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Characterization of Cytokine-Induced Myeloid-Derived Suppressor Cells from Normal Human Peripheral Blood Mononuclear Cells

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Tumor immune tolerance can derive from the recruitment of suppressor cell populations, including myeloid-derived suppressor cells (MDSCs). In cancer patients, increased MDSCs correlate with more aggressive disease and a poor prognosis. Expression of 15 immune factors (TGFβ, IL-1β, IL-4, IL-6, IL-10, GM-CSF, M-CSF, IDO, fms-related tyrosine kinase 3 ligand, c-kit ligand, inducible NO synthase, arginase-1, TNF-α, cyclo-oxygenase 2, vascular endothelial growth factor [VEGF]) by MDSC-inducing human solid tumor cell lines was evaluated by RT-PCR. Based upon these data, cytokine mixtures were then tested for their ability to generate suppressive CD33+ cells from healthy donor PBMCs in vitro by measuring their ability to inhibit the proliferation of, and IFN-γ production by, fresh autologous human T cells after CD3/CD28 stimulation. Induced MDSCs were characterized with respect to their morphology, surface phenotype, and gene expression profile. MDSC-inducing cancer cell lines demonstrated multiple pathways for MDSC generation, including overexpression of IL-6, IL-1β, cyclo-oxygenase 2, M-CSF, and IDO. CD33+ cells with potent suppressive capacity were best generated in vitro by GM-CSF and IL-6, and secondarily by GM-CSF + IL-1β, PGE2, TNF-α, or VEGF. Characterization studies of cytokine-induced suppressive cells revealed CD33+ CD11b+Cd66b+Hla-Drlow IL-13Rα2+ large mononuclear cells with abundant basophilic cytoplasm. Expression of inducible NO synthase, TGFβ, NADPH oxidase, VEGF, and arginase-1 was also upregulated, and Transwell studies showed suppression of autologous T cells to be contact dependent. Suppressive CD33+ cells generated from PBMCs by GM-CSF and IL-6 were consistent with human MDSCs. This study suggests that these cytokines are potential therapeutic targets for the inhibition of MDSC induction in cancer patients. The Journal of Immunology, 2010, 185: 2273–2284.

Myeloid-derived suppressor cells (MDSCs) have recently been recognized as a subset of innate immune cells whose function can alter adaptive immunity and produce immunosuppression (1). These cells are a heterogeneous population of immature cells derived from the lineage of dendritic cells (DCs), macrophages, and granulocytes (2). In mice, MDSCs are identified by CD11b+, IL-4Rα+, and GR-1+ expression, with recognized granulocytic and monocytic subsets (2). Human MDSCs, in contrast, are less well defined, but are generally agreed to be suppressive, myeloid derived (CD33+), CD11b+, and nonlineage determined (Lin−; CD3− CD19− CD56− CD14+) with poor Ag presentation (HLA-DR−) (3, 4). Granulocyte marker CD66b has also been reported on human MDSCs (5).

MDSCs are absent or rare in healthy hosts, but may naturally accumulate in situations of trauma and sepsis to temper immune responses (2, 6). MDSCs are also observed to accumulate in the setting of many tumors as key contributors to tumor immune tolerance (2). In murine tumor models and cancer patients, MDSCs are found in increased numbers in the tumor microenvironment, peripheral blood, liver, and tumor-draining lymph nodes, and their concentrations correlate with increased stage and metastatic disease (7, 8). The specific pathways by which tumors recruit, expand, and activate MDSCs remain unknown, but increasing evidence exists for the involvement of IL-1β, IL-6, cyclo-oxygenase 2 (COX2)-generated PGE2, high concentrations of GM-CSF, M-CSF, vascular endothelial growth factor (VEGF), IL-10, TGFβ, IDO, fms-related tyrosine kinase 3 ligand (FLT3L), and stem cell factor (c-kit ligand or stem cell factor (c-kit Lk)) (2, 9, 10). Coculture of immune competent cells with tumor cell lines has been shown to induce tolerogenic DCs or MDSCs in vitro (11-13) (M.G. Lechner, D.J. Liebertz, B. Bingham, C. Megiel, T. Woo, and A.L. Epstein, submitted for publication). Thus, further examination of the immune modulatory factors expressed by MDSC-inducing cancers and tumor cell lines could identify the cytokine(s) necessary for generation of this suppressor cell population and provide therapeutic targets for MDSC inhibition.

Because MDSCs comprise a heterogeneous population of cells, identification of unique surface markers for MDSCs, particularly in humans, has been elusive, and definition of MDSCs by suppressive function remains important (4). However, expression of C/EBPβ, a member of basic region-leucine zipper transcription factors that regulate immune and inflammatory response genes, has been proposed as a transcriptional marker of activated and functionally suppressive MDSCs in mice, analogous to FoxP3 expression in regulatory T (Treg) cells (14, 15). MDSCs mediate T cell suppression through a variety of mechanisms, including arginase-1 (ARG-1)-mediated local arginine depletion, inducible NO synthase (iNOS) and NADPH oxidase (NOX2) production of reactive oxygen and nitrogen species, VEGF expression, and cysteine depletion (16–19). In addition to direct T cell suppression, recent evidence sug-
gests a role for MDSCs in the expansion of CD4+CD25+FoxP3+ Treg cells in the tumor microenvironment through both TGFβ-dependent and independent pathways (11, 20). The expression of programmed death ligand (PDL) 1 is increased on the surface of MDSCs in some murine tumor models, although the role of this and related ligands in MDSC-mediated suppression remains unclear (21, 22). Through these mechanisms, MDSCs have been observed to mediate both Ag-specific and nonspecific immune tolerance in cancer (23).

Previously, we demonstrated the in vitro generation of human MDSCs from healthy donor PBMCs through direct coculture with human solid tumor cell lines (13) (M.G. Lechner et al., submitted for publication). The tumor-educated CD33+ cells showed a phenotype typical of human MDSCs (CD33+ HLA-DRlow CD66b+) and exhibited potent suppressive ability against autologous CD8+ T cells in an Ag-nonspecific manner. In this study, we examine the ways in which select cytokines induce MDSCs from healthy donor PBMCs. Using the data generated by tumor-induced MDSCs, specific cytokine mixtures were developed for the in vitro generation of human CD33+ MDSCs from nonsuppressive PBMCs. Characterization of the morphology, phenotype, suppressive function, and gene expression of the resulting suppressor cells showed a population consistent with human MDSCs. These studies shed light on potential therapeutic targets to abrogate MDSC accumulation in the setting of tumor immune tolerance.

**Materials and Methods**

**Cell lines and cell culture**

Tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA) or gifted to the Epstein laboratory. Notable gifts include the SW cell lines from the Scott and White Clinic (Temple, TX) and the Panc 4.14 cell line from E. Jaffe (Johns Hopkins Medical Center, Baltimore, MD). Tumor cell line authenticity was performed by cytogenetic and surface marker analysis performed at American Type Culture Collection or in our laboratory. All cell lines were maintained at 37˚C in complete medium (RPMI 1640 with 10% FCS [HyClone, Logan, UT], 2 mM l-glutamine 100 U/ml penicillin, and 100 μg/ml streptomycin) in T-25 flasks in humidified 5% CO2 incubators and passaged two to three times per week by light trypsinization.

**Cytokine induction of human MDSCs**

In vitro generation of human MDSCs

Cytokine induction. Human PBMCs were isolated from healthy volunteer donors by venipuncture, followed by differential density gradient separation (Ficoll-Hypaque; Sigma-Aldrich, St. Louis, MO). PBMCs were cultured in T-25 flasks at 5 × 10⁷ cells/ml in complete medium supplemented with cytokines, as indicated. Recombinant human cytokines used for induction include the following: IL-1β (10 ng/ml; Sigma-Aldrich), IL-6 (10 ng/ml; Sigma-Aldrich), PGE2 (1 μg/ml; Sigma-Aldrich), TGFB1 (2 μg/ml; R&D Systems, Minneapolis, MN), TNF-α (10 ng/ml; R&D Systems), VEGF (10 ng/ml; Sigma-Aldrich), and GM-CSF (10 ng/ml; R&D Systems). For combination–cytokine induction experiments, the cytokines used are indicated in Results. GM-CSF was added to the mixtures of cytokines to support cell viability, as described previously (24). PBMCs cultured in medium alone were run in parallel as a control for each donor. Cultures were run in duplicate, and medium and cytokines were refreshed every 2–3 d. For all studies, University of Southern California Institutional Review Board approval was obtained, and a total of 18 male and 7 female donors was used under protocol HS-06-00579.

MDCS isolation. After 1 wk, all cells were collected from PBMC cultures. Adherent cells were removed using nonprotease cell detachment solution Detachin (Genlantis, San Diego, CA). CD33+ cells were isolated from each culture using anti-CD33 magnetic microbeads and LS column separation (Miltenyi Biotec, Bergisch Gladbach, Germany), per manufacturer’s instructions. The purity of isolated cell populations was found to be >90% by flow cytometry.

**Quantitative RT-PCR for gene expression of tumor cell lines and MDSCs**

For gene expression studies, tumor cell lines were collected from culture flasks by trypsinization and cytokine-induced CD33+ cells isolated from whole PBMC cultures by FACS after cytokine induction described above. RNA was isolated from cells using Qiagen (Valencia, CA) RNeasy kits (RNeasy Mini to isolate tumor RNA, and RNeasy Micro to isolate MDSC RNA), followed by DNase (TurboDNase; Applied Biosystems, Foster City, CA) treatment. For quantitative real-time PCR (qRT-PCR), 100 ng DNase-treated RNA was amplified with gene-specific primers using one-step Power SYBR Green RNA-to-Ct kit (Applied Biosystems) and run in an Mx300P Stratagene thermocycler. Data were acquired and analyzed using the Maxpro software (Stratagene). Gene expression was normalized to a housekeeping gene (GAPDH) or myeloid cell marker (CD33), and fold change was determined relative to human standard RNA (Stratagene). Primer sequences for induction include the following: IL-1b (10 ng/ml; R&D Systems), VEGF (10 ng/ml; Sigma-Aldrich), and GM-CSF (10 ng/ml; R&D Systems). For combination–cytokine induction experiments, the cytokines used are indicated in Results. GM-CSF was added to the mixtures of cytokines to support cell viability, as described previously (24). PBMCs cultured in medium alone were run in parallel as a control for each donor. Cultures were run in duplicate, and medium and cytokines were refreshed every 2–3 d. For all studies, University of Southern California Institutional Review Board approval was obtained, and a total of 18 male and 7 female donors was used under protocol HS-06-00579.

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NCF1, neutrophil cytosolic factor 1.
Characterization of MDSCs
Suppression assay. The suppressive function of cytokine-induced CD33+ cells was measured by their ability to inhibit the proliferation of autologous T cells, as described previously (13). M.G. Lechner et al., submitted for publication. Fresh T cells isolated from PBMCs of autologous donors by anti-CD3 microbeads and magnetic LS column separation (Miltenyi Biotec) were CFSE labeled (2.5 μM; Sigma-Aldrich) and seeded in 96-well plates at 2 × 10^5 cells/well. CD33+ cells from the above cytokine induction cultures were added to these wells at a 1:4 or 1:2 ratio. T cell stimulation was provided by anti-CD3/CD28 stimulation beads (Invitrogen, Carlsbad, CA) and IL-2 (100 U/ml; R&D Systems). Suppression assay wells were analyzed by flow cytometry for proliferation of T cells after 3 d. For each donor and assay run, controls included T cells cultured alone with and without T cell stimulation, and T cells cultured with CD33+ cells from medium-only culture. Each CD33+ sample was run in duplicate, and data were acquired as percentage of proliferation for 15,000 events of live, lymphoid-gated cells. Samples were run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data acquisition and analysis were performed using CellQuestPro software (BD Biosciences) at the University of Southern California Flow Cytometry core facility.

IFN-γ cytokine bead array
IFN-γ production by T cells in the MDSC suppression assay was measured as a correlate of T cell activation. Supernatant was collected at the conclusion of the suppression assay from each sample and stored at −80°C until analysis. Protein levels of IFN-γ were measured using BD Cytometric Bead Array Kit Human IFN-γ Flex set and human soluble protein master bead kit (BD Biosciences), per manufacturer’s instructions. Samples were run on a FACS Calibur flow cytometer (BD Biosciences), and data acquisition and analysis were performed using CellQuestPro and FCAP Array software (BD Biosciences).

Morphology of MDSCs
Wright-Giemsa staining (Protocol Hema 3; Fisher, Kalamazoo, MI) of CD33+ cell cytospin preparations was performed to assess the morphology of cytokine-induced MDSCs. Freshly isolated PBMCs and CD33+ cells from healthy donors were prepared in parallel for comparison. Observation, evaluation, and image acquisition were performed using Leica DM2500 microscope (Leica Microsystems, Deerfield, IL) connected to an automated, digital SPOT RTk camera and SPOT Advanced Software (SPOT Diagnostic Instrument, Sterling Heights, MI). Images were resized and brightened for publication using Adobe Photoshop software (Adobe Systems, San Jose, CA).

Flow cytometry analyses of cell phenotypes
The phenotype of in vitro generated MDSCs was evaluated for expression of CD33, HLA-DR, CD11b, CD11c, CD66b, CD68, CD14, and IL-13Rα2 and compared with whole PBMCs and noninduced CD33+ cells. Changes in PBMC subpopulations during cytokine induction and the expansion of Treg cells in CD33+ T cell cultures also were measured by flow cytometry. For staining, cells were collected from flasks using DetachThe Gel (Genlantis) to minimize cell surface protein digestion and washed twice with FACS buffer (2% FCS in PBS), and 10^6 cells were resuspended in 100 μl FACS buffer. Cells were stained for 1 h on ice with mixtures of fluorescently conjugated mAbs or isotype-matched controls, then washed twice with FACS buffer, and resuspended in FACS buffer for analysis. For intracellular staining, cell surface staining was performed first, followed by buffer fixation/permeabilization (eBioscience, San Diego, CA) and intracellular staining. Abs used from BD Biosciences were as follows: CD4 (RPA-T4), CD8 (RPA-T8), CD11b (ICRF44), CD11c (B-ly6), CD14 (MSE2), CD25 (M-A251), CD3 (HIM-4, WM53), CD66b (G10F5), FoxP3 (259D/C7), and HLA-DR (G46-6); Santa Cruz Biotechnology (Santa Cruz, CA); IL-13Rα2 (B-1D3); Miltenyi Biotec: CD25 (4E5); and eBio-science; CD1a (HI149), CD3 (OKT3), CD20 (2H7), CD36 (MEM188), CD68 (Y1/82A), and isotype controls. Samples were run on a FACS Calibur flow cytometer (BD), and data acquisition and analysis were performed, as above. Samples were run in duplicate, and PBMCs cultured in medium alone were run in parallel for comparison. Data were acquired as the fraction of labeled cells within a live-cell gate set for 15,000 events.

Transwell assays
The MDSC suppression assay was modified to test contact dependency of MDSC-mediated T cell suppression. For these studies, cytokine-induced CD33+ cells were cocultured with CFSE-labeled T cells in Transwell inserts to inhibit direct T cell–CD33+ cell contact. Fresh T cells isolated from PBMCs of autologous donors were used for these studies, with T cells cultured in plate wells and CD33+ cells cultured in Transwell inserts to inhibit direct T cell–CD33+ cell contact.

Cytokine induction of CD33+ MDSCs from T cell-depleted PBMCs
To determine the role, if any, of T cells, the cytokine induction assay was repeated for select cytokine mixtures using T cell-depleted PBMCs from healthy volunteers, rather than whole PBMCs. For these studies, PBMCs obtained after density gradient separation were depleted of T cells using anti-CD3 magnetic microbeads and LD column separation (Miltenyi Biotec, per manufacturer’s instructions). Subsequent culture, cell isolation, and function evaluation of cytokine-induced CD33+ cells from these cultures were performed, as described in the MDSC isolation and suppression assays above.

Statistical analysis
For MDSC suppression assays, CFSE fluorescence was measured by flow cytometry to determine the percentage of proliferation for each sample relative to T cell controls. Where possible, mean relative proliferation and SD were calculated and graphed using Microsoft Excel software (Microsoft, Redmond, WA). A change in mean T cell proliferation in the presence or absence of tumor-educated MDSCs was tested for statistical significance using the Student t test for independent samples. For all analyses in which multiple experimental samples were compared against one another (e.g., qRT-PCR), a one-way ANOVA was performed, followed by Dunnett post-test analysis using GraphPad Prism (La Jolla, CA). For all statistical tests, significance level α = 0.05.

Results
Human solid tumor cell lines overexpress multiple putative MDSC-promoting factors
In this study, cancer cell line induction of human CD33+ MDSCs was used as a model for tumor induction of human MDSCs. Previously, we screened 100 human solid tumor cell lines for their ability to induce functionally suppressive CD33+ MDSCs from healthy donor PBMCs using a novel coculture method (13) (M.G. Lechner et al., submitted for publication). In that study, we reported 46 cancer cell lines capable of generating CD33+ human MDSCs. In this study, we selected 17 of the 46 MDSC-inducing cancer cell lines, as well as 6 non-MDSC–inducing control cancer cell lines, to examine the mechanisms by which tumors generate MDSCs. Gene expression levels of putative MDSC-inducing immune modulatory factors were compared for both groups of cancer cell lines using qRT-PCR techniques (Fig. 1). Gene expression by tumor cell lines was examined for 15 immune modulatory factors: ARG-1, IL-1β, IL-4, IL-6, IL-10, iNOS, c-kit L, COX2, FLT3L, GM-CSF, IDO, M-CSF, TGFβ, TNFα, and VEGF.

All tumor cell lines examined were found to have a statistically significant increase in expression of at least one of these factors relative to a reference human RNA standard; however, no single factor was shown to be critical for MDSC induction (Fig. 1). MDSC-inducing tumor cell lines showed increased expression of COX2, IL-1β, IL-6, M-CSF, and IDO (Fig. 1). FLT3L and c-kit L expression appeared to be increased in both groups of tumor cell lines, suggesting that these are not singular distinguishing factors for MDSC induction. TGFβ, GM-CSF, and ARG-1 showed consistent downregulation among MDSC-inducing tumor cell lines. These results suggest multiple pathways of MDSC induction for the tested human cancer cell lines.

Human CD33+ MDSCs are generated in vitro by soluble immune modulatory factors
Using MDSC induction by cancer cell lines as a model, cytokine mixtures were designed for the in vitro generation of human MDSCs from healthy donor PBMCs. Briefly, PBMCs were cultured for 1 wk in the presence or absence of immune modulatory factors GM-CSF.
and IL-1β, IL-6, VEGF, TGFB, TNF-α, or PGE2 [as a proxy for COX2 overexpression (26)], and then CD33+ cells were isolated and tested for suppressive function by MDSC suppression assays. The ability of cytokine-induced CD33+ to inhibit autologous T cell proliferation and IFN-γ production was evaluated at cell ratios of 1:2 and 1:4 (Fig. 2A, 2B). Multicytokine combinations were also tested for the induction of CD33+ MDSCs.

Results from these studies show the generation of potent CD33+ MDSCs after incubation of nonresponsive PBMCs with select cytokine combinations. Notably, GM-CSF + IL-6 and GM-CSF + IL-6 + VEGF cytokine combinations generated MDSCs with the ability to suppress autologous T cell proliferation by 80.6% and 74.5%, respectively (mean, n = 4), at a 1:4 ratio (Fig. 2A). Furthermore, GM-CSF alone, GM-CSF + IL-1β, GM-CSF + TNF-α, and GM-CSF + VEGF cytokine conditions yielded MDSCs with significant suppressive function (range 43–57% suppression at 1:4 ratio). CD33+ cells treated with GM-CSF + PGE2 exhibited weak suppressive ability. Whereas these data suggest a positive role for IL-6, VEGF, IL-1β, TNF-α, and GM-CSF in MDSC generation, TGFB does not appear to have a major role in the promotion of suppressive function in CD33+ cells because TGFB treatment consistently decreased the potency of other cytokines in the induction of suppressive function (Fig. 2A). GM-CSF was found to be critical to maintain myeloid cell viability, as reported previously (24), and was included in all cytokine mixtures, although at lower concentrations than used by Ko et al. (24) (10 ng/ml versus 50 ng/ml). However, even this low level of GM-CSF alone appeared to induce some suppressive function. The low sample number for these studies. As reported previously (11, 20), although conclusions are limited by the small number of CD33+ cells from medium-only cultures showed remarkably homogeneous morphology and were

FIGURE 1. Expression of putative MDSC-inducing factors by human solid tumor cell lines and PBMCs. The fold change in gene expression of putative MDSC-inducing immune modulatory factors was determined by qRT-PCR techniques for MDSC-inducing and noninducing human solid tumor cell lines. In addition, the expression of these factors by freshly isolated PBMCs from healthy volunteer donors was analyzed in parallel. Due to the large variation in gene expression levels among tumor cell lines, the log10 fold change is shown (e.g., fold increases of 10 and 100 are represented as log10 changes of 2.0 and 3.0, respectively). Genes whose expression was higher in the tumor cell line than the reference sample are shown in red (90th percentile); those whose expression was lower than the reference sample are shown in green (10th percentile). Note the differential expression of factors COX2, IL-1β, and IL-6 in MDSC-inducing and noninducing cell lines and the high expression of GM-CSF in normal donor PBMCs compared with tumor cell lines.
distinctly different from starting PBMCs and freshly isolated CD33+ cells. The morphology of starting PBMCs following differential density gradient separation was typical of healthy human donors, showing numerous lymphocytes and mixed monocyte populations with rare granulocytes and eosinophils. Noninduced CD33+ cells isolated from PBMCs demonstrated a lymphocyte-depleted monocyte population of small cells, as expected. In contrast, cytokine-induced CD33+ cells were consistently large mononuclear cells with basophilic, granular-appearing cytoplasm (Fig. 3A). As reported previously, binucleate cells were commonly observed in CD33+ cells cultured under numerous conditions (27). With cytokine exposure, cells appeared to change from blast-
FIGURE 3. The morphology and phenotype of cytokine-induced CD33+ MDSCs resemble tumor-induced MDSCs. 

A. Morphology of cytokine-induced CD33+ populations compared with PBMCs by Wright-Giemsa staining (original magnification ×400). Starting PBMC population (1) shows small granulocytic and monocytic cells scattered among lymphocytes. CD33+ cells isolated after 1-wk culture with complete medium alone (2) or following cytokine induction (3–8) appear as large mononuclear cells with abundant cytoplasm. Non-suppressive cytokine-induced CD33+ cells (3, 4) are similar in morphology to suppressive cytokine-induced CD33+ cells (5–8), although the cytoplasm of the latter frequently appears more basophilic. Cytokine mixtures used for induction were (3) TGFβ + PGE2 + GM-CSF, (4) TGFβ + PGE2 + IL-1β + GM-CSF, (5) GM-CSF alone, (6) PGE2 + GM-CSF, (7) IL-6 + GM-CSF, and (8) VEGF + GM-CSF. 

B. Phenotypes of cytokine-induced CD33+ cells compared with whole PBMCs and CD33+ cultured in medium alone were determined by flow cytometry. The expression of putative MDSC markers (CD33, CD11b, IL-13Ra2, CD66b) and markers of mature APCs (HLA-DR, CD11c, CD14, CD68) was evaluated for each sample (black line) relative to isotype controls (gray). In addition, forward and side scatter analyses of cells were performed to compare size and granularity of cytokine-induced CD33+ cells to controls. For GM-CSF + PGE2–induced CD33+ cells, two discrete populations of cells were noted. For this cytokine mixture, only the granulocytic population (shown gated) expressed a phenotype consistent with human MDSCs. 

C. Expression of transcription factor C/EBPβ by CD33+ cells induced under different cytokine milieu was evaluated by qRT-PCR techniques. There was a statistically significant difference in mean C/EBPβ gene expression only between GM-CSF + TGFβ– and medium-treated CD33+ cells (p < 0.05), with no difference among any other groups.
like cells with scant cytoplasm (Fig. 3A1, 3A2) to cytoplasm predominant cells (Fig. 3A3–8). However, no discernable difference was observed in morphology between suppressive and nonsuppressive cytokine-treated CD33 cells by Wright-Giemsa staining.

**Phenotype of cytokine-induced CD33**<sup>+</sup> MDSCs resembles tumor-induced MDSCs**

The surface phenotype of cytokine-treated CD33<sup>+</sup> cells was analyzed by flow cytometry and compared with the phenotypes of whole PBMCs and CD33<sup>+</sup> cells cultured in medium alone (Fig. 3B). Flow cytometry analyses of cell forward and side scatter demonstrated a primarily granulocytic CD33<sup>+</sup> population induced by cytokines, as compared with the largely monocytic morphology seen in PBMCs and medium-only CD33<sup>+</sup> cell populations. Staining for CD33 confirmed a high purity of target cells following anti-CD33 magnetic bead labeling and column separation. The reported phenotype of human MDSCs is CD33<sup>+</sup> HLA-DR<sup>low</sup> CD11b<sup>+</sup> CD66b<sup>+</sup>, with low expression of differentiated macrophage and DC markers (4, 5). CD33<sup>+</sup> cells from all cytokine-treated cultures showed similar increases in CD11b and CD66b expression relative to isotype controls, with low to intermediate expression of monocyte/macrophage-associated markers CD68 and CD14. Low expression of Ag presentation protein HLA-DR appeared to distinguish suppressive from nonsuppressive cytokine-treated CD33<sup>+</sup> cells. A small increase in IL-13Ra2 expression was also observed in suppressive, but not nonsuppressive or control, CD33<sup>+</sup> cells. For GM-CSF + PGE<sub>2</sub>-induced CD33<sup>+</sup> cells, two discrete populations of cells were noted. For this cytokine mixture, only the granulocytic population (shown gated separately in Fig. 3B) expressed a phenotype consistent with human MDSCs. Overall, suppressive cytokine-induced CD33<sup>+</sup> cells generated by in vitro culture demonstrated a phenotype consistent with that previously reported for human MDSCs (4).

**Expression of C/EBPβ by cytokine-induced CD33<sup>+</sup> cells does not correlate with suppressive function**

Expression of transcription factor C/EBPβ by CD33<sup>+</sup> cells induced under different cytokine milieu was evaluated by qRT-PCR techniques (Fig. 3C). Although C/EBPβ has been proposed as a transcriptional marker upregulated in activated murine MDSCs (15), there was not a statistically significant difference in the expression of this transcription factor between suppressive and nonsuppressive human cytokine-induced CD33<sup>+</sup> cells (Fig. 3C).

**Cytokine-induced CD33<sup>+</sup> MDSCs have upregulated iNOS, TGFβ, VEGF, and NOX2**

MDSC-mediated suppression of effector T cell responses has been shown to correlate with increased expression of ARG-1, iNOS, NOX2, VEGF, and TGFβ by suppressor cells (16–20). To better characterize the nature of suppressive cytokine-induced CD33<sup>+</sup> cells, gene expression of these putative mechanisms of MDSC suppression was evaluated by qRT-PCR techniques (Fig. 4A). Cytokine-induced CD33<sup>+</sup> suppressor cells demonstrate significant upregulation of iNOS, NOX2, VEGF, and/or TGFβ compared with freshly isolated, noninduced CD33<sup>+</sup> cells. iNOS, VEGF, and TGFβ are consistently upregulated by various inducing cytokine mixtures, whereas upregulation of NOX2 relates most closely with TNF-α induction. GM-CSF + IL-6-induced CD33<sup>+</sup> cells appeared to mediate their potent suppressive function through ARG-1, iNOS, VEGF, and TGFβ, with minimal contribution from NOX2 (Fig. 4A). Also, different cytokine-induced myeloid suppressor cell groups demonstrated various patterns of gene expression (Fig. 4A). Previous reports have shown increased expression of inhibitory B7 homologs on murine MDSCs derived from some experimental tumor models, although the functional significance of these markers is debated (21, 22). Gene expression levels of PDL1 (B7H1), PDL2 (B7H2), and B7H4 were compared between suppressive and nonsuppressive cytokine-induced CD33<sup>+</sup> cells by qRT-PCR studies (Fig. 4B). PDL1 expression was generally observed to be upregulated in all groups of cytokine-induced CD33<sup>+</sup> cells, although the increase was statistically significant only for GM-CSF–induced cells (p < 0.05). Cytokine-induced CD33<sup>+</sup> cells showed consistent downregulation of PDL2 and B7H4 genes relative to CD33<sup>+</sup> from medium-only cultures, with no differences between suppressive and nonsuppressive cells (Fig. 4B). These results are consistent with reports from experimental tumor models in mice (21, 22), and do not support a role for PDL1, PDL2, or B7H4 in suppression mediated by these human cytokine-induced CD33<sup>+</sup> cells.

**Suppression by human CD33<sup>+</sup> MDSCs is contact dependent**

A variety of suppressive mechanisms has been reported for human and murine MDSCs, although it is unclear whether they require cell-to-cell contact with target T cells. In our assays, separation of suppressive cytokine-induced CD33<sup>+</sup> cells from responder T cells by Transwell inserts (0.4-μm pores) was found to abrogate the suppressive effects of all cytokine-induced MDSCs (Fig. 4C). These data suggest that many, if not all, suppressive mechanisms employed by these cells are contact dependent.

**Cytokine induction expands CD56<sup>+</sup>, CD33<sup>+</sup>, and CD14<sup>+</sup> cell populations**

Suppressive CD33<sup>+</sup> cells with MDSC-like morphology, phenotype, and gene expression were successfully generated from whole PBMCs derived from healthy volunteer donors. To shed light on the cellular context of CD33<sup>+</sup> suppressor cell induction, changes in cell types and numbers during cytokine induction were measured by flow cytometry analyses (Fig. 5A). All cytokine mixtures, with the exception of GM-CSF + VEGF, showed expansion of CD56<sup>+</sup> (NK cells), CD33<sup>+</sup> (myeloid), CD14<sup>+</sup> (monocytes), and to a lesser extent CD11a<sup>+</sup> (DC) cell populations relative to PBMCs cultured in medium alone. This effect occurs with GM-CSF alone, suggesting that this cytokine promotes proliferation of cells in the myeloid and granulocyte compartment, consistent with its known function in normal hematopoiesis (28). Expansion of CD66b<sup>+</sup> cells above the levels observed with GM-CSF alone occurred with the addition of IL-1β or PGE<sub>2</sub> to cytokine mixtures. T cell numbers and CD4/CD8 ratio appeared to be largely unaffected by cytokine induction. Cytokine mixtures also did not appear to affect the frequency of B cells (CD20<sup>+</sup>) or macrophages (CD68<sup>+</sup>). Cytokine mixtures inducing CD33<sup>+</sup> suppressor cells did not produce changes in cell types and numbers distinct from those observed for cytokine mixtures that did not induce MDSCs.

**T cells are not needed for cytokine induction of CD33<sup>+</sup> MDSCs from normal donor cells**

T cells comprise a large fraction of PBMCs, and depletion of this population may increase the yields of CD33<sup>+</sup> cells from cytokine-induced cultures. To determine whether T cells were necessary for the induction of suppressive CD33<sup>+</sup> cells by the cytokine mixtures used above, cytokine induction experiments were repeated using T cell-depleted PBMCs. The suppressive function of CD33<sup>+</sup> cells from whole PBMCs or from T cell-depleted PBMC cultures treated with cytokine mixtures was then compared, as shown in Fig. 5B. CD33<sup>+</sup> MDSCs generated in the absence of T cells demonstrated a comparable suppressive capacity to those generated from whole PBMCs for most cytokine mixtures examined. Interestingly,
induction studies using GM-CSF alone, GM-CSF + TNF-α, and GM-CSF + VEGF showed that CD33+ generated in the absence of T cells was more suppressive than those generated from whole PBMCs ($p < 0.05$).

GM-CSF expression by PBMCs is upregulated by coculture with tumor cell lines

Cytokine induction studies showed that GM-CSF alone or in combination with IL-6, IL-1β, and VEGF generates potent CD33+
suppressor cells. Whereas this cytokine appears to be critical in human myeloid suppressor cell induction, MDSC-inducing tumor cell lines were found to have very low GM-CSF expression (Fig. 1 and ELISA data not shown). PBMCs, however, do have strong expression of GM-CSF (Fig. 1) and thus may provide a source of GM-CSF for MDSC induction in the tumor microenvironment.

GM-CSF expression by PBMCs in the presence or absence of inducing tumor cell line was measured by qRT-PCR (Fig. 5C). These data showed strong upregulation of GM-CSF production by normal donor PBMCs following direct coculture with MDSC-inducing cell lines 4-998 (osteogenic sarcoma) or SCCL-MT1 (head and neck squamous cell carcinoma), but not culture in medium alone. Anal-

FIGURE 5. Cellular context for cytokine induction of CD33⁺ MDSCs from normal donor PBMCs. A, Changes in cell types and frequencies during cytokine induction were measured by flow cytometry. Significant expansion of CD56⁺ (NK), CD33⁺ (myeloid), and CD14⁺ (monocyte) cell populations was observed in PBMCs treated with all cytokine mixtures, with the exception of GM-CSF + VEGF. The addition of IL-1β or PGE₂ appeared to increase the frequency of CD66b⁺ cells beyond that observed with GM-CSF treatment alone. The number and CD4/CD8 ratio of T cells did not appear to be affected by the cytokine combinations examined in this study, nor did the frequency of B cells (CD20⁺) or macrophages (CD68⁺). Cytokine mixtures inducing CD33⁺ suppressor cells did not produce changes in cell types and numbers distinct from those observed for cytokine mixtures that did not induce MDSCs. B, The suppressive function of CD33⁺ cells from whole PBMCs or from T cell depleted PBMC cultures treated with cytokine mixtures was compared to determine the requirement of T cells in the induction of MDSCs. CD33⁺ MDSCs generated in the absence of T cells demonstrated a comparable suppressive capacity to those generated from whole PBMCs for most cytokine mixtures examined. However, for cytokine mixtures GM-CSF alone, GM-CSF + TNF-α, and GM-CSF + VEGF, CD33⁺ generated in the absence of T cells were more suppressive than those generated from whole PBMCs (p < 0.05). C and D, To shed light on possible sources of GM-CSF for the induction of suppressor cells in tumor–PBMC cocultures, gene expression of GM-CSF in PBMCs following direct coculture with inducing tumor cell lines was measured by qRT-PCR techniques and compared with cells cultured in medium alone (mean shown + SD). GM-CSF expression by PBMCs was strongly upregulated following tumor cell line coculture. Analysis of GM-CSF expression in the CD33⁺ cell fraction suggests that the increase in GM-CSF observed is not localized to this subpopulation.
ysis of the CD33+ cell fraction showed that the increase in GM-CSF production was not localized to this population (Fig. 5D).

Discussion

Immune suppressor cells, including MDSCs, contribute to tumor immune tolerance and promote tumor progression. In this study, a group of MDSC-inducing human solid tumor cell lines was evaluated for their expression of factors previously implicated in the generation and activation of MDSCs. These data were then used to identify specific cytokine combinations sufficient to induce functionally suppressive myeloid (CD33+) cells from healthy donor PBMCs as an in vitro model of tumor–MDSC induction. Characterization of the morphology, phenotype, suppressive capacity, and gene expression of the cytokine-induced suppressive cells showed a population consistent with human MDSCs. This study also demonstrated that the induction of CD33+ MDSCs from immune competent PBMCs by select cytokine combinations occurs in a context of CD33+/CD14+/CD56+ cell expansion and does not require T cells.

A key first step to the current study was the demonstration that human cancer cell lines produced several factors required for the induction of MDSCs after coculture with normal volunteer PBMCs. The production of 15 putative MDSC-inducing factors by cancer cell lines was evaluated using previously identified MDSC-inducing and noninducing human solid tumor cell lines (Fig. 1) (13) (M.G. Lechner et al., submitted for publication). From these studies, it was clear that multiple immune modulatory factors are responsible for MDSC generation, notably IL-6, IL-1β, IDO, COX2, and M-CSF (Fig. 1). The notion is consistent with the plethora of reported tumor-specific tumor-generated MDSC subtypes.

Using cancer cell line MDSC induction as a guide, six cytokine combinations were identified for the in vitro induction of potent CD33+ MDSC-like suppressor cells from normal donor PBMCs, as follows: GM-CSF, GM-CSF + IL-1β, GM-CSF + IL-6, GM-CSF + PGE2, GM-CSF + TNF-α, and GM-CSF + VEGF (Fig. 2). Of these cytokine mixtures, IL-6 and GM-CSF consistently generated the most suppressive CD33+ cells, as measured by the ability to suppress proliferation of, and IFN-γ production by, autologous T cells at different ratios (Fig. 2). Whereas hypoxia was not directly examined in this study, IL-6 and TNF-α have been shown to increase hypoxia-inducible factor-1α expression even in the absence of hypoxia (31). Thus, this study is in agreement with previous reports suggesting a role for hypoxia and hypoxia-inducible factor-1α in IL-6–induced MDSCs (32). These results confirm the importance of IL-6 as a therapeutic target for cancer immunotherapy and MDSC inhibition and provide a means to study specific tumor-generated MDSC subtypes.

Wright-Giemsa staining and flow cytometry analyses of cytokine-induced CD33+ cells and freshly isolated CD33+ cells demonstrate a shift from small, mononuclear cells to a homogeneous population of large mononuclear cells with abundant basophilic, granular cytoplasm (Fig. 3A). As reported previously for tolerogenic DCs (27), binucleated cells were commonly observed in CD33+ cells cultured under different conditions. Importantly, these studies demonstrate that cell morphology alone does not distinguish between CD33+ MDSCs and nonsuppressive CD33+ cells. This study concurs with previous reports from cancer patients and murine tumor models in reporting a granulocytic MDSC population (by flow cytometry analyses; Fig. 3B) that may derive from the expansion and conversion of mononuclear precursor cells (33). In characterizing the surface phenotype of cytokine-induced MDSCs, this study reports on the expression of several MDSC-associated surface markers not previously examined together (Fig. 3B). These results corroborate CD66b and CD11b as markers of human MDSCs in conjunction with CD33 positivity (4, 5). Importantly, a comparison of cytokine-induced CD33+ cells with and without suppressive function highlights differential expression of HLA-DR and IL-13Rα2 (Fig. 3B). These two phenotypic features, HLA-DRlow and IL-13Rα2int, may provide ways to distinguish between suppressive and nonsuppressive myeloid cells, in much the same way that FoxP3+ and CD39+ may be used to distinguish CD4+CD25+ Treg from activated T effector cells (34). Transcription factor C/EBPβ has also been suggested as a correlate to suppressive ability in murine MDSCs (15), but this study did not find a statistically significant difference in mean C/EBPβ expression between functionally suppressive and nonsuppressive cytokine-induced human CD33+ cells (Fig. 3C).

CD33+ MDSCs induced by GM-CSF and IL-6 mediated potent suppression of autologous T cell proliferation and IFN-γ production. Suppressive myeloid cells were also generated by cytokine mixtures of IL-1β, VEGF, TNF-α, and PGE2, with GM-CSF added to support cell viability, but the resulting suppressor cells were noticeably less potent (Fig. 2A, 2B). In characterizing the sup-
pression function of cytokine-induced MDSCs, this study evaluated the expression of putative suppression genes and the requirement for cell contact with target cells. As reported previously for murine MDSCs, suppression of non-Ag-specific autologous T cell responses by human CD33+ MDSCs was contact dependent (35). This study did not evaluate Ag-specific suppression, and future studies in this area are warranted. In vitro generated suppressive MDSCs exhibited increased expression of iNOS, NOX2, TGFβ, and VEGF (Fig. 4A). Interestingly, slight variations in the gene expression profile of, or Treg expansion by, suppressive CD33+ cells occurred with different inducing cytokines (Figs. 2C, 4A). IL-6- and GM-CSF–induced MDSCs appeared to exert strong suppression through upregulation of ARG-1, iNOS, VEGF, and TGFβ3, with minimal contribution from NOX2 expression (Fig. 4A). This diversity reflects the variation in suppressive mechanisms reported for MDSCs from cancer patients and murine tumor models (16–20) and further suggests the existence of MDSC subpopulations. As noted above, MDSC-inducing tumor cell lines were observed to upregulate expression of different factors that may be responsible for MDSC generation (Fig. 1), which may explain the presence of different MDSC populations among tumors. In this study, the generation of suppressive CD33+ MDSCs using multiple distinct cytokine mixtures also supports the existence of diverse MDSC subpopulations (Fig. 2). The MDSC suppression assay described in this study tested the ability of CD33+ cells to inhibit T cell proliferation and IFN-γ production in the presence of IL-2 (100 IU/ml) and CD3/CD28 stimulation. This method is similar to suppressive assays frequently used to evaluate the function of Treg cells (34). It is important to note that many investigators use APC populations as feeder cells in Treg suppression assays. Just as Treg may be expanded from responder CD3+ T cell populations and contribute to suppression in MDSC suppression assays (Fig. 2C), Treg induction of MDSCs from APCs present in Treg suppression assays may contribute to inhibition of T cell responses. As demonstrated in this study, CD33+ cells may be induced from normal donor cells by select cytokines and mediate potent suppression of autologous T cells (Fig. 2A, 2B).

Previously, we reported the in vitro generation of human CD33+ MDSCs from PBMCs by direct coculture with select human solid tumor cell lines (13) (M.G. Lechner et al., submitted for publication). In this study, cytokines IL-6 and GM-CSF, and secondarily GM-CSF in combination with IL-1β, PGE2, TNF-α, or VEGF, are shown to be sufficient for the induction of functionally suppressive CD33+ cells from healthy donor PBMCs. MDSCs are not typically present in healthy individuals (2), and the ability to generate these suppressor cells in vitro from nonsuppressive populations represents an important discovery. Suppressor cells induced by IL-6 and GM-CSF should provide an important model for studying tumor-associated human MDSCs and may enable the generation of tolerogenic myeloid cells for the adoptive immunotherapy of autoimmune disease. The cytokine induction combinations identified in this study allow derivation of human MDSCs from a starting population of common, healthy PBMCs. This is in contrast to reports of in vitro generation of murine MDSC populations from embryonic, splenic, or bone marrow–derived cells (36), cell fractions that are less readily available from human donors. Whereas many similarities exist between murine and human immune systems, studies in Treg highlight the need to be cognizant of interspecies differences in surface markers and biology of immune suppressor cells (34, 37). Thus, the discovery of human-specific MDSC induction methods is a valuable tool for the further study of these suppressor cells. Based upon these results, future investigations are warranted to study the potential of IL-6 and GM-CSF as therapeutic targets for the inhibition of MDSC induction in cancer patients.

Acknowledgments

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Disclosures

A.L.E. is co-founder and part owner of Cancer Therapeutics Laboratories. All other authors have no financial conflicts of interest.

References


Corrections


In Fig. 1, the cell line CAL-27 should be grouped with the myeloid-derived suppressor cell-inducing cell lines and was mistakenly grouped with the noninducing cell lines. The findings and conclusion of the paper are unchanged. The published figure legend is correct, but is printed below for reference.

![Figure 1](https://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1090100)

**FIGURE 1.** Expression of putative MDSC-inducing factors by human solid tumor cell lines and PBMCs. The fold change in gene expression of putative MDSC-inducing immune modulatory factors was determined by qRT-PCR techniques for MDSC-inducing and noninducing human solid tumor cell lines. In addition, the expression of these factors by freshly isolated PBMCs from healthy volunteer donors was analyzed in parallel. Due to the large variation in gene expression levels among tumor cell lines, the log₁₀ fold change is shown (e.g., fold increases of 10 and 100 are represented as log₁₀ changes of 2.0 and 3.0, respectively). Genes whose expression was higher in the tumor cell line than the reference sample are shown in red (90th percentile); those whose expression was lower than the reference sample are shown in green (10th percentile). Note the differential expression of factors COX2, IL-1β, and IL-6 in MDSC-inducing and noninducing cell lines and the high expression of GM-CSF in normal donor PBMCs compared with tumor cell lines.

![Table](https://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1090100)

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**Correction:** The cell line CAL-27 should be grouped with the myeloid-derived suppressor cell-inducing cell lines and was mistakenly grouped with the noninducing cell lines.

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