Human Monocytes, but not Dendritic Cells Derived from Them, Are Defective in Base Excision Repair and Hypersensitive to Methylating Agents

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Abstract

Monocytes and dendritic cells are key players in the immune response. Because dendritic cells drive the tumor host defense, it is important that monocytes and dendritic cells survive cytotoxic tumor therapy. Although most of the anticancer drugs target DNA, the DNA repair capacity of monocytes and dendritic cells has not yet been investigated. We studied the sensitivity of monocytes and monocyte-derived dendritic cells against various genotoxic agents and found monocytes to be more sensitive to overall cell kill and apoptosis upon exposure to methylating agents (e.g., *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, methyl methanesulfonate, and the anticancer drug temozolomide). On the other hand, upon treatment with the cross-linking chemotherapeutics fotemustine, mafosfamide, and cisplatin, monocytes and dendritic cells responded in the same way. Monocytes were also more sensitive than lymphocytes. The data indicate a defect in the repair of DNA methylation damage in monocytes. Because the expression of the repair protein O^6-methylguanine-DNA methyltransferase was higher in monocytes than in dendritic cells, and because its inhibition by O^6-benzylguanine had no effect on the sensitivity of monocytes, we investigated the base excision repair (BER) pathway. In contrast to dendritic cells, monocytes are unable to perform BER following exposure to methylating agents. Expression studies revealed that monocytes lack XRCC1 and ligase IIIα, whereas dendritic cells, similar to human lymphocytes, express these repair proteins at a high level. The data revealed a DNA repair defect in a specific human cell population. The BER defect in monocytes may cause them to be selectively killed during tumor therapy with alkylating agents, provoking hematotoxicity and sustained immunosuppression. [Cancer Res 2007;67(1):26–31]

Introduction

Monocytes and dendritic cells play an important role in the immune system (1). Dendritic cells present antigenic peptides on the surface of the cell as MHC, thus stimulating T helper cells and CTLs as well as, via T cells, macrophages (2). Using antigen-loaded dendritic cells in tumor therapy for vaccination, dendritic cell–mediated cancer immunotherapy has gained broad clinical application (3). Given the key role of monocytes and dendritic cells in the immune response and during tumor therapy, it would be desirable to protect monocytes and monocyte-derived dendritic cells from the side effects of cytotoxic anticancer drugs. Most anticancer agents target DNA (4). Therefore, the DNA repair status is of utmost importance for drug sensitivity of tumors, and, at the same time, for protection of the normal tissue, notably the immune system. Although DNA repair has been studied in many tumors (5), the DNA repair status of monocytes and dendritic cells has not yet been investigated. Here, we elucidated the repair status and drug sensitivity of monocytes and dendritic cells. We focused on anticancer drugs with a well-known mechanism of action, such as alkylating agents and cross-linking drugs. We show that monocytes are more sensitive than dendritic cells, which were derived from them, to methylating genotoxins, and that monocytes undergo apoptosis at a high frequency upon methylation. This hypersensitivity of monocytes is due to a defect in base excision repair (BER), which removes *N*-alkylation lesions from DNA. We also show that during dendritic cell maturation, the expression of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT) is down-regulated, whereas the BER capacity becomes enhanced. To our best knowledge, this is the first report on the expression of repair enzymes in monocytes and dendritic cells and a demonstration of a defect of BER in a given cell type in the body that correlates with lack of protection against methylating genotoxins, including anticancer drugs. The findings bear important implications. They show that precursors of immunologically relevant cells (i.e., monocytes) can be killed preferentially upon exposure to alkylating anticancer drugs and also endogenous and environmental carcinogens, which may provoke immunosuppression. The results provide an explanation for the delayed and sustained immunosuppression caused by alkylating chemotherapeutic intervention.

Materials and Methods

Cells, culture media, and cytokines. Monocytes and dendritic cells were cultivated on Petri dishes in X-VIVO-15 (Bio Whittaker Europe, Verviers, Belgium) supplemented with 1.5% autologous plasma. The final concentration of human recombinant cytokines was 800 units/mL of granulocyte macrophage colony-stimulating factor (GM-CSF; Leukomax; Novartis Pharmaceuticals, Basel, Switzerland); 1,000 units/mL interleukin-4 (IL-4) and IL-6, IL-1β; 10 ng/mL tumor necrosis factor-α (Strathmann Biotech, Hanover, Germany); and 1 μg/mL prostaglandin E_2 (Minprostin; Pharmacia-Upjohn, Heppenheim, Germany).

Antibodies. The following antibodies were used for immunofluorescence staining. Mouse IgG: CD14, CD19, CD80 (MAB104), CD86 (IT2.2), CD83 (HB15A; Coulter/Immunotech, Hamburg, Germany); rat IgG: HLA-DR (Serotec/Camon, Wiesbaden, Germany); and mouse- and rat-specific isotypes (Coulter/Immunotech). Conjugated secondary reagents were FITC-conjugated goat anti-mouse IgG and phycoerythrin-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Generation of dendritic cells from peripheral blood mononuclear cells. Dendritic cells were generated from monocytes as previously described (6). The phenotypic characterization of monocytes and immature and mature dendritic cells was based on expression of specific surface

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markers measured by fluorescence-activated cell sorting. Monocytes are characterized by high expression of CD14 (>80%) and CD86 (40%). Immature dendritic cells express costimulatory molecules, including CD80, CD86, and CD83. Mature dendritic cells exhibited the highest expression level of CD86, CD80, and CD83 in >85% of the population. The surface marker CD14/CD19 was only marginally expressed (data not shown). The purified cell populations were not contaminated with B lymphocytes exhibiting CD19.

Flow cytometric analysis. For phenotyping, monocytes and dendritic cells were carefully washed in cold PBS with 0.5% autologous serum and incubated for 20 min at 4°C with each monoclonal antibody (mAb; 5 μg/mL). After washing with cold PBS/serum, the cells were incubated with FITC- and phycoerythrin-conjugated second-step mAb for 20 min at 4°C, washed thrice, and analyzed by flow cytometry (FACSCalibur, CellQuest software, BD Biosciences, Mountain View, CA).

Survival experiments. Monocytes and immature dendritic cells were cultured at a density of 0.5×10^6/mL and were treated with different doses of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG; Sigma, Munich, Germany), methyl methanesulfonate (Sigma), temozolomide (Schering-Plough, Kenilworth, NJ), cisplatin (Sigma), and maphosphamide (Asta Medica, Frankfurt, Germany). After 72 h, cell survival was quantified by measuring cell viability by trypan blue exclusion. Data were expressed as percentage of surviving cells compared with the nontreated control.

Quantification of apoptosis. Apoptosis was measured by flow cytometry (sub-G_1 DNA content). After treatment with MNNG or temozolomide, monocytes and immature dendritic cells were washed in PBS, fixed in 70% ethanol, and stained with propidium iodide (16.5 μg/mL) in PBS after RNase (0.03 μg/mL) digestion. Samples were analyzed on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). The number of apoptotic cells per sample was calculated using a computer program (Cellquest software).

Single-cell gel electrophoresis. DNA single-strand breaks were determined and quantified by the highly sensitive alkaline single-cell gel electrophoresis assay (comet assay) as previously described (7). Analysis of DNA migration was done by using the image analysis system of Kinetic Imaging Ltd. (komet 4.0.2; Optilas). The mean tail moment (defined as percentage of DNA in the tail × tail length) of 50 cells per sample was determined.

Preparation of cell extracts and Western blot analysis. For whole-cell extracts, cells were harvested, washed once with ice-cold PBS, and resuspended in sonification buffer [20 mmol/L Tris-HCl (pH 8.5), 1 mmol/L EDTA, 5% glycerin, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride]. After sonification, the remaining cell debris was removed by centrifugation at 14,000 × *g* for 15 min; supernatants were collected; and protein concentration was determined (8). Nuclear extracts were prepared as described (9). Cell extract (20 μg) was separated on a 10% SDS-polyacrylamide gel for 2 h at 30 mA and was blotted onto a nitrocellulose membrane for 2 h at 350 mA using buffer composed of 25 mmol/L Tris-HCl, 86 mmol/L glycine, and 20% methanol. The antibodies used were MGMT (Chemicon, Temecula, CA), *N*-methylpurine-DNA glycosylase (MPG; Rabin), apurinic endonuclease (APE; Novus, Littleton, CO), polymerase β (Polβ; Neomarkers, Fremont, CA), proliferating cell nuclear antigen (PCNA; Calbiochem, La Jolla, CA), flap endonuclease 1 (FEN1; Cell Signaling, Danvers, MA), XRCC1 (Neomarkers), ligase IIIα (Lig IIIα; BD Biosciences), p53 (PharMingen, San Diego, CA), and glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX) as protein loading control.

Determination of MGMT and APE activity. MGMT activity in cell extracts was determined as previously described (10). Whole-cell extracts were assayed for APE activity using a double-stranded oligonucleotide containing a single apurinic site. A uracil-containing oligonucleotide was ^{32}P-end labeled by T4 kinase and then annealed with the complementary strand. After purification with QIAquick Nucleotide Removal kit, uracil glycosylase treatment was done according to the manufacturer's protocol (Boehringer, Mannheim, Germany). This double-stranded oligonucleotide containing a single apurinic site (2 pmol of ^{32}P-labeled substrate) was incubated with 5 μg of cellular extract at 37°C in a total volume of 60 μL. After different periods of time, aliquots of 10 μL were taken and separated on a 20% sequencing gel.

Results and Discussion

To determine the sensitivity of monocytes and monocyte-derived dendritic cells to genotoxic agents, cells were treated with different genotoxic agents, and overall cell viability was determined by trypan blue exclusion. As shown in dose-response curves, monocytes were significantly more sensitive to MNNG (Fig. 1*A*), and to the corresponding similar-acting anticancer drug temozolomide (Fig. 1*B*), than dendritic cells. This difference was not observed when cells were treated with cisplatin (Fig. 1*C*) or the cyclophosphamide

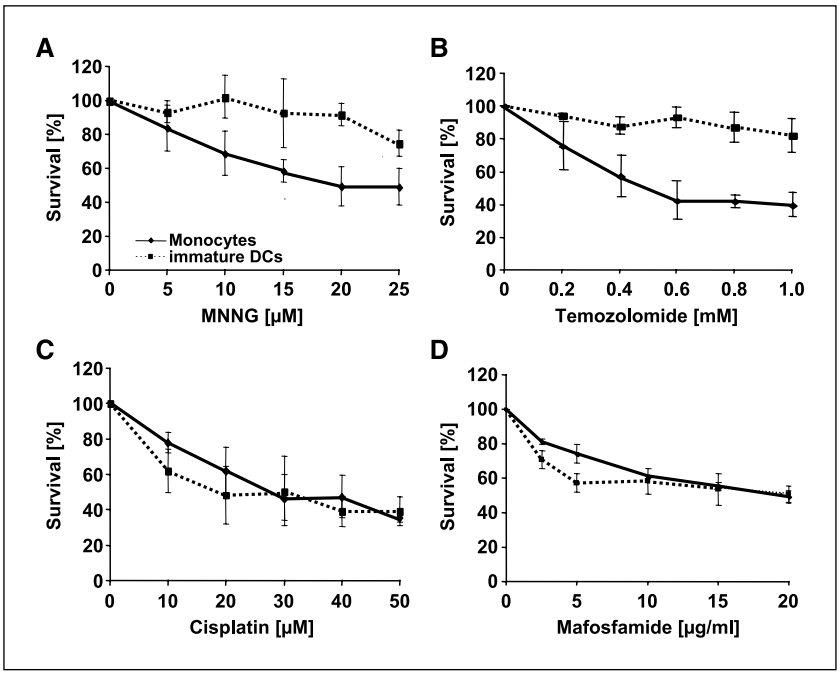

Figure 1. Survival of monocytes and monocyte-derived dendritic cells (*DC*) upon treatment with different genotoxic agents. Monocytes and immature dendritic cells were treated with different doses of (*A*) MNNG, (*B*) temozolomide, (*C*) cisplatin, and (*D*) mafosfamide, and survival was quantified by trypan blue exclusion 72 h later. The genotoxins were added from a stock solution to the medium onto the plates. The medium was not changed to avoid side effects (permanent treatment with the agents). *Points,* mean of at least three independent experiments; *bars,* SD.

analogue mafosfamide (Fig. 1*D*). Monocytes and dendritic cells also did not differ in their sensitivity to the chloroethylating anticancer drug fotemustine (data not shown), which indicates that their hypersensitivity specifically pertains to a subgroup of anticancer drugs, the methylating agents.

Methylating genotoxins are powerful inducers of apoptosis in stimulated human lymphocytes (11). Therefore, it was of interest to see whether monocytes and dendritic cells undergo apoptosis in response to MNNG. As shown in Fig. 2*A*, MNNG induces apoptosis in monocytes and dendritic cells, which are both nonproliferating as determined by flow cytometry 72 h after treatment. Quantification of the sub-G_1 fraction showed high apoptosis levels in monocytes, whereas apoptosis was induced only at low levels in immature dendritic cells (Fig. 2*A*). This was corroborated in a kinetic study which showed that apoptosis in monocytes starts at 24 h after MNNG treatment and further increases with time. At all

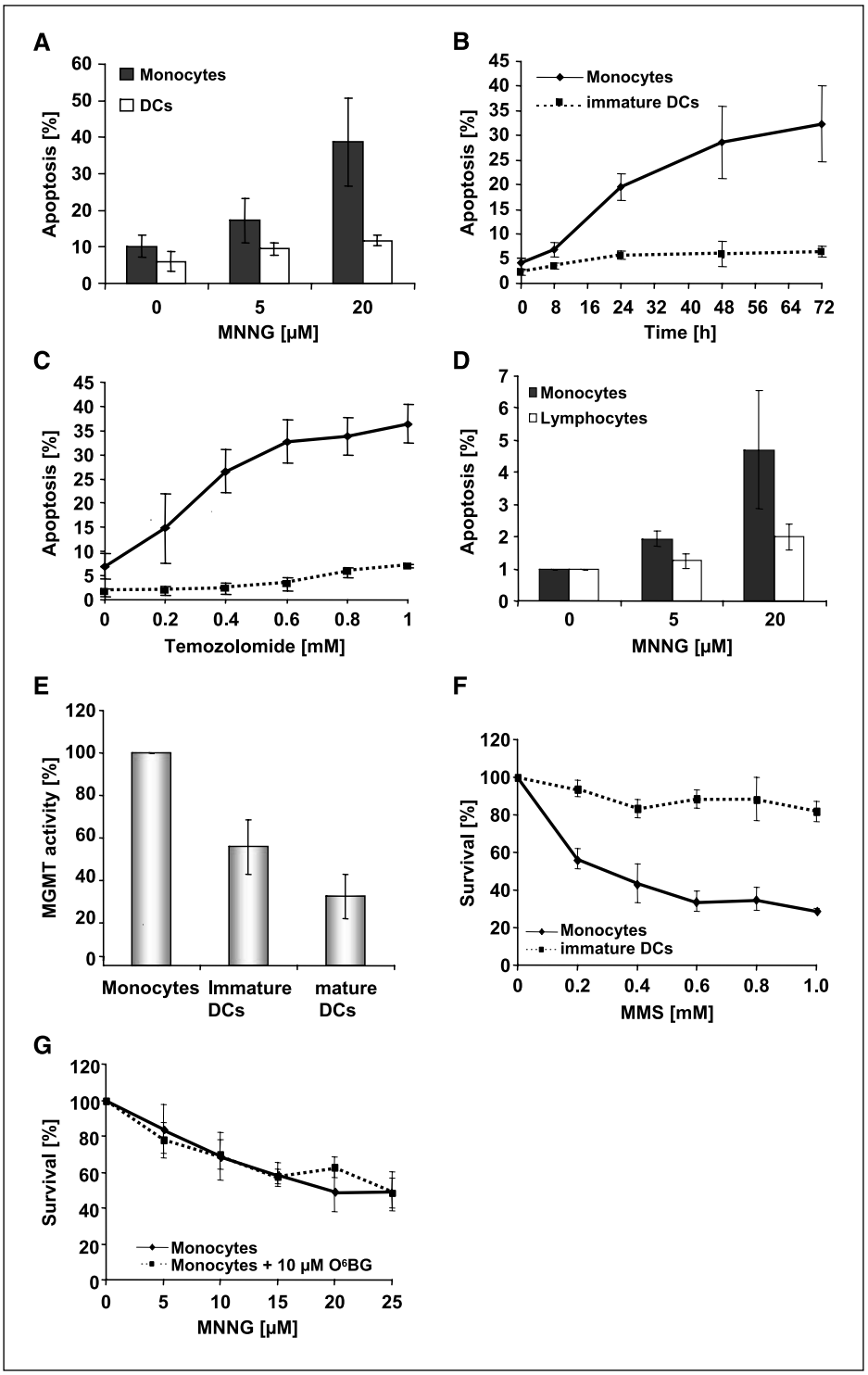

Figure 2. Cell death of monocytes and immature dendritic cells after treatment with methylating agents. *A,* apoptosis quantified by sub-G_1 fraction upon exposure of cells to 5 or 20 μmol/L MNNG after 72 h. *B,* apoptosis as a function of postexposure time up to 72 h of cells treated with 20 μmol/L MNNG. Data of three independent experiments are pooled. *C,* apoptosis of monocytes and dendritic cells treated with different doses of temozolomide and measured after 72 h. *D,* frequency of apoptosis in monocytes and PBLCs treated with 20 μmol/L MNNG. Apoptosis was measured 72 h later. MGMT activity and survival response: *E,* MGMT activity was measured in monocytes and immature and mature dendritic cells from five different healthy donors. Relative MGMT activity is shown as percentage of activity of monocytes. *F,* sensitivity of monocytes and monocyte-derived dendritic cells after methyl methanesulfonate (*MMS*) treatment. Survival was measured by trypan blue exclusion assay. *G,* survival of monocytes in which MGMT was not depleted and depleted by treatment for 1 h with 10 μmol/L O^6-benzylguanine (O^6BG), followed by treatment with different doses of MNNG. Genotoxins and O^6-benzylguanine were added from a stock solution directly to the medium onto the plates, without changing the medium. *Columns/points,* mean of at least three independent experiments; *bars,* SE.

time points, apoptosis upon methylation was much higher in monocytes than in dendritic cells derived from them (Fig. 2*B*). This was also found for temozolomide, which was used as a representative methylating anticancer drug (Fig. 2*C*). To elucidate whether monocytes respond differently from human peripheral blood lymphocytes (PBLC), we studied PBLCs as well. As shown in Fig. 2*D*, the frequency of apoptosis induced by MNNG in monocytes was clearly higher than in nonproliferating PBLCs obtained from the same donor (Fig. 2*D*). The data show that monocytes represent a blood cell population that is exceptionally sensitive to DNA-methylating agents.

A major determinant of resistance of cells to MNNG and other O^6-methylating drugs is the DNA repair protein MGMT (12). Therefore, we assumed that the sensitivity of the cell was due to a low MGMT level. However, this was not the case. To our surprise, monocytes expressed a significantly higher MGMT level than dendritic cells (Fig. 2*E*). Thus, the average MGMT level was 357 $\pm$ 191 fmol/mg protein in monocytes, 155 $\pm$ 39 fmol/mg in immature dendritic cells, and 63 $\pm$ 21 fmol/mg in mature dendritic cells. The data show that during maturation of monocytes into dendritic cells, MGMT becomes down-regulated. Therefore, the high sensitivity of monocytes to O^6-methylating agents (such as MNNG) cannot be explained based on the diminished repair of O^6-methylguanine. In line with this is the observation that monocytes are also significantly more sensitive than dendritic cells to methyl methanesulfonate (Fig. 2*F*), which induces only small amounts of O^6-methylguanine in DNA (13). Furthermore, inhibition of MGMT by O^6-benzylguanine did not sensitize monocytes to the killing effect of MNNG (Fig. 2*G*). Collectively, the data show that O^6-methylguanine is not responsible for the hypersensitivity of monocytes to the killing effect of alkylating agents.

Most of the lesions induced by MNNG, temozolomide, and other methylating agents are *N*-methylpurines that can also cause cell death (14). These lesions are repaired by BER; therefore, a reasonable hypothesis would be that monocytes are defective in BER. This prompted us to study BER in monocytes and monocyte-derived dendritic cells. Treatment of monocytes and dendritic cells with MNNG resulted in the appearance of DNA single-strand breaks that occurred during the treatment period (60 min) at a much higher level in monocytes than in dendritic cells (Fig. 3*A*). These single-strand breaks disappeared within 4 h postincubation in dendritic cells, whereas in monocytes, the amount of single-strand breaks kept on increasing (Fig. 3*B*). A significant higher level of single-strand breaks was also observed in monocytes compared with immature dendritic cells (obtained from the same donor) when treatment occurred with temozolomide (Fig. 3*C*). This was also true for PBLCs that responded in the same way as dendritic cells (Fig. 3*C*). Because single-strand breaks following alkylation result from BER, arising from cleavage of apurinic sites by apurinic endonuclease (15), we conclude that monocytes but not monocyte-derived dendritic cells and PBLCs are defective in a late step of BER.

To elucidate at which step monocytes are defective in BER, the expression of BER proteins was measured in monocytes, monocyte-derived immature dendritic cells, and mature dendritic cells. As shown in Fig. 4*A*, MPG, APE1, DNA Polβ, PCNA, and FEN1 were expressed at similar levels in monocytes and dendritic cells. The APE1 blot consistently showed two bands in monocytes that may indicate the existence of a splice variant. The most dramatic difference was observed, however, for XRCC1 and Lig IIIα, which were hardly detectable in monocytes, whereas in immature and mature dendritic cells, they were expressed at a high level (Fig. 4*B*). Interestingly, p53 that is reported to stimulate BER (16) was also not expressed in monocytes, whereas it is constitutively up-regulated in monocyte-derived dendritic cells (Fig. 4*B*). Human PBLCs clearly express XRCC1 and Lig IIIα (Fig. 4*C*), which is in line with the finding that PBLCs are able to perform BER after methylation. Because XRCC1 in a complex with Lig IIIα is required for the ligation step in BER (17), lack of XRCC1 will necessarily lead to an accumulation of DNA single-strand breaks that represent DNA repair intermediates. This has indeed been observed to occur (Fig. 3*A* and *B*). Based on the data, we conclude that lack of expression of XRCC1 and Lig IIIα is likely responsible for the observed BER defect of human monocytes.

The two bands of APE, shown in the representative Western blot in Fig. 4*A*, could be taken to indicate that monocytes exhibit higher APE activity than dendritic cells. Therefore, APE enzyme activity was determined using a cleavage assay that is based on conversion of a double-stranded oligonucleotide containing a single apurinic site into a cleaved fragment (see Fig. 4*D* for a

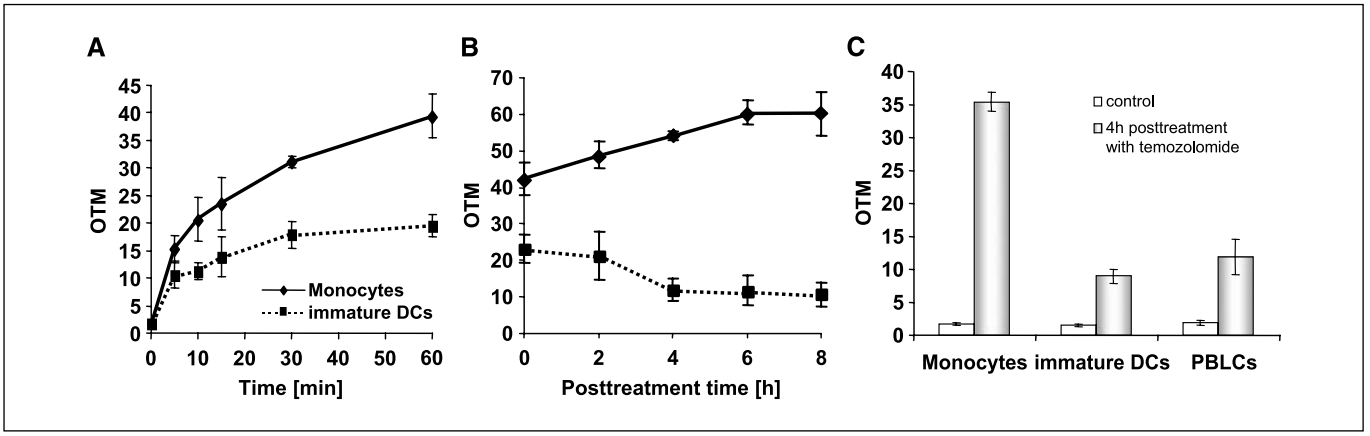

Figure 3. Formation of DNA single-strand breaks in monocytes, dendritic cells, and PBLCs. Cells were treated with 20 μmol/L MNNG. DNA break formation was determined using alkaline single-cell gel electrophoresis. The level was expressed by the olive tail moment (*OTM*; ref. 7). *A,* frequency of single-strand breaks that were induced during the MNNG treatment period of 60 min. *B,* frequency of single-strand breaks as a function of time after MNNG treatment for 60 min. *C,* frequency of single-strand breaks in monocytes, immature dendritic cells, and PBLCs exposed to temozolomide (1 mmol/L), measured 4 h after treatment. *Points/columns,* mean of at least three independent experiments; *bars,* SE.

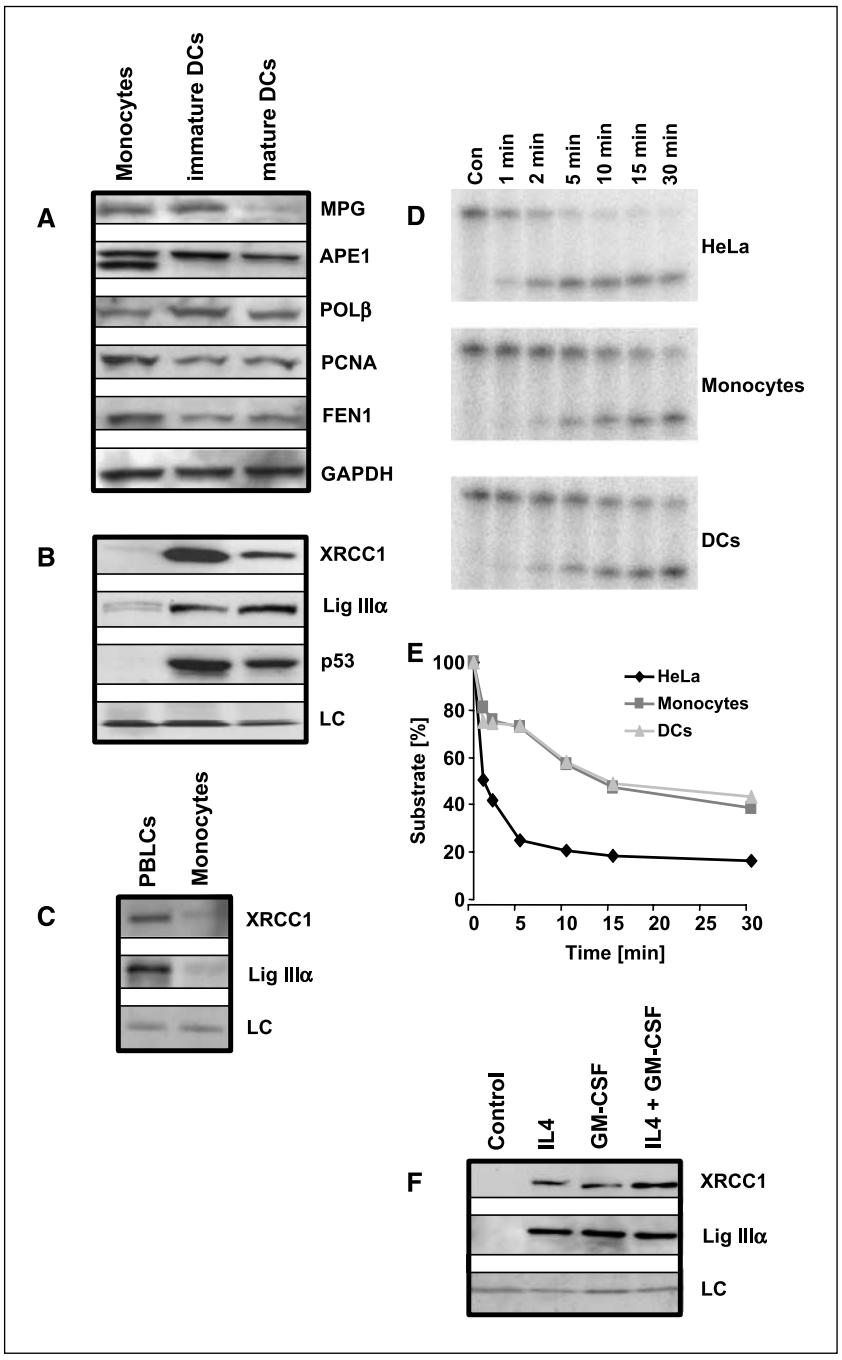

Figure 4. Expression of BER proteins in monocytes and monocyte-derived dendritic cells. *A,* protein expression of MPG, APE1, Polβ, PCNA, and FEN1 in total cell extracts. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as loading control. *B,* expression of XRCC1, Lig IIIα, and p53 in nuclear extracts. *Bottom,* Ponceau staining of protein on the gel (loading control, *LC*). *C,* expression of XRCC1 and Lig IIIα in monocytes and PBLCs. *D,* activity of APE1 in HeLa cells, monocytes, and dendritic cells. Extracts of HeLa cells, monocytes, and dendritic cells were assayed for APE activity as described in Materials and Methods. After incubation at different times, aliquots were taken and separated on a 20% sequencing gel. Control (*Con*) was incubated for 30 min in the absence of cell extract. *Top band,* uncleaved 35-mer substrate; *bottom band,* converted 15-mer radiolabeled oligonucleotide. *E,* quantification of radioactively labeled oligonucleotides converted into cleavage products as assayed in time course experiments by densitometric analysis. *F,* expression of XRCC1 and Lig IIIα in monocytes not treated (*Control*) or treated with IL-4, GM-CSF, or both, for 1 wk.

representative experiment with HeLa cells, monocytes, and dendritic cells). The quantification revealed that the rate of cleavage of the substrate was significantly higher in HeLa cells than in monocytes and dendritic cells. More importantly, there was no difference in APE activity between monocytes and their dendritic cell offspring (Fig. 4*E*). This further supports the conclusion that the BER defect in monocytes is due to a lack of expression of XRCC1 and Lig IIIα.

The differentiation of monocytes into dendritic cells occurs by treatment with IL-4 and GM-CSF. Therefore, we wondered whether either one of these cytokines, or their combination, is able to trigger up-regulation of XRCC1 and Lig IIIα. As shown in Fig. 4*F*, treatment with either IL-4 or GM-CSF gives rise to a clear increase in the level of XRCC1 and Lig IIIα. For XRCC1, the effect seems additive if the cytokines were applied together. The data indicate that after differentiation of monocytes into dendritic cells by IL-4 and GM-CSF, the cells are able to express these repair proteins.

XRCC1 and Lig IIIα form dimeric complexes (18) that are recruited to the repair site after APE cleavage and Polβ-mediated nucleotide insertion (19). XRCC1 may also interact with Polβ, but this only seems to occur if Polβ is bound on DNA during the repair reaction (19). XRCC1 stabilizes Lig IIIα (17); therefore, lack of XRCC1 observed in monocytes may cause Lig IIIα to become degraded. Irrespective of the mechanism involved, because XRCC1 is essential for BER by recruiting Lig IIIα to the lesion, the lack of expression of XRCC1 and Lig IIIα in monocytes results in a defect

in the last step of BER, making monocytes vulnerable to simple alkylating agents. This seems to be a specific property of the monocyte cell population because dendritic cells and also macrophages derived from them[1] as well as PBLCs are competent for BER.

The finding of a high induction level of apoptosis in monocytes compared with dendritic cells and PBLCs implicates that during tumor therapy with methylating anticancer drugs, the monocyte population may be specifically depleted, whereas PBLCs and other blood compartments are protected. The hypersensitivity of monocytes may finally result in a lack of dendritic cells, causing severe attenuation of the antitumor immune response. Because methylating drugs are widely used in the therapy of glioblastomas, malignant melanomas, and endocrine carcinomas, alkylating drug-induced death of monocytes may have significant impact on the effectiveness of the therapy of these tumors. It should also be noted that DNA-methylating agents are widely distributed environmental carcinogens, are present in food and tobacco smoke, and are also endogenously formed (20). Therefore, the possibility that the immune response during carcinogen exposure could be impaired should also be taken into consideration.

To our best knowledge, this is the first report on the expression of repair enzymes in monocytes and dendritic cells. It is also the first demonstration of down-regulation of BER in a specific cell type in the body that correlates with lack of protection against methylating genotoxins. The data bear important implications. Thus, monocytes that are precursors of immunologically relevant cells, such as dendritic cells, might be killed preferentially upon exposure to anticancer drugs and also to endogenous and environmental carcinogens, provoking immunosuppression. The data obtained provide a firm basis for *in vivo* studies aimed at explaining the delayed and sustained immunosuppression caused by chemotherapeutic intervention with alkylating anticancer drugs.

[1] Our unpublished data.

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