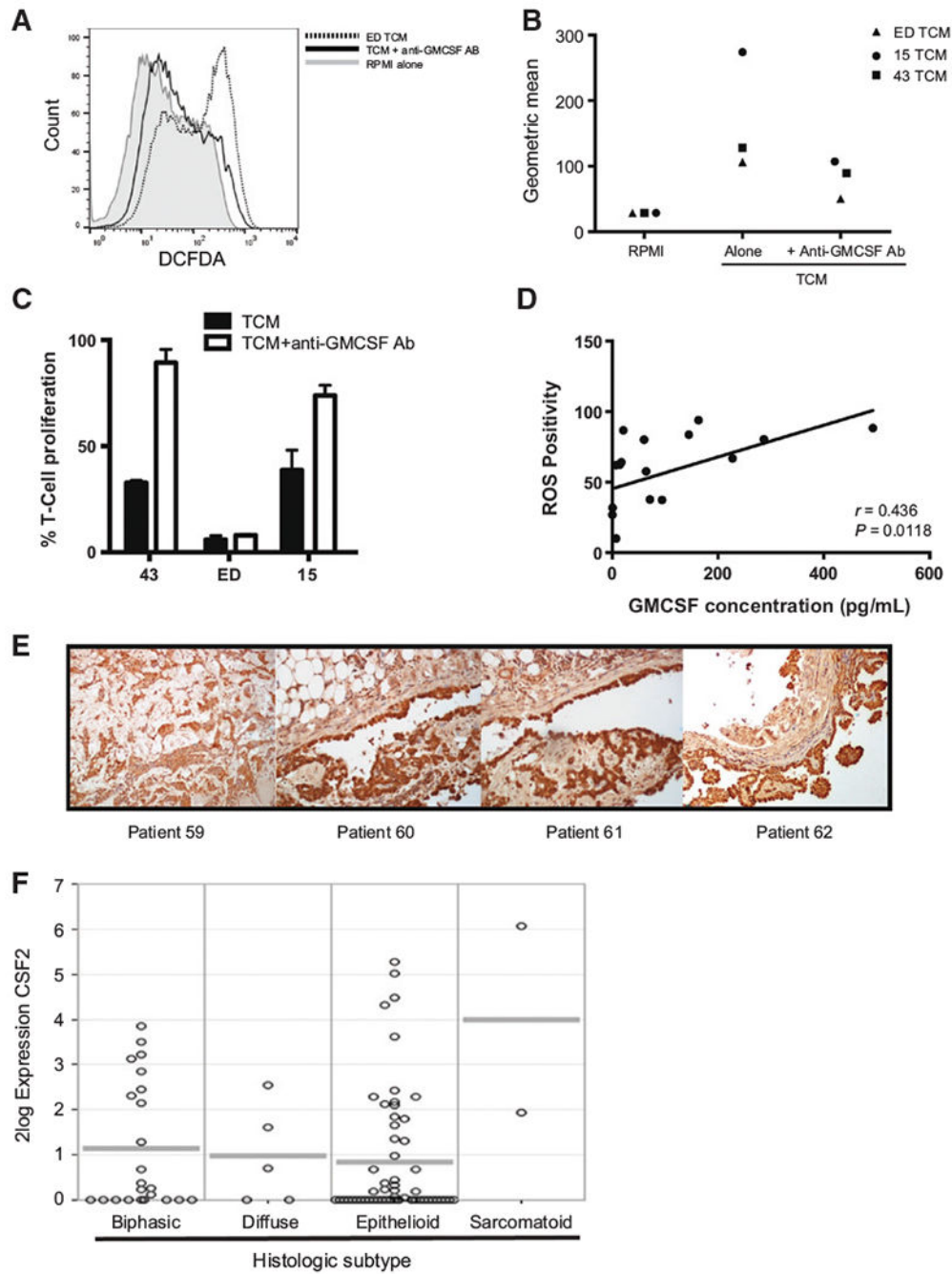
Figure 6.
Anti-GM-CSF–neutralizing antibody can prevent granulocyte suppressive function. **A,** The addition of anti-GM-CSF–neutralizing antibody to mesothelioma cell line conditioned media prevents granulocyte ROS expression, as measured by DCFDA staining. Representative histograms for ED cell line shown. **B,** The addition of anti-GM-CSF–neutralizing antibody to mesothelioma cell line conditioned media prevents granulocyte ROS expression. Geometric means for DCFDA staining shown following flow cytometric detection. **C,** The addition of anti-GM-CSF–neutralizing antibody to mesothelioma cell line conditioned media

prevents granulocyte-suppressive activity compared with granulocytes cultured in tumor-conditioned media alone. **D,** Correlation between GM-CSF concentrations in tumor-conditioned media and expression of ROS in conditioned granulocytes, as measured by DCFDA mean fluorescence intensity by flow cytometry. Linear correlation line shown. **E,** Immunohistochemistry staining demonstrating the presence of GM-CSF in mesothelioma tumor sections. Mesothelioma of epithelioid (first image), adenomatoid (second and third images), and mixed/biphasic (fourth image) type demonstrated cytoplasmic positivity of tumor cells in a diffuse pattern. Images taken at 20× magnification. **F,** Interrogation of 87 primary tumor samples within the R2: database demonstrated that GM-CSF is expressed in over 50% of the samples and did not correlate with histological subtype.