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Increased Production of Immature Myeloid Cells in Cancer Patients: A Mechanism of Immunosuppression in Cancer¹

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Defective dendritic cell (DC) function caused by abnormal differentiation of these cells is an important mechanism of tumor escape from immune system control. Previously, we have demonstrated that the number and function of DC were dramatically reduced in cancer patients. This effect was closely associated with accumulation of immature cells (ImC) in peripheral blood. In this study, we investigated the nature and functional role of those ImC. Using flow cytometry, electron microscopy, colony formation assays, and cell differentiation in the presence of different cell growth factors, we have determined that the population of ImC is composed of a small percentage (<2%) of hemopoietic progenitor cells, with all other cells being represented by MHC class I-positive myeloid cells. About one-third of ImC were immature macrophages and DC, and the remaining cells were immature myeloid cells at earlier stages of differentiation. These cells were differentiated into mature DC in the presence of 1 μ M all-*trans*-retinoic acid. Removal of ImC from DC fractions completely restored the ability of the DC to stimulate allogeneic T cells. In two different experimental systems ImC inhibited Ag-specific T cell responses. Thus, immature myeloid cells generated in large numbers in cancer patients are able to directly inhibit Ag-specific T cell responses. This may represent a new mechanism of immune suppression in cancer and may suggest a new approach to cancer treatment. *The Journal of Immunology*, 2001, 166: 678–689.

Professional APCs, dendritic cells (DC),⁴ play a central role in induction of antitumor immune responses. These cells can take up, process, and present tumor-specific Ags, and they are responsible for activation of tumor-specific T cells (1). The important role of DC in cancer is underscored by the number of reports in which the presence of DC in tumor tissues was associated with good clinical prognosis of the disease (reviewed in Ref. 2).

In recent years several groups have described defective function of DC in tumor-bearing mice and in cancer patients (3–6). It is now considered one of the important mechanisms of tumor escape from immune system control. The major findings in these studies were the lack of expression of costimulatory molecules in tumor-associated DC consistent with the phenotype of immature DC. A population of DC isolated from the peripheral blood of patients with breast and head and neck cancer demonstrated significantly reduced ability to cluster and stimulate allogeneic and Ag-specific T cell responses (7, 8). These cells have a substantially lower level of expression of MHC class II (HLA-DR) and costimulatory molecules than DC isolated from control donors. In agreement with

these reports DC isolated from tumor-bearing mice also had a decreased expression of B7-2 and MHC class II as well as some adhesion molecules. These cells were unable to induce effective peptide-specific and antitumor cytotoxic immune responses and were ineffective as a tumor vaccine (9).

Thus, DC in tumor-bearing hosts are functionally defective. Previous studies have shown that several tumor-derived factors (vascular endothelial growth factor, IL-6, M-CSF, and gangliosides) affect DC maturation from hemopoietic progenitor cells (HPC) in vitro. However, mature DC were functionally competent (10). This was consistent with the fact that functionally competent DC can be generated in the absence of tumor-derived factors from bone marrow progenitor cells of tumor-bearing mice and from peripheral blood progenitors of cancer patients (8, 11). Thus, there is now enough evidence that tumor-derived factors affect DC differentiation. This may result in a substantial decrease in the number of mature DC in cancer patients. This hypothesis has been directly confirmed in two recent studies in which the number of DC was significantly decreased in cancer patients (12, 13). The decrease in the presence of DC in peripheral blood from cancer patients closely correlated with the stage and duration of the disease. We have previously demonstrated that this decrease was also closely associated with appearance of a large number of cells lacking markers of mature lymphoid and myeloid cells in peripheral blood (13). We termed those cells immature cells (ImC). We hypothesized that those ImC might have an impact on immune response in cancer. In this study we characterized the nature of ImC, demonstrated that these cells actively suppressed Ag-specific T cell responses, and identified factors able to differentiate these cells in vitro. This may suggest a new approach to improve immune response in cancer.

Materials and Methods

Patients

Forty-four patients, 32–79 years of age, with histologically confirmed cancer were enrolled in the study. Of these 44 patients, 13 had squamous cell carcinoma of the head and neck (HNSCC), 21 had non-small cell lung

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⁴ Abbreviations used in this paper: DC, dendritic cell(s); ImC, immature cell(s); HNSCC, squamous cell carcinoma of the head and neck; CCM, complete culture medium; ATRA, all-*trans*-retinoic acid; LMMA, *N*^G-monomethyl-L-arginine; TT, tetanus toxoid; ELISPOT, enzyme-linked immunospot; PBS-T, PBS containing 0.05% Tween 20; HPC, hemopoietic progenitor cells; Lin, lineage.

carcinoma, and 10 had breast cancer. The vast majority of patients were newly diagnosed, but a few had recurrent disease with no prior therapy for at least 1 year before the study. All patients had advanced diseases (stages III–IV) in accordance with the American Joint Committee on Cancer Criteria. Seven healthy volunteers served as controls.

Reagents

Complete culture medium (CCM) included RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS and antibiotics. Ficoll-Paque was obtained from Pharmacia-Biotech (Uppsala, Sweden). PE-, FITC-, or Quantum Red-conjugated anti-human MHC class I (W6/32), anti-HLA-DR, CD3, CD13, CD14, CD19, CD57, and CD15 Abs as well as isotype-matched mouse Ig were purchased from Sigma (St. Louis, MO). FITC-conjugated anti-CD1a, anti-CD86, and anti-CD40 Abs were obtained from PharMingen (San Diego, CA), FITC-conjugated anti-CD34 Abs were purchased from Becton Dickinson (Franklin Lakes, NJ) and Coulter (Hialeah, FL), and FITC-conjugated anti-CD33 Ab was obtained from Serotec (Raleigh, NC). SRBC were obtained from Cocalico (Reamstown, PA), and metrizamide was purchased from Nyegaard (Oslo, Norway) and Sigma. Recombinant human GM-CSF, IL-4, G-CSF, M-CSF, and CD40 ligand were purchased from RDI (Flanders, NJ). Recombinant human TNF- α , TGF- β , and Flt-3 ligand were purchased from R&D Systems (Minneapolis, MN). All-*trans*-retinoic acid (ATRA) was obtained from Sigma. A stock solution of ATRA in absolute ethanol (10^{-3} M) was stored at -30°C . N^G -monomethyl-L-arginine (LMMA) was obtained from Calbiochem (San Diego, CA).

Peptide GILGFVFTL derived from the matrix of the influenza virus was synthesized by SynPep (Dublin, CA). This peptide has a high affinity with HLA-A2.

Cell isolation

DC and T cells were isolated from peripheral blood as previously described (14) with some modifications. Briefly, mononuclear cells obtained after centrifugation of peripheral blood over a Ficoll-Paque gradient were incubated with 2-aminoethylisothiuronium bromide (Sigma)-treated SRBC. Cells that adhered to the red cells (R^+) and those that did not (R^-) were separated on a Ficoll-Paque gradient. RBCs were then incubated for 18 h in CCM. Nonadherent cells were centrifuged over a metrizamide gradient (7.25 g of metrizamide in 50 ml of CCM) to obtain enriched fraction of DC. These cells were used in additional experiments.

In some experiments R^- mononuclear cells were cultured for 2 h in CCM. After that time nonadherent cells were removed, and adherent cells were cultured in CCM supplemented with 30 ng/ml GM-CSF and 10 ng/ml IL-4. The same amount of cytokines in 0.5 ml of CCM was added on day 3. After 5–6 days of culture DC were collected, washed, and used in further studies.

R^+ cells were further processed by osmotic lysis of red cells to obtain an enriched T cell fraction followed by overnight incubation in CCM at 37°C . More than 90% of nonadherent cells were T cells, as estimated by flow cytometry.

MLR and Ag-specific T cell proliferation

The ability of DC to stimulate allogenic T cells was tested in MLR. Fifty thousand T cells were plated in each well of 96-well round-bottom plates, and DC and T cells were cultured at ratios of 1:20, 1:40, 1:80, and 1:160 for 5 days. One microcurie of [^3H]thymidine (sp. act., 25Ci/mmol) was added to each well 18 h before harvesting the cells. [^3H]thymidine uptake was counted in a liquid scintillation counter (Beckman, Palo Alto, CA).

Ag-specific T cell response was measured using tetanus toxoid (TT). DC were cultured with autologous T cells in the presence of 1.0 $\mu\text{g}/\text{ml}$ TT. [^3H]thymidine was added after 4 days of culture, and uptake was counted 18 h later in a liquid scintillation counter. Background levels of T cell proliferation (without TT) were subtracted.

Generation of peptide-specific CTLs

Flu peptide-specific CTLs were generated from the peripheral blood of an HLA-A2-positive donor. DC (2×10^5 cells) isolated as described above were pulsed for 2 h with the peptide (10 μM), washed, and incubated in CCM with 2×10^6 T cells in 24-well plates in the presence of IL-7 (25 ng/ml). IL-2 (1.5 ng/ml) was added 2 days later. T cells were restimulated with peptide-pulsed DC on days 7, 14, and 21. IL-7 (25 ng/ml) was added immediately after restimulation, and IL-2 (1.5 ng/ml) was added 2 days later. CTLs were harvested on day 21 or day 28 and either used immediately or cryopreserved in liquid nitrogen until being tested in an enzyme-linked immunospot (ELISPOT) assay.

The peptide-specific CTLs were tested by cytotoxicity of the peptide-loaded target T2 cells. Briefly, T2 cells ($1.6 \times 10^5/\text{well}$) were loaded with 10 $\mu\text{g}/\text{ml}$ of β_2 -microglobulin (Sigma) and 100 μM of FLU peptide. After overnight incubation cells were washed, labeled with ^{51}Cr , and used as targets in a standard 6-h chromium release assay. As a control, T2 cells were incubated with β_2 -microglobulin alone.

IFN- γ ELISPOT assay

The 96-well multiscreen filtration plates (Millipore, Bedford, MA) were coated with 50 μl of mouse anti-human IFN- γ mAb (MAB285, R&D Systems; final concentration, 12.5 $\mu\text{g}/\text{ml}$). After overnight incubation at 4°C , wells were washed four times with PBS. The remaining protein binding sites were blocked by incubating plates for 2 h at 37°C with 200 $\mu\text{l}/\text{well}$ RPMI 1640 supplemented with 10% human serum. T cells (10^5 cells/well) were incubated in final volume of 200 μl with 10^4 sorted DC or ImC in the presence of 10 $\mu\text{g}/\text{ml}$ specific peptide and 2 ng/ml IL-2. After 24-h incubation at 37°C wells, were washed six times with PBS containing 0.05% Tween 20 (PBS-T). Wells were then incubated overnight at 4°C with 100 μl (5 $\mu\text{g}/\text{ml}$) of biotinylated goat anti-human IFN- γ Ab (BAF285; R&D Systems), washed six times with PBS-T, and incubated for 2 h at room temperature with 50 μl (1.25 $\mu\text{g}/\text{ml}$) of avidin-alkaline phosphatase (Sigma). Wells were washed three times with PBS-T and three times with PBS and then incubated with 50 μl of substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium, Sigma) for 10–15 min. The reactions were stopped by discarding the substrate and washing the plates under tap water. The plates were then air-dried, and colored spots were counted using a stereomicroscope.

Flow cytometry

Cells were labeled with PE-, FITC-, or Quantum Red-conjugated Abs by incubation on ice for 30 min followed by washing with PBS. Data acquisition and analysis were performed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. For intracellular labeling cells were fixed for 60 min with 2% paraformaldehyde, permeabilized for 20 min with 0.2% Tween 20, and stained with FITC-conjugated anti-HLA DR Ab. Nonspecific binding was measured using FITC-conjugated isotype-matched mouse Ig. Cell sorting was performed on FACStar cell sorter (Becton Dickinson)

Assessment of colony formation

Colony formation by HPC was measured using semisolid 1% methylcellulose medium supplemented with recombinant cytokines (erythropoietin, stem cell factor, GM-CSF, G-CSF, IL-6, and IL-3) supporting the optimal growth of erythrocyte bone-forming unit, GM-CFU, M-CFU, G-CFU, and GEMM-CFU colonies (Methocult H4436; Stem Cell Technologies, Vancouver, Canada). Sorted peripheral blood cells were seeded at 15,000 cells/plate. Colonies were scored on days 12–13.

Electron microscopy

The cells were fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), washed in buffer, and embedded as a pellet in 1.5% low gelling temperature agarose (Sigma). The cells were given two additional washes with the sodium phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h, washed with water, block stained in 2% aranyl acetate for 2–4 h, washed again, and dehydrated using an acetone gradient. They were gradually infiltrated with Araldite resin over 8–24 h, embedded in the resin, and cured for 18 h at 65°C . The blocks were sectioned using a Reichert-Jung Ultracut E ultramicrotome (Vienna, Austria), and ultrathin (100 nm) sections on copper grids were stained with Reynold's lead citrate, carbon coated, and viewed using a JEOL JEM-1200 Ex electron microscope (Peabody, MA). The cells present were identified, and at least 100 cells were counted for each sample. The cell diameters (37–45 cells/sample) were measured at their widest points from electron micrograph prints, and the mean and SEM were calculated.

Statistical analysis

Statistical analysis was performed using parametric and nonparametric methods and JMP statistical software (SAS Institute, Cary, NC).

Results

In previous studies we have observed a significantly reduced number of DC in peripheral blood from cancer patients. This decrease closely correlated with the stage and duration of the disease (13). The decreased presence of DC in the circulation was closely associated with the accumulation of cells that copurified with DC,

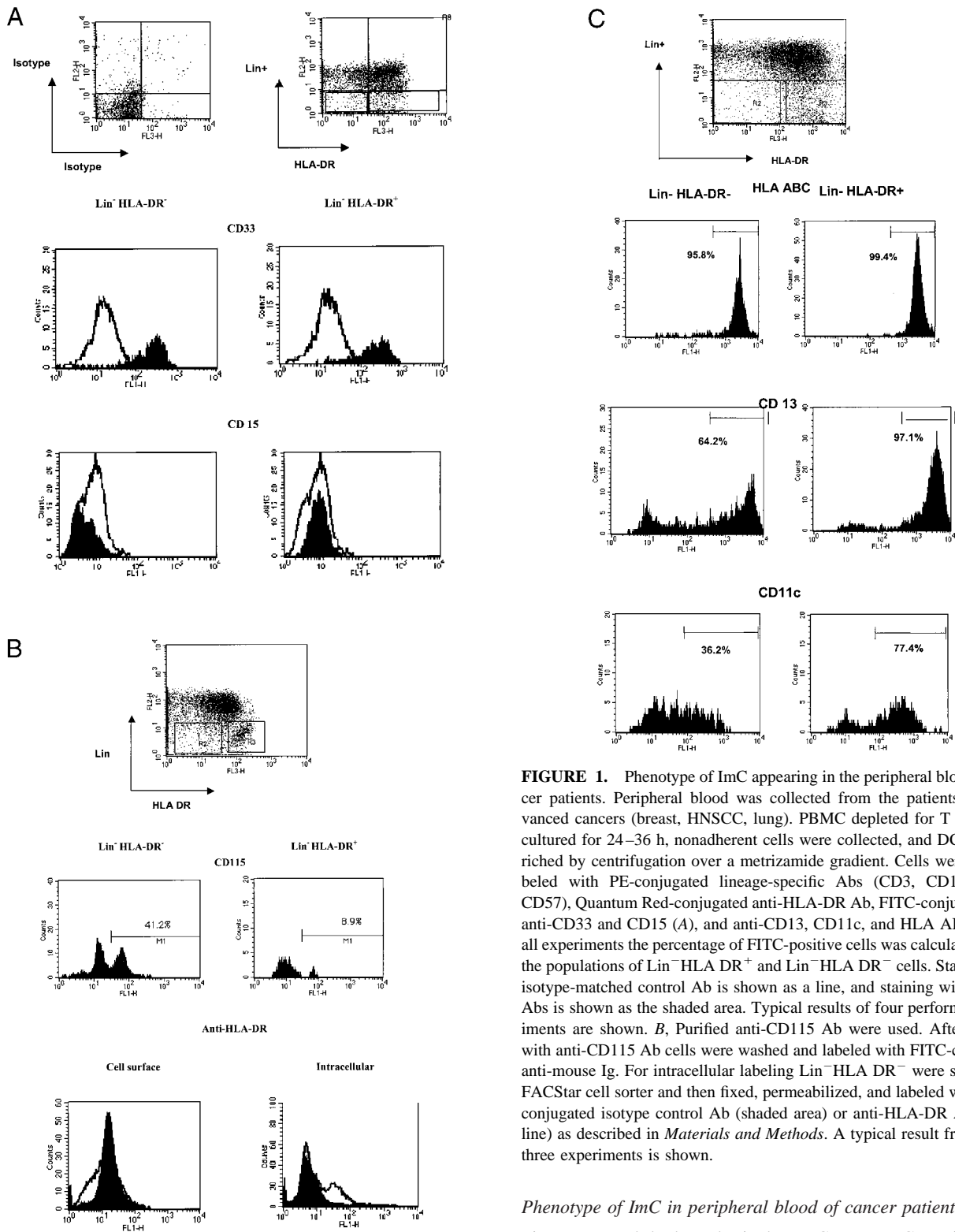


FIGURE 1. Phenotype of ImC appearing in the peripheral blood of cancer patients. Peripheral blood was collected from the patients with advanced cancers (breast, HNSCC, lung). PBMC depleted for T cells were cultured for 24–36 h, nonadherent cells were collected, and DC were enriched by centrifugation over a metrizamide gradient. Cells were then labeled with PE-conjugated lineage-specific Abs (CD3, CD14, CD19, CD57), Quantum Red-conjugated anti-HLA-DR Ab, FITC-conjugated Abs anti-CD33 and CD15 (A), and anti-CD13, CD11c, and HLA ABC (C). In all experiments the percentage of FITC-positive cells was calculated within the populations of $\text{Lin}^- \text{HLA-DR}^+$ and $\text{Lin}^- \text{HLA-DR}^-$ cells. Staining with isotype-matched control Ab is shown as a line, and staining with specific Abs is shown as the shaded area. Typical results of four performed experiments are shown. B, Purified anti-CD115 Ab were used. After labeling with anti-CD115 Ab cells were washed and labeled with FITC-conjugated anti-mouse Ig. For intracellular labeling $\text{Lin}^- \text{HLA-DR}^-$ were sorted on a FACStar cell sorter and then fixed, permeabilized, and labeled with FITC-conjugated isotype control Ab (shaded area) or anti-HLA-DR Ab (single line) as described in *Materials and Methods*. A typical result from one of three experiments is shown.

Phenotype of ImC in peripheral blood of cancer patients

but did not express markers specific for mature T or B, lymphocytes, NK cells, monocytes, or DC (CD3, CD19, CD57, CD14, HLA-DR, B7-2, and CD40) (13). We termed these cells ImC. In healthy volunteers ImC represented only a minor part of the DC fraction (<3%). In patients with early stages of cancer the presence of ImC increased >5-fold. In patients with advanced disease the number of ImC became higher than that of DC (13). To characterize the nature and possible role of ImC in cancer we studied patients with advanced stages of breast, lung, or HNSCC cancers.

First, we tested the hypothesis that ImC were HPC. Enriched DC fractions from healthy volunteers and cancer patients were labeled with PE-conjugated lineage (Lin)-specific Abs (anti-CD3, -CD14, -CD19, and -CD57), Quantum Red-conjugated anti-HLA-DR Ab, and FITC-conjugated anti-CD34 Ab (two different clones obtained from Becton Dickinson and Coulter were used). CD34 molecules are expressed on HPC. The presence of CD34⁺ cells was analyzed in the $\text{Lin}^- \text{HLA-DR}^-$ ImC population. In four experiments CD34⁺ cells represented from 0.9–1.8% of this cell population.

To investigate the presence of colony-forming HPC $\text{Lin}^- \text{HLA-DR}^-$ ImC and $\text{Lin}^- \text{HLA-DR}^+$ DC from three cancer patients and

three control donors were sorted on a cell sorter (FACStar, Becton Dickinson). Cells were then cultured in semisolid methylcellulose medium supplemented with growth factors supporting the growth of myeloid and erythroid colonies (stem cells). $\text{Lin}^- \text{HLA-DR}^+$ obtained from cancer patients and control donors gave rise to 195.4 ± 25.9 colonies/100,000 cells, whereas $\text{Lin}^- \text{HLA-DR}^-$ gave rise to $1,750.5 \pm 250.6$ colonies/100,000 cells ($p < 0.05$). Most of these colonies were erythrocyte bone-forming units and GM-CFU. No differences in the total number of colonies and the types of the colonies were found between control donors and cancer patients (data not shown). Thus, the $\text{Lin}^- \text{HLA-DR}^-$ population of ImC was enriched for progenitor cells. However, the proportion of colony-forming cells among ImC was only about 1.7%, which was consistent with the number of CD34^+ cells. Thus, these data demonstrate that HPC represent only a minor fraction of ImC.

To investigate the nature of ImC further, DC fractions isolated from cancer patients were labeled with PE-conjugated lineage-specific Abs, Quantum Red-conjugated anti-HLA-DR Ab, and FITC-conjugated Abs specific for different cell lineages. At least four experiments have been performed for each marker. More than 99% of $\text{Lin}^- \text{HLA-DR}^+$ DC and $>95\%$ of $\text{Lin}^- \text{HLA-DR}^-$ ImC expressed myeloid cell marker CD33 (Fig. 1A). Only $0.8 \pm 0.4\%$ of $\text{Lin}^- \text{HLA-DR}^+$ DC and $0.9 \pm 0.3\%$ of $\text{Lin}^- \text{HLA-DR}^-$ ImC expressed granulocyte marker CD15 ($p > 0.1$; Fig. 1A). At the same time, $8.3 \pm 1.0\%$ of $\text{Lin}^- \text{HLA-DR}^+$ DC and $32.4 \pm 8.7\%$ of $\text{Lin}^- \text{HLA-DR}^-$ ImC expressed M-CSF receptor (CD115) specific for mature and ImC of the monocyte/macrophage cell lineage ($p < 0.05$; Fig. 1B). About two-thirds of all ImC expressed myeloid cell marker CD13, and more than one-third of these cells expressed CD11c marker specific for macrophages/DC (Fig. 1C). In three independent experiments almost all ImC ($\text{Lin}^- \text{HLA-DR}^-$) expressed MHC class I molecule (Fig. 1C).

As has been reported, immature DC contain intracellular MHC class II molecules associated with MHC class II compartments (15). In mature DC these molecules are transported to the cell surface. To investigate whether these ImC contain intracellular MHC class II molecules, $\text{Lin}^- \text{HLA-DR}^-$ cells were sorted on a flow cell sorter (FACStar, Becton Dickinson), fixed, permeabilized, and stained with anti-HLA-DR Ab. In three separate experiments $>20\%$ of all $\text{Lin}^- \text{HLA-DR}^-$ cells contained high levels of intracellular HLA-DR (Fig. 1B).

Thus, together these data demonstrate that with the exception of small proportion of HPC, ImC were represented by MHC class I-positive myeloid cells. About one-third of the ImC were relatively immature monocytes/macrophages and DC. The remaining cells probably represented earlier stages of cell differentiation.

Ultrastructure of immature myeloid cells

Two populations of cells, ImC ($\text{Lin}^- \text{HLA-DR}^-$) and DC ($\text{Lin}^- \text{HLA-DR}^+$) cells, were sorted on a FACStar cell sorter and analyzed by electron microscopy. Two separate experiments were performed. These two populations of cells did not differ with regard to the presence of apoptotic or necrotic cells; the total numbers of dying cells in the two experiments were, respectively, 11 and 4% for $\text{Lin}^- \text{HLA-DR}^-$ and 10 and 3% for $\text{Lin}^- \text{HLA-DR}^+$ cells. Relatively small ImC represented a significant proportion of the $\text{Lin}^- \text{HLA-DR}^-$ cells (Fig. 2, A and B). In the two experiments, the mean diameter at the widest points of all cells in this $\text{Lin}^- \text{HLA-DR}^-$ population were 6.1 ± 0.2 and 6.4 ± 0.2 μm compared with 7.8 ± 0.2 and 7.8 ± 0.3 μm in the $\text{Lin}^- \text{HLA-DR}^+$ population ($p < 0.05$). In the two samples, 25 and 29% of the total $\text{Lin}^- \text{HLA-DR}^-$ cells were identifiable as morphologically immature monocytes and DC (Fig. 2, C–E). These cells are small, only 4–6 μm in diameter. Many vacuoles are present. Although DC can

also express vacuoles, this is generally a monocyte/macrophage characteristic, particularly if different sizes of vacuoles are present and abundant. Another characteristic is the horseshoe-shaped nucleus that may be developing in this cell (Fig. 2C). The volume of the cytoplasm compared with that of the nucleus is small and somewhat similar to that in lymphocytes. However, the cell surface is irregularly shaped and is not like that in lymphocytes or lymphoblasts, but is in the form of developing veils (Fig. 2D). As the DC develop further the volume of the cytoplasm in relation to the nucleus increases, and distinct veiled projections can be seen. The cytoplasm has fewer inclusions than macrophages. Despite this veiled morphology, the immaturity of these cells is evident from the size and the still heterochromatic nucleus (Fig. 2E). Such ImC were rarely found in the population of $\text{Lin}^- \text{HLA-DR}^+$ that contained cells of a more mature appearance. These cells are larger, with larger veiled projections. The chromatin is becoming more euchromatic, suggesting that maturation is occurring. The cytoplasm is paler than that in macrophages with fewer organelles and occasional large apparent vacuoles, probably produced from the invaginations of the veiled surface morphology (Fig. 2F). Thus, these data are in agreement with the results of surface marker expression and suggest that ImC belong to the myeloid cell lineage.

Differentiation of ImC in the presence of growth factors and cytokines

We asked whether these ImC could be differentiated into mature cells in vitro. T cell-depleted mononuclear cells obtained from patients with advanced disease were cultured overnight, and non-adherent cells were collected and either analyzed immediately or cultured in the presence of various cytokines. Half the medium was replenished, and fresh cytokines were added on day 3 in all experiments. After 5–6 days of culture cells were collected, washed, and labeled with a cocktail of Abs as described above. Less than 10% of the cells survived 5–6 days of incubation in medium alone. In preliminary experiments we tested two cytokines important for the survival of different populations of myeloid cells: GM-CSF and IL-3. All cells survived a 5- to 6-day incubation with 20–30 ng/ml GM-CSF or 20–50 ng/ml IL-3 (data not shown). However, neither of these cytokines significantly changed the proportion of ImC. The culture of ImC for 5–6 days with IL-3 (1–20 ng/ml) or with a combination of GM-CSF and IL-3 also did not affect the proportion of ImC (Fig. 3). We used GM-CSF in all subsequent experiments to maintain cell viability. Several combinations of factors were used: GM-CSF and IL-4 (10 ng/ml), TNF- α (1 ng/ml), TGF- β (10 ng/ml), FLT-3 ligand (100 ng/ml), LPS (1 $\mu\text{g}/\text{ml}$), and CD40L (200 ng/ml). At least three experiments with each combination were performed. Only the combination of GM-CSF and IL-4 significantly increased the proportion of DC (Fig. 3). The proportion of DC was further increased (almost to control levels) when a combination of three cytokines (GM-CSF, IL-4, and CD40L) or (GM-CSF, IL-4, and TNF- α) was used (data not shown). However, previous studies have shown that the combination of GM-CSF and IL-4 induced generation of DC from plastic adherent progenitors in peripheral blood (16, 17). These cells actively proliferate during the first couple of days in culture. A substantial proportion of these cells might have been present in the culture, and DC generated from those progenitors might then significantly decrease the proportion of ImC. To eliminate this possibility $\text{Lin}^- \text{HLA-DR}^-$ cells were first sorted and then incubated with GM-CSF (control), GM-CSF and IL-4, or GM-CSF, IL-4, and TNF- α for 6 days as described above. In three experiments the combination of these cytokines decreased the presence of ImC ($\text{Lin}^- \text{HLA-DR}^-$, B7-2 $^-$, or CD40 $^-$) by only 15–20% (data not

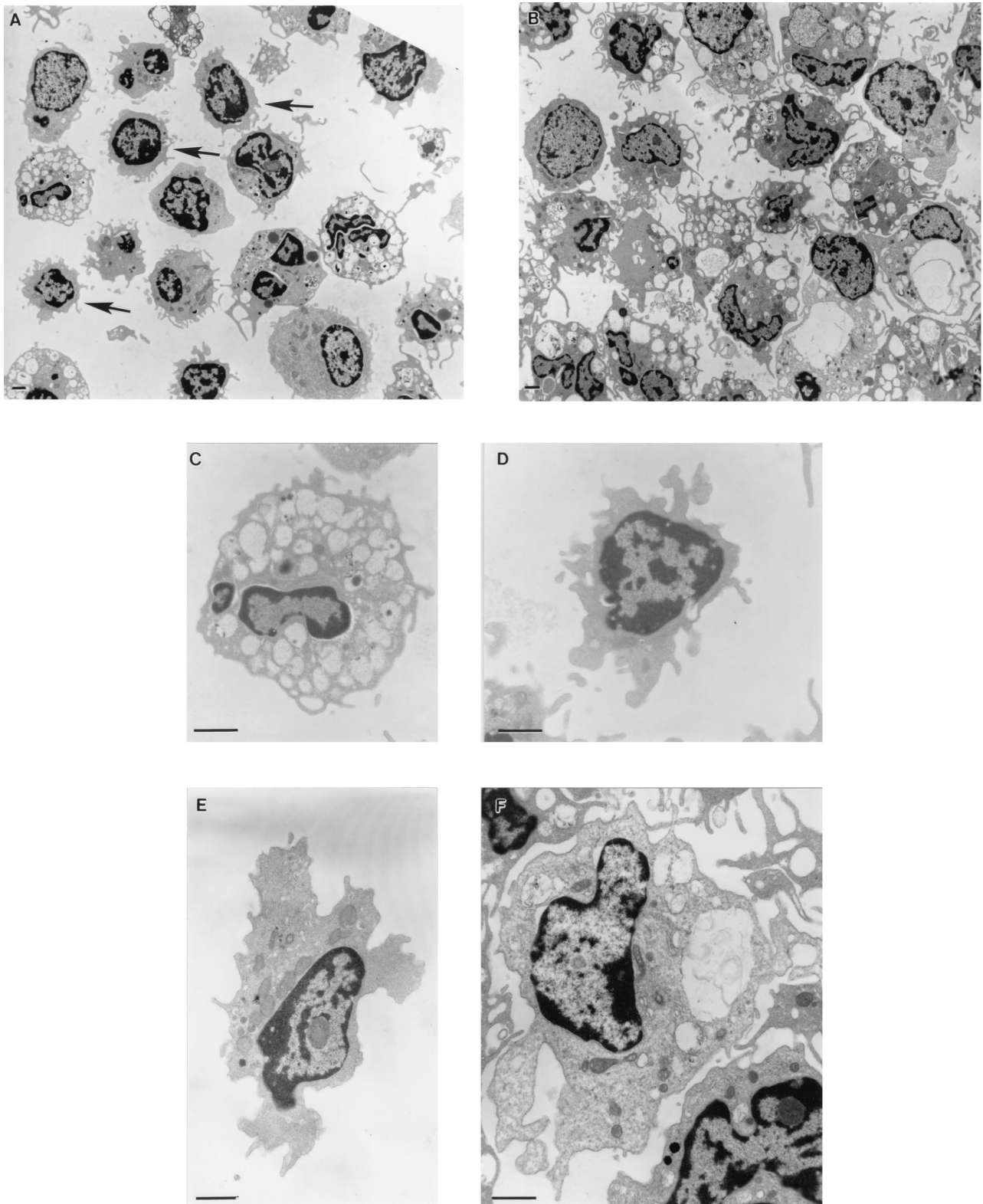


FIGURE 2. Electron microscope pictures of lineage-negative cells. Lin-HLA DR⁻ and Lin-HLA DR⁺ cells were sorted on the FACStar cell sorter and examined by electron microscopy. *A*, Lin-HLA DR⁺ cells contained small ImC (arrows) and immature DC and monocytes (magnification, $\times 3500$). *B*, Lin-HLA DR⁺ cells were larger, and mostly had many long veils (magnification, $\times 3500$). *C–E*, Lin-HLA DR⁻ cells showing, respectively, an immature monocyte, an immature DC, and an immature DC with short veils. All cells have heterochromatic nuclei (magnification, $\times 11,500$). *F*, Lin-HLA DR⁺ mature DC with veils, a plain cytoplasm, and a nucleus becoming euchromatic (magnification, $\times 11,500$).

shown). These data indicate that the considerable decrease in the proportion of ImC observed in the presence of these cytokines in previous experiments was due to accumulation of DC generated from the progenitors but not from ImC.

We then asked whether the immature myeloid cells could be differentiated into granulocytes or monocytes/macrophages in the presence of appropriate growth factors. Lin⁻HLA-DR⁻ cells were sorted and then cultured with GM-CSF, G-CSF (20 ng/ml), or

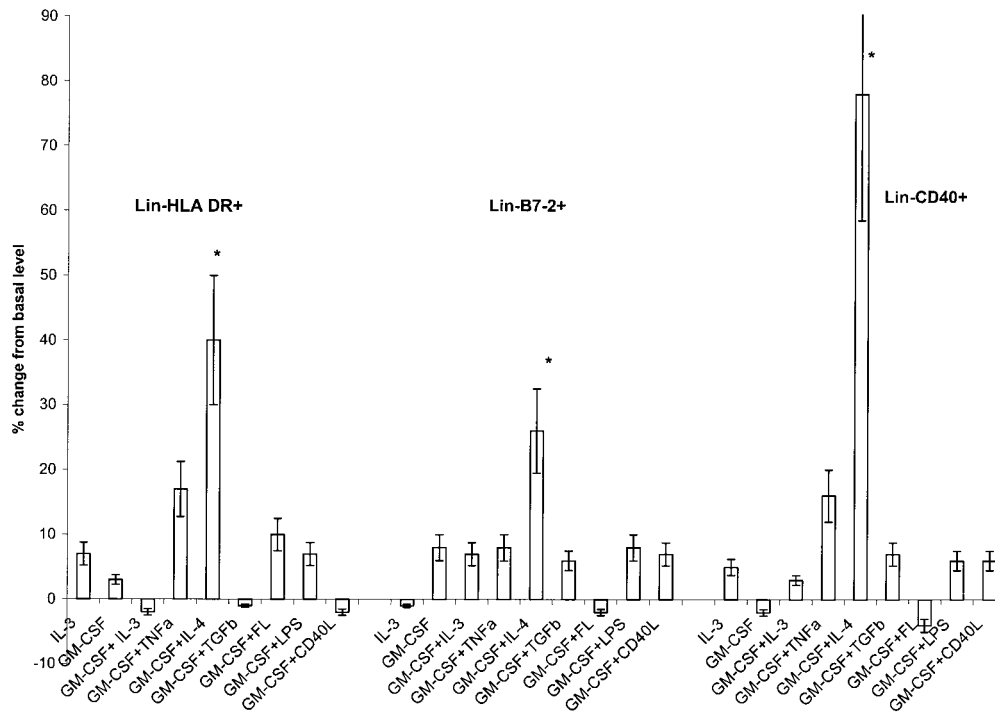


FIGURE 3. Differentiation of ImC into DC in the presence of a combination of different growth factors and cytokines. PBMC were collected from patients with advanced head and neck, breast, and lung cancer. Enriched fractions of the DC were prepared as described in *Materials and Methods*. Cells were cultured for 5–6 days with growth factors and cytokines as indicated on the *x*-axis. After that time cells were washed and labeled with a PE-conjugated mixture of lineage-specific Abs and FITC-conjugated anti-HLA-DR, B7-2, and CD40 Abs. The proportion of Lin⁺ HLA DR⁺, Lin⁺ B7-2⁺, and Lin⁺ CD40⁺ cells was calculated. The pretreatment (basal) level of DC in each sample is expressed as zero. The percentage of changes in the proportion of DC after 6 days of incubation is shown. Combined results from at least three experiments for each combination of cytokines and growth factor are shown. *, Statistically significant differences from the a basal level ($p < 0.05$).

M-CSF (20 ng/ml). After 5–6 days in culture the proportions of CD15⁺ and CD14⁺ cells were determined. Neither of these growth factors increased the presence of CD15⁺ cells. GM-CSF and G-CSF did not significantly change the proportion of CD14⁺ cells. However, treatment with M-CSF increased the presence of CD14⁺ cells. Almost 30% of cells expressed this marker after 5–6 days in culture (three separate experiments with the same results were performed). Thus, about 30% of ImC found in the peripheral blood of cancer patients could be differentiated into monocytes/macrophages in the presence of M-CSF.

Functional significance of immature myeloid cells

To investigate the possible functional role of ImC, Lin⁺ HLA-DR⁻ and Lin⁺ HLA-DR⁺ cells were isolated from the peripheral blood of patients with advanced cancer using cell sorting. Cells were then cultured with allogeneic T cells isolated from healthy volunteers. DC isolated from healthy individuals were used as controls. As reported earlier, the cells in the DC fraction from patients with advanced cancer demonstrated a profound defect in their ability to stimulate allogeneic T cells (8, 13). Here, in four experiments Lin⁺ HLA-DR⁻ cells were not able to stimulate allogeneic T cells (Fig. 4A). At the same time, Lin⁺ HLA DR⁺ DC demonstrated control levels of T cell stimulation (Fig. 4A). Thus, it appears that removal of ImC completely restored the ability of the DC population to stimulate allogeneic T cells.

To investigate whether ImC might affect Ag-specific T cell responses in cancer, two different experimental system have been used. T cells, Lin⁺ HLA-DR⁺ DC, and Lin⁺ HLA-DR⁻ ImC were isolated from peripheral blood of patients with advanced cancer as described in *Materials and Methods*. To investigate the ImC effect on T cell response to TT (predominantly MHC class II-associated

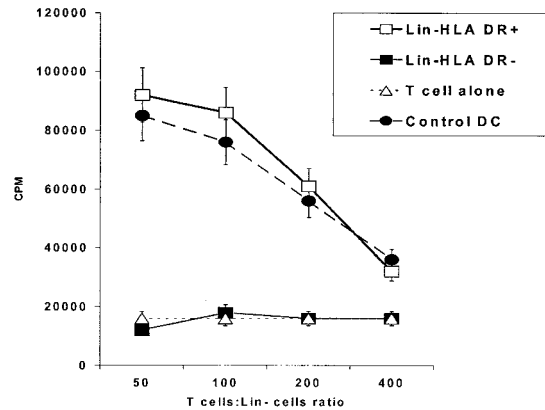
response) T cells (50,000/well) were incubated in 96-well plates with Lin⁺ HLA-DR⁺ DC (5,000 cells/well) with or without 1 μ g/ml of TT. Different numbers of ImC (1,000–5,000/well) were added to the mixture, and T cell proliferation was measured after 5 days. In all four experiments the addition of ImC significantly reduced TT-specific T cell proliferation (Fig. 3B). At an ImC/DC ratio of 1:1 (usually seen in cancer patients (13)) the T cell response was reduced >3-fold (Fig. 4B). No effect of ImC on T cell proliferation in absence of TT was found (data not shown). It was possible that in these experiments ImC might compete with T cells for nutrients in the medium. To exclude this possibility we used sorted autologous CD14⁺ cells instead of ImC. The addition of as many as 5000 CD14⁺ monocytes to the culture with DC did not significantly affect TT-specific T cell proliferation (data not shown).

For analysis of the MHC class I-restricted response, a CTL line was generated from HLA-A2-positive healthy volunteer by stimulation of T cells with influenza virus-derived peptide with high affinity to HLA-A2 molecule. The peptide specificity of the CTLs was confirmed in cytotoxicity assay with peptide-loaded T2 cells (data not shown). ImC and DC were isolated from peripheral blood of HLA-A2-positive cancer patients and were incubated with the specific CTLs with or without peptide. Production of IFN- γ by T cells was analyzed 24 h later using an ELISPOT assay. DC from a cancer patient increased the number of peptide-specific IFN- γ producing cells >3-fold. However, the presence of ImC in the mixture at an ImC/DC ratio of 1:1 almost completely abrogated that effect (Fig. 4C). These data demonstrated that ImC were able to inhibit Ag-specific T cell responses.

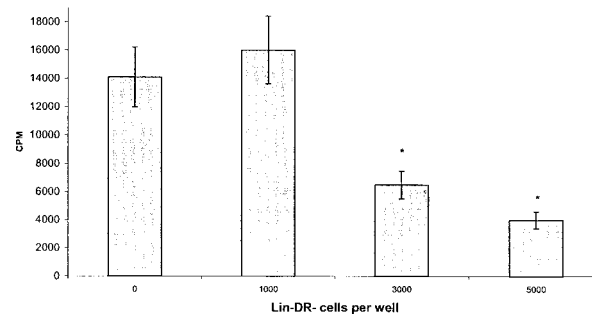
What could be a mechanism of the effects of ImC? These cells are comprised of immature macrophages and myeloid cells known

FIGURE 4. Functional activity of ImC. *A*, Peripheral blood was collected from cancer patients with advanced diseases. The DC fraction was prepared as described in *Materials and Methods*. Lin⁻HLA DR⁻ and Lin⁻HLA DR⁺ cells were sorted on a cell sorter and incubated in triplicate with 50,000 T cells isolated from control donors at the indicated ratios. Control DC were isolated from healthy individuals. T cell proliferation was measured by [³H]thymidine uptake after 5 days of culture. The results of one experiment are shown. Four experiments with similar results were performed. *B*, T cells, Lin⁻HLA-DR⁻ ImC and Lin⁻HLA-DR⁺ DC were isolated from the peripheral blood of cancer patients as described above. Five thousand DC were mixed together with 50,000 T cells with and without TT (1 μg/ml). The indicated number of ImC was added to the DC-T cell mixture and incubated for 5 days. T cell proliferation was measured in triplicate as described above. The values of T cell proliferation in the absence of TT were subtracted. The average ± SE in one experiment is shown. Four experiments with the same results were performed. *, Statistically significant differences from T cell proliferation without ImC ($p < 0.05$). *C*, Lin⁻HLA-DR⁻ ImC and Lin⁻HLA DR⁺ DC were isolated from the peripheral blood of HLA-A2-positive cancer patients as described above. CTLs specific for influenza virus-derived peptide were generated from an HLA-A2-positive donor as described in *Materials and Methods*. DC (10⁴) were mixed with 10⁵ CTLs and cultured for 24 h with and without specific peptide. In parallel wells CTLs were cultured with 10⁴ ImC or with a mixture of 10⁴ DC and 10⁴ ImC. The number of IFN-γ-producing cells per 10⁶ cells was measured in duplicate using an ELISPOT assay as described in *Materials and Methods*. The averages ± SE in four experiments are shown. *, Statistically significant differences from the results with cells incubated without peptide ($p < 0.05$); #, statistically significant differences from the results obtained without ImC ($p < 0.05$).

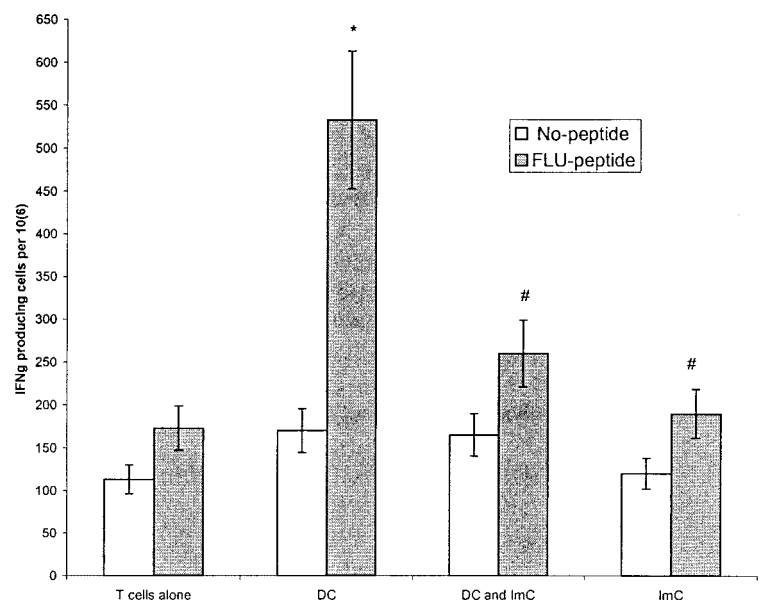
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B



C



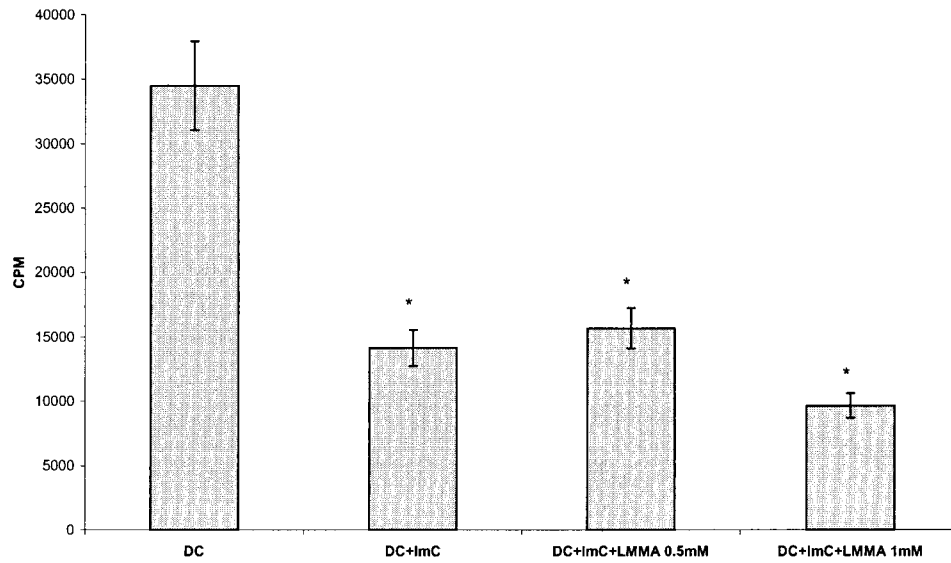
to produce NO. NO is a well-described factor that inhibits T cell function (18). To investigate the role of NO in the observed effects of ImC we used a competitive inhibitor of NO synthase, LMMA (19). LMMA did not significantly affect the inhibitory effect of ImC on TT-dependent T cell proliferation or on FLU peptide-specific IFN-γ-producing cells (Fig. 5). To study the role of soluble factors released by ImC, these cells and DC were cultured for 24 h in U-bottom 96-well plates at a concentration of 5×10^5 cells/ml. Cell supernatants were collected and immediately used in

experiments with TT-dependent proliferation and FLU peptide-specific response. In these experiments DC and T cells isolated from HLA-A2-positive healthy volunteer were used. The presence of supernatants at a final concentration as high as 20% (v/v) did not significantly affect the T cell response (data not shown).

Differentiation of immature myeloid cells with ATRA

ATRA is a natural oxidative metabolite of vitamin A and is known to be a regulator of cell differentiation (20, 21). ATRA induces

A



B

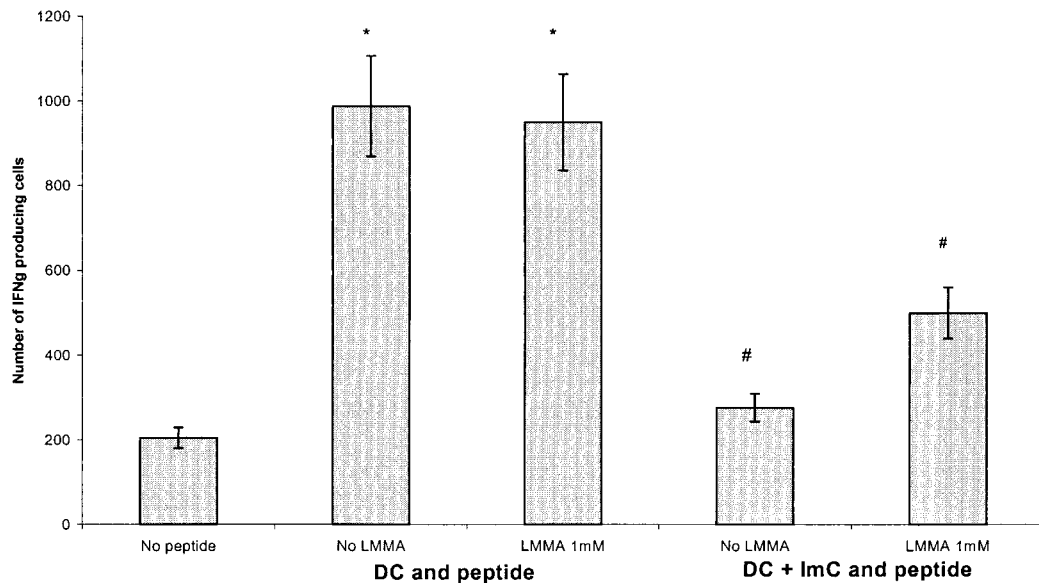


FIGURE 5. An inhibitor of NO production (LMMA) does not change the effect of ImC. T cells, ImC, and DC were isolated from cancer patients as described in *Materials and Methods*. **A**, Ten thousand DC were mixed together with 100,000 T cells with and without TT (1 μ g/ml). Ten thousand ImC were added to the DC-T cell mixture and incubated for 5 days. LMMA was dissolved in PBS and added at the start of the culture at a final concentration of 0.5 or 1 mM. T cell proliferation was measured in triplicate as described above. The values of T cell proliferation in the absence of TT were subtracted. The averages \pm SE in one experiment are shown. Two experiments with the same results were performed. *, Statistically significant differences from T cell proliferation without ImC ($p < 0.05$). **B**, Lin⁻HLA-DR⁻ ImC and Lin⁻HLA DR⁺ DC were isolated from the peripheral blood of HLA-A2-positive cancer patients. CTLs specific for influenza virus-derived peptide were generated from HLA-A2-positive donor as described in *Materials and Methods*. DC (10^4) were mixed with 10^5 CTLs and cultured for 24 h with and without specific peptide (DC). In parallel wells CTLs were cultured with 10^4 ImC and 10^4 DC. LMMA was added at the start of the culture at a final concentration of 1 mM. The number of IFN- γ -producing cells per 10^6 cells was measured in triplicate using an ELISPOT assay as described in *Materials and Methods*. The average \pm SE are shown. Two experiments with similar results were performed. *, Statistically significant differences from the results with cells incubated without peptide ($p < 0.05$); #, statistically significant differences from the results obtained without ImC ($p < 0.05$).

terminal differentiation of promyelocytes into mature neutrophils in patients with M3 (acute promyelocytic) leukemia (22). Because the majority of ImC were represented by immature myeloid cells at early stages of differentiation, we hypothesized that these cells can be differentiated into mature cells by ATRA. To test this hypothesis cells from DC fractions of cancer patients were cultured with 30 ng/ml of GM-CSF and different concentrations of ATRA (5 nM to 10 μ M). After 5 days the total number of cells and cell viability were measured. ATRA at concentrations of 5 and 10 μ M was toxic for the cells. Cell viability returned to a control level at 1 μ M and remained at the same level at all other tested concentrations (Fig. 6A). The total number of recovered cells remained at the control (no ATRA) level at concentrations from 10^{-6} to 5×10^{-8} M and increased at lower concentrations of ATRA (Fig. 6B). In all subsequent experiments we used ATRA at concentrations of 1 μ M and lower. Five-day incubation of cells with ATRA dramatically reduced the presence of ImC and increased the presence of DC. In four experiments the proportion of Lin⁻HLA-DR⁺ DC increased from $30.4 \pm 4.8\%$ (in the presence of GM-CSF alone) to $70.2 \pm 5.6\%$ (at an ATRA concentration of 1 μ M) and was slightly lower at an ATRA concentration of 0.5 μ M. A further

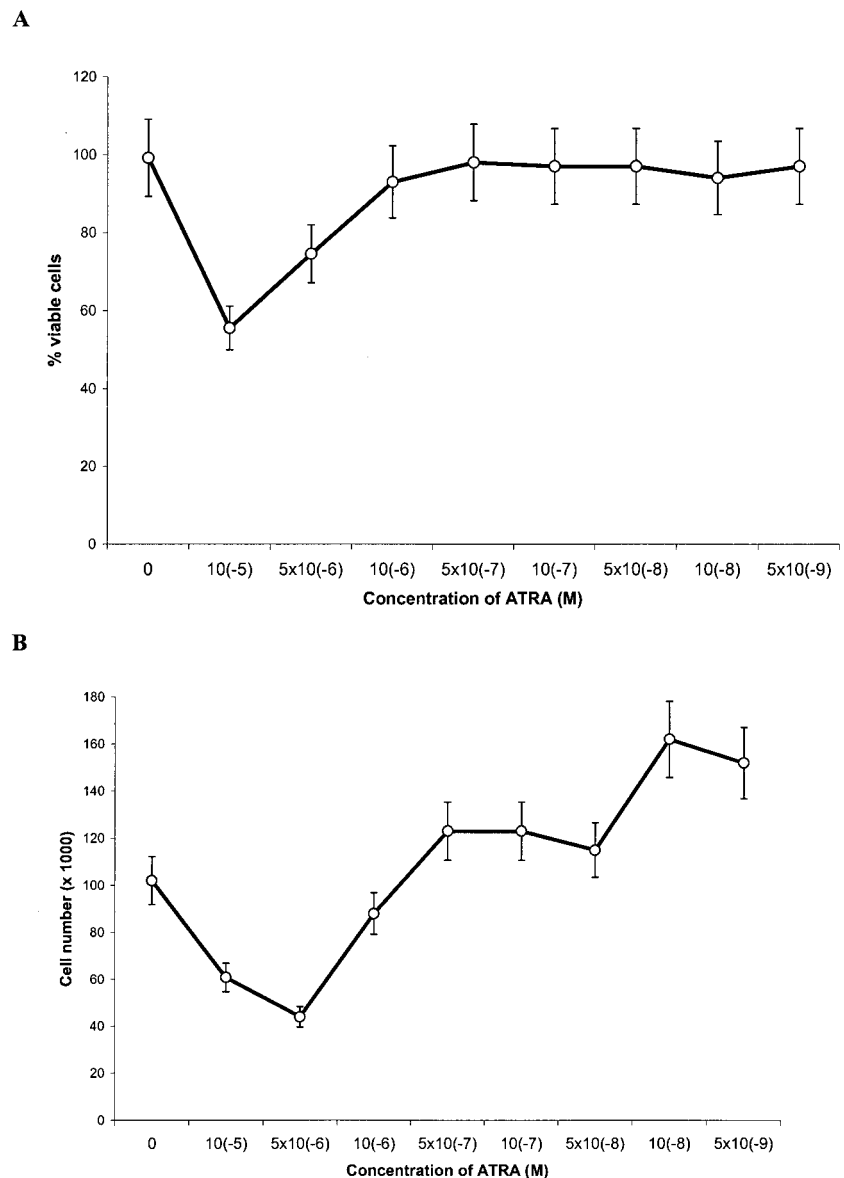
decrease in the ATRA concentration cancelled the effect (Fig. 7A). Similar dramatic changes were observed in the presence of Lin⁻B7-2⁺ DC (Fig. 7A). The proportion of CD83⁺ (marker specific for mature DC) cells in the DC fraction increased in the presence of 1 μ M ATRA by almost 4-fold. To confirm that these cells were indeed DC we used an allogeneic MLR (a specific function of relatively mature DC). In five independently performed experiments ATRA dramatically increased the ability of these cells to stimulate control allogeneic T cells (Fig. 7B). These data indicate that ATRA was able to differentiate the majority of ImC to DC.

Discussion

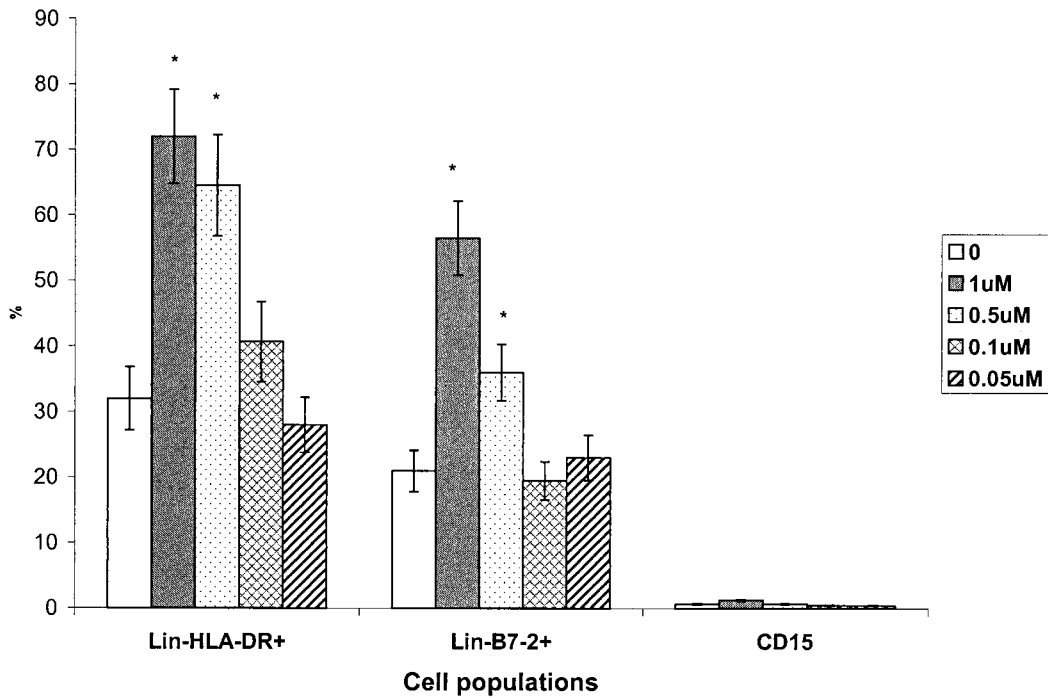
The evidence presented in this paper shows that the population of ImC that is increased in the blood of patients with cancer consists of early stage myeloid cells and immature monocytes and DC; these cells were also able to block T cell stimulation.

Effective function of the DD is an important element of antitumor immunity. The presence of functionally competent DC is critical for effective antitumor control and for the success of cancer immunotherapy. There is ample evidence of inadequate function of these cells in tumor-bearing hosts. At the same time, mature DC

FIGURE 6. Effect of ATRA on cell viability and recovery. Cells in DC fractions obtained from cancer patients were incubated in duplicate in U-bottom 96-well plates (10^5 cells/well) in 200 μ l of CCM containing 30 ng/ml of GM-CSF and different concentrations of ATRA. After 6 days of incubation, the percentage of viable cells was measured using trypan blue exclusion (A). The number of recovered cells per well was calculated (B). Two experiments with the same results were performed. The average \pm SE in one experiment are shown.



A



B

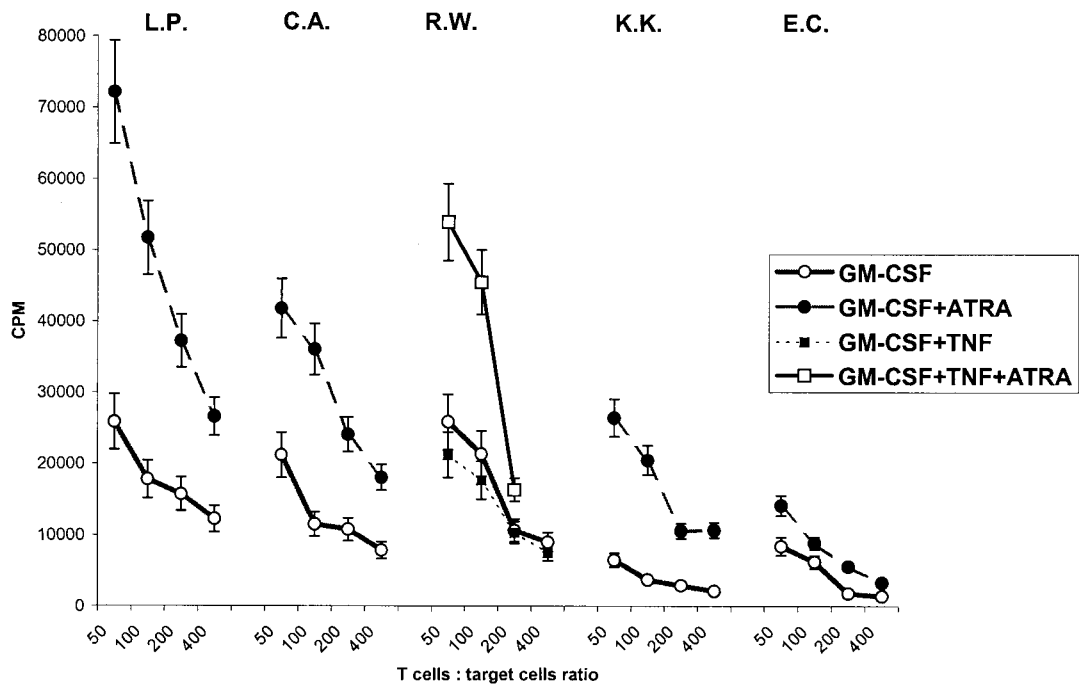


FIGURE 7. Effect of ATRA on ImC differentiation. A, Cells from enriched fractions of DC were cultured with 30 ng/ml GM-CSF and different concentrations of ATRA for 6 days. After that time cells were collected and labeled with PE-conjugated lineage-specific markers and FITC-conjugated anti-HLA-DR, B7-2, or CD15 Abs as described in Fig. 2. The proportions of Lin⁻HLA-DR⁺ and Lin⁻B7-2⁺ DC as well as Lin⁻CD15⁺ cells were calculated. The average ± SE in three performed experiments are shown. *, Statistically significant differences from the group of cells treated only with GM-CSF (*p* < 0.05). B, Cells obtained from five patients with advanced stages of lung cancer were treated with GM-CSF (30 ng/ml), GM-CSF and TNF-α (5 ng/ml), and the combination of these cytokines with 1 μM ATRA as described above. After 6 days in culture, cells were collected, irradiated at 20 Gy, and incubated in triplicate with 50,000 T cells obtained from healthy volunteers. Different T cell/target cell ratios were used. T cell proliferation was measured using [³H]thymidine as described in *Materials and Methods*. The average ± SE for each experiment are shown.

remaining in tissues demonstrate normal levels of functional activity and functionally potent DC can be generated from progenitors in patients even with advanced stages of cancer (3–6, 8–10). Tumor-derived factors dramatically affect DC differentiation *in vitro* (23–27). Decreased DC production was associated with accumulation of monocytes/macrophages and immature myeloid cells. In addition, a significant decrease in the proportion and absolute numbers of DC in peripheral blood from cancer patients has been recently reported (12, 13). All these data support the idea that abnormal differentiation of DC forms a basis for defects of DC in cancer. We have previously demonstrated that the decreased presence of DC in cancer patients was closely associated with the accumulation in peripheral blood of cells lacking markers specific for mature cells of lymphoid and myeloid lineages (13). These cells were termed ImC. The presence of these cells dramatically increased in patients with early stages of cancer. In patients with advanced disease the number of ImC was significantly higher than the number of DC (13). The presence of ImC dropped considerably within 3–4 wk after surgical removal of the tumor (13). This drop is consistent with the hypothesis that the generation of these cells was due to the production of soluble tumor-derived factors. Thus, the appearance of ImC in peripheral blood of cancer patients was clinically relevant.

DC belong to the myeloid cell lineage, and DC and macrophages share a common progenitor. Therefore, it was not surprising that inhibition of DC differentiation *in vitro* results in the appearance of immature myeloid cells and monocytes (23, 24). Here we investigated the nature of the ImC generated in cancer patients. First, we tested the hypothesis that these cells could be CD34⁺ HPC. Increased production of these cells in patients with head and neck cancer has been previously reported (28). The population of ImC indeed contained an increased proportion of CD34⁺ HPC. However, the total percentage of these cells was <2%. The rest of the cells were MHC class I-positive myeloid cells. ImC did not express granulocyte markers. However, about 30% of these cells expressed M-CSF receptor (CD115), specific for cells of monocyte/macrophages lineage. The same proportion of cells expressed another marker of this cell lineage, CD11c. About 60% of ImC were CD13 positive. This molecule is expressed on a portion of immature myeloid cells, monocyte/macrophage, and DC. About 20% of the cells expressed intracellular HLA-DR, which might be considered characteristic of immature DC. Similar data were obtained by electron microscopy. This was confirmed by *in vitro* maturation experiments with growth factors. About one-third of the ImC became CD14⁺, plastic-adherent macrophages in the presence of M-CSF. About 20% of cells became Lin-HLA-DR⁺ DC in the presence of GM-CSF and IL-4. The presence of G-CSF or GM-CSF did not lead to differentiation of these cells into granulocytes. Taken together these data indicate that about one-third of ImC are represented by ImC of the macrophage/DC cell lineages. The remaining two-thirds of ImC are probably the cells at earlier stages of myeloid differentiation.

The exact mechanism of increased production of immature myeloid cells in cancer patients is not clear. However, it is known that tumor cells may produce several growth factors and cytokines able to stimulate myelopoiesis (GM-CSF, M-CSF, and IL-6). In addition, vascular endothelial growth factor produced by many tumors is able to affect myelopoiesis (29). It is possible that increased production of these growth factors may affect the normal pathway of cell differentiation resulting in the accumulation of immature myeloid cells.

We also asked whether these immature myeloid cells might affect immune function. Elimination of ImC using cell sorting completely restored the functional potency of the DC fraction. This is

consistent with previously reported observation that DC sorted based on phenotypic characteristics were functionally competent in tumor-bearing mice (10). These data indicate that the appearance of ImC may be responsible for the decreased function of DC in cancer patients observed in previous studies. To investigate the effect of ImC on the Ag-specific T cell response we used two experimental systems. In both these systems (MHC class II-associated TT-specific T cell proliferation and MHC class I-restricted IFN- γ production) ImC actively inhibited the T cell response in the presence of functionally competent DC. This effect was seen at an ImC/DC ratio of 1:1. This or higher ratios were observed in almost all patients with advanced stages of cancer and in some patients with early stages of the disease. These data demonstrate that ImC may actively suppress immune responses and thus contribute to tumor nonresponsiveness. Several mechanisms may be responsible for the observed effects of ImC. Our preliminary data ruled out a direct role of apoptosis; ImC did not induce apoptosis of T cells (unpublished observations). Supernatants from ImC also did not significantly affect either T cell proliferation in response to TT or IFN- γ production in response to FLU peptide. The experiments with the NO inhibitor LMMA suggest that NO was not actively involved in the observed effects of ImC. Thus, it is more likely that ImC exert their effects via direct cell-cell contact. The fact that almost all ImC express MHC class I molecule suggests that ImC might induce T cell anergy by engaging the TCR complex on T cells in the absence of costimulatory signals. At this time we are investigating this possibility.

Thus, it appears that ImC can be an important factor in immunosuppression in cancer. Hyperproduction of these cells might impede any attempt to induce a strong Ag-specific T cell response in cancer patients. It could be one of the factors that make cancer vaccines ineffective in patients with advanced stages of the disease. Therefore, it will be beneficial to find ways to eliminate these cells. One of the approaches would be to differentiate ImC into relatively mature cells. We have used several combinations of growth factors that promote differentiation of DC, macrophages, and granulocytes. They demonstrate only a minor effect on the population of ImC. The precise mechanism of this nonresponsiveness is not clear. It could be explained by the fact that the majority of ImC were represented by immature myeloid cells at early stages of differentiation. It is possible that these cells may have an altered differentiation program. This may be due to the lack of expression of the growth factors' receptors or to affected molecular mechanisms responsible for cell differentiation. These mechanisms are under investigation at this time. Because of the nature of ImC, we have used ATRA in an attempt to differentiate these cells into granulocytes. A naturally occurring isomer of retinoic acid, ATRA is a well-known factor capable of induction of differentiation of the human leukemia cell line HL-60 and freshly isolated acute promyelocytic leukemia cells (30, 31). It is successfully used in differentiation induction therapy in patients with acute promyelocytic leukemia (32, 33). ATRA may also affect the growth of normal hemopoietic progenitors and blast progenitors in acute myelogenous leukemia. However, these effects of ATRA depend on culture conditions (34–36). To our surprise, in the presence of GM-CSF and ATRA the majority of ImC were differentiated into relatively mature DC. The ability of GM-CSF to enhance the effect of ATRA on differentiation of human myeloblastic leukemia ML-1 cells into granulocytes has been previously described (37). No information is available about the ability of ATRA, in combination with any other growth factor, to induce differentiation of the DC. However, early studies in maturing splenic DC from mice showed that exposure to low doses of retinoid resulted in DC with an enhanced capacity to stimulate T cell proliferation; higher amounts

resulted in a lower stimulatory capacity (37). It is likely that this effect depends on the nature of ImC. More studies are underway to identify the mechanism of the effects of ATRA on DC differentiation.

In conclusion, here we have demonstrated a significant accumulation of immature myeloid cells in peripheral blood of cancer patients. These cells actively suppressed the Ag-specific T cell response in cancer patients and thus could be involved in immunosuppression in cancer. ATRA was able to differentiate the majority of these cells into relatively mature DC. This observation may suggest a new approach to treatment of solid tumors and may be useful in the immunotherapy of cancer, especially in patients with advanced disease.

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