Protumoral role of monocytes in human B-cell precursor acute lymphoblastic leukemia: involvement of the chemokine CXCL10

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Myelomonocytic cells play a key role in the progression of many solid tumors. However, very little is known about their contribution to the progression of hematopoietic cancers. We investigated the role of monocytes in the progression of human B-cell precursor acute lymphoblastic leukemia (BCP-ALL). We demonstrated that coculturing human monocytes in vitro with CD19$^+$ BCP-ALL blasts from patients "conditioned" them to an inflammatory phenotype characterized by significant up-regulation of the chemokine, CXCL10. This phenotype was also observable ex vivo in monocytes isolated from BCP-ALL patients, which show elevated CXCL10 production compared with monocytes from healthy donors. Functionally, the "conditioned" monocytes promoted migration and invasive capacity of BCP-ALL cells. Increased invasion was mediated by matrix metalloproteinase 9 expression and activity in the BCP-ALL cells induced by the monocyte-derived CXCL10. However, neither the "conditioned" monocytes nor the CXCL10 produced by these cells had any effect on the proliferation/viability of BCP-ALL cells and angiogenesis. Collectively, our results strongly suggest a protumoral role for human monocytes in BCP-ALL, orchestrated by CXCL10 and its effect on tumor cell migration and invasion. These observations highlight the importance of the CXCL10/CXCR3 chemokine circuit in BCP-ALL progression. (*Blood*. 2012;119(1): 227-237)

Introduction

Monocytes/macrophages are versatile innate immune cells that play a key role in host defense by performing a wide variety of functions.[1,2] Depending on the microenvironmental cue, monocytes/macrophages can display distinct phenotypes and functions.[1,3,4] For example, microbial stimuli (eg, lipopolysaccharide) and IFN-γ induce these cells to an M1 or "classical" activation state, which is characterized by up-regulation of inflammatory cytokines (eg, IL-12, IL-23, and TNF) and potent microbicidal as well as tumoricidal properties. In contrast, IL-4 and IL-13 induce an M2 (or "alternative") activation state, which is characterized by high expression of anti–inflammatory cytokines (eg, IL-10), scavenging receptors (eg, mannose receptor, CD163), and efficient phagocytic ability. These cells facilitate the resolution of inflammation, tissue repair or remodeling, and tumor-promoting activities.[5] However, it may be pointed out that M1 and M2 phenotypes represent 2 extremes of a spectrum of macrophage functional states,[3,4] and cells with overlapping M1-M2 characteristics have been noted under certain pathophysiologic settings.[6,7]

In recent years, several studies have focused on the contribution of myelomonocytic cells in cancer progression.[3,8] Monocytes/macrophages are recruited to tumor tissues where they promote tumor growth, proliferation, angiogenesis, and metastasis through the release of factors, such as TNF, IL-6, VEGF, IL-8, and matrix metalloproteinases (MMPs).[7,9,10] In line with this fact, a pro-angiogenic population of monocytes (termed as Tie-2–expressing monocytes) has been recently identified as the principal regulators of tumor angiogenesis.[11] Monocytes/macrophages may also contribute to tumor progression by promoting immunosuppression through the release of immunosuppressive cytokines, such as IL-10 and TGF-β.[12] Indeed, characterization of these cells from tumor-bearing animals shows them to possess an IL-12^{low}/IL-10^{high} phenotype with reduced antigen presentation capacity.[12-14] Based on these properties, tumor associated macrophages (TAMs) were suggested to be an M2-polarized population, although variations to this theme have been observed depending on the type and stage of the cancer.[7,15]

Although the majority of the evidence demonstrating the role of monocytes/macrophages in cancer progression come from mouse tumor models, relatively little is known about their contribution in human cancers. Moreover, most of these studies have focused on studying the role of myelomonocytic cells in the progression of solid tumors or epithelial cancer. Thus, little is known about the contribution of these cells in the progression of hematologic cancers (leukemia or lymphoma).

Acute lymphoblastic leukemia (ALL) is a malignant disorder of lymphoid progenitor cells. It is the most common childhood malignancy and accounts for almost 20% of acute leukemias in adults.[16] B-cell precursor ALL (BCP-ALL) is the most common type of childhood ALL. Based on a few recent studies suggesting the interaction of B cells with tumor-associated monocytic cells in solid tumors and the prognostic relevance of TAMs in B-cell lymphoma,[17-19] we questioned whether monocytes/macrophages

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might play an integral role in the progression of human BCP-ALL. Addressing this point, the current investigation reports, for the first time, a tumor-promoting role of monocytes in human BCP-ALL and the molecular mechanism(s) involved therein.

Methods

Cell lines, reagents, and cell culture

The human cell lines REH, RS4;11, NALM6,[20-22] and DAUDI were cultured in IMDM (Invitrogen) containing 4mM L-glutamine, 25mM HEPES buffer, and 3 g/L sodium bicarbonate. Medium was supplemented with 10% human serum (Gemini Bioproducts) and 100 U/mL penicillin-streptomycin. HUVEC cells were purchased from ATCC. USA. RS4;11 cells were obtained from ATCC and a kind gift from Dr Allen Yeoh Eng Juh (National University of Singapore, Singapore). DAUDI cells were a kind gift from Dr Josè Golay (Laboratory of Cell Therapy "G. Lanzani," Riuniti Hospital, Bergamo, Italy). REH and NALM6 cells were purchased from DSMZ. The following reagents were purchased from the manufacturers indicated in the parentheses: Lipid A, *Escherichia coli* Serotype R515(e) (Alexis Biochemicals), and recombinant CXCL10 and CXCL10 neutralizing antibody (R&D Systems).

Isolation and culture of human blood monocytes

Monocytes were obtained from healthy subjects as follows. Briefly, PBMCs were isolated from buffy coats obtained from the Bloodbank of Health Sciences Authority, Singapore, using Ficoll-Hypaque density gradient centrifugation. Monocyte isolation was performed using the $CD14^+$ Monocyte isolation kit from Miltenyi Biotec according to the manufacturer's instructions. Aliquots of the isolated monocytes were then stained with anti–CD14 (61D3; BioLegend) and tested by flow cytometry to determine their purity. Monocytes were plated in 12-well tissue culture plates (Nunclon) at 1.5×10^6 cells per well in incomplete IMDM (ie, medium without serum) for 1 hour at 37°C in the tissue culture incubator. Thereafter, the adherent monocytes were cultured in complete medium (ie, IMDM medium containing 10% [volume/volume] human serum, Gemini Bio-Products).

Patient information and collection of blasts

BM cells were collected at diagnosis from 24 children with BCP-ALL, including 15 common ALL (cALL) and 9 pre-B ALL (Clinica Pediatrica, Ospedale San Gerardo, Monza, Italy). The median age was 6.2 years (range, 2-15 years); 12 patients were male and 12 were female. In all patients, blast infiltration was greater than 80%. Blast samples expressing 90% or more of $CD19^+$ antigen were used for our experiments. Flow cytometric data on CD10 staining, cytogenetic, and molecular analysis of the patient BM cells are provided in supplemental Table 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The study was approved by the local institutional review board (Monza, Italy), and informed consent was obtained from patients and their guardians in accordance with the Declaration of Helsinki.

Luminex and ELISA for cytokines

Plasma obtained from patients and healthy donors was analyzed for cytokines/chemokines using a 27-plexed Bioplex assay (Bio-Rad) based on the Luminex xMAP Multiplexing Technology. Cell-free supernatants from monocytes cocultured with patient blasts were tested for CXCL10, using a CXCL10 ELISA kit (R&D Systems) as per the manufacturer's instructions.

Tumor cell invasion and migration assay

Monocyte/BCP-ALL cell coculture. $CD14^+$ monocytes (1.5×10^6) were seeded in the lower wells of a 24-well tissue culture plate and cocultured with BCP-ALL cells (or cell lines) placed in 0.4-μm transwell inserts at a ratio of 1:2.5 for 24 hours. After coculture, inserts containing BCP-ALL cells were removed and the monocytes were replenished with fresh medium. After 24 hours, these tumor-conditioned monocyte supernatants were used for the subsequent invasion and migration experiments.

The invasion assay was conducted using transwell cell culture inserts (24 wells, 3-μm pore size; BD Biosciences). Briefly, upper inserts were coated with 200 μL of Matrigel (Invitrogen), diluted with PBS (1:6), and allowed to set for 1 hour at 37°C. A total of 3×10^5 BCP-ALL cells were resuspended in 200 μL of complete medium and added to the upper chamber. Complete medium was added to the lower chamber. Once the cells were attached, the media (upper and lower chambers) were changed to serum-free media. Supernatants from the tumor-conditioned monocytes were then added to the lower chamber at 50% volume/volume concentration. Twenty-four hours later, cells on the upper surface of the insert membrane were removed with cotton swabs. The invaded cells that were attached to the bottom surface of the membranes were detached and counted together with the cells in the bottom chamber for each insert, using a hemocytometer. Migration experiments were performed in the same way except that the transwell inserts were not coated with Matrigel.

Quantitative PCR

Cells were lysed with Trizol (Invitrogen), and total RNA was prepared using the RNAeasy kit (QIAGEN) according to the manufacturer's instructions. A total of 1 μg of total RNA was reverse transcribed and the cDNA used for quantitative PCR analysis on an iCycler iQ5 Real-Time PCR detection system (Bio-Rad) as described earlier.[12]

MMP9 activity by zymography

Human BCP-ALL cells were cultured in 12-well tissue culture plate overnight and treated with different doses (1, 50, and 100 ng/mL) of recombinant human CXCL-10 for 4 hours. After the treatment, cells were centrifuged and supernatant collected and analyzed for matrix metalloproteinase 9 (MMP9) activity (92 kDa) by gelatin zymography. Briefly, a total of 20 μL of cell supernatant was mixed with equal amounts of 2× Novex Tris-Glycine SDS sample buffer and loaded onto Novex 10% Zymogram (0.1% gelatin) gel (Invitrogen) and run using the Novex Tris-Glycine SDS Running buffer at 125 V for 1.5 hours. After electrophoresis, the gel was incubated in the 100 mL of Novex Zymogram Renaturing Buffer for 30 minutes at room temperature with gentle agitation. Thereafter, the gel was processed with the Novex Zymogram Developing buffer and stained with Simply Blue Safestain as per the manufacturer's instructions. The stained gel was dried and scanned for documentation. For better image visualization, a black and white color inversion setting was used.

Data analysis for correlation studies

Three datasets initially sourced from Oncomine (Compendia Bioscience) were used to study the potential gene coexpression relationship between *CXCL10* and *MMP9* in B-cell ALL. The datasets were chosen because they all use the same or similar Affymetrix chip set, HG133+. Dataset 1 consisted of 103 B-precursor ALL patient samples selected from Bhojwani et al (GSE3912).[23] Dataset 2 consisted of 145 B-lineage ALL patient samples selected from Holleman et al (GSE635).[24] Dataset 3 consisted of 99 B-precursor ALL patient samples selected from Bhojwani et al (GSE7440).[25] For each of the datasets, expression data files were normalized by Affymetrix MAS, Version 5.0. The normalized expression values were log2-transformed before correlation calculation. Pearson method was used to calculate correlation coefficients and *P* values.

Statistical analysis

Data were analyzed using GraphPad Prism4 software. The statistical significance of differences among 2 groups was determined by a Student *t* test, whereas that for 3 or more groups was done using a 1-way ANOVA, Tukey test. In all cases, data were considered significant when $P < .05$.

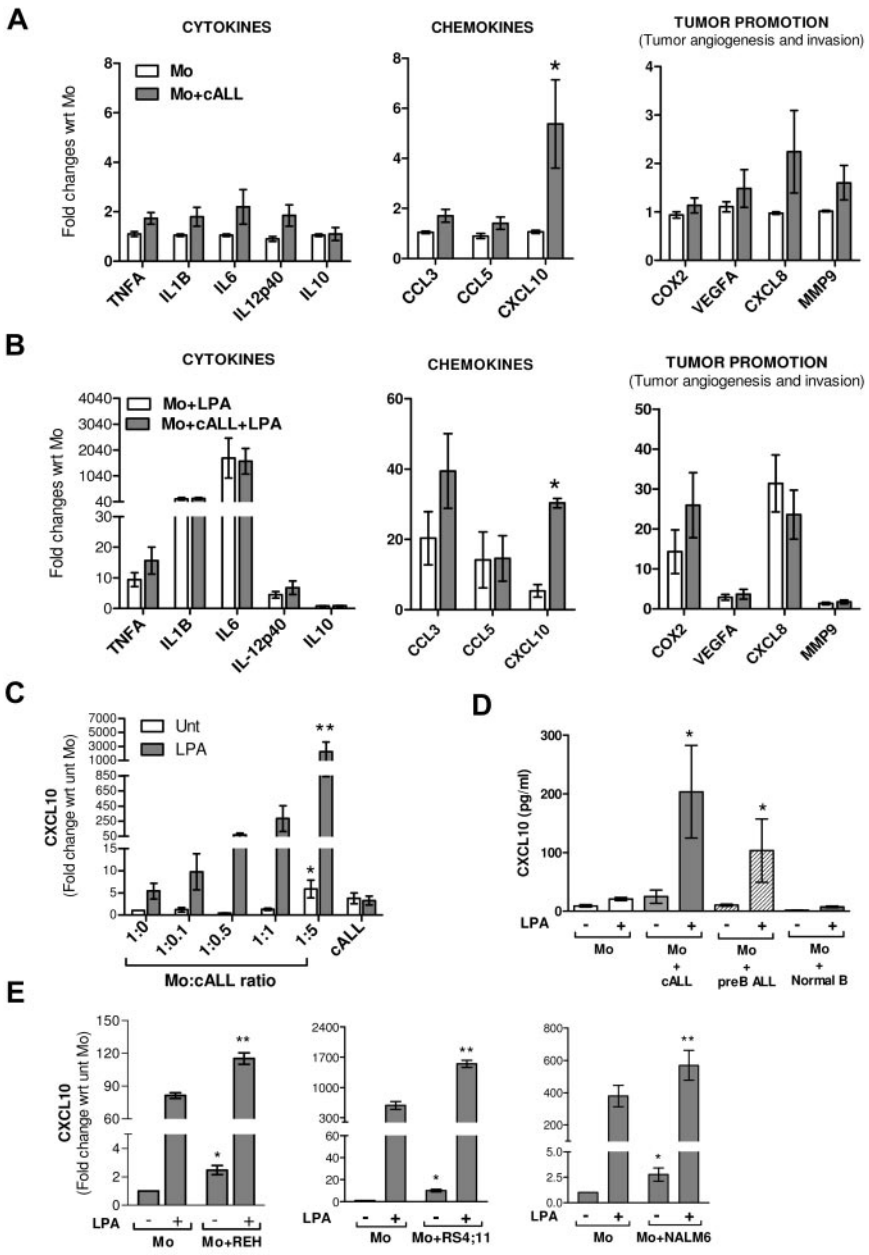

Figure 1. Human monocytes cocultured in vitro with BCP-ALL cells show increased expression of chemokine CXCL10. (A) Human monocytes (Mo) were cocultured with $CD19^+$ cALL blasts isolated from cALL patients in a transwell system for 18 hours. Thereafter, the cALL-containing transwells were removed, the monocyte monolayers were washed, lysed, and use for accessing the expression of a panel of cytokine, chemokine, and tumor promotion (tumor angiogenesis and invasion) related genes. Mo indicates monocytes alone; and Mo + cALL, monocytes cocultured with cALL tumor blasts. A Mo/cALL ratio of 1:5 was used in this experiment. $^*P < .04$, versus Mo. (B) Monocytes were cultured alone or cocultured with cALL cells as described in panel A. Thereafter, the cALL-containing transwells were removed; the monocyte monolayers were washed, incubated in fresh media for 1 hour, and stimulated with LPA (100 ng/mL) for 4 hours. Expression of the same panel of genes as described in panel A was assessed by quantitative PCR. Mo + LPA indicates monocytes cultured alone stimulated with LPA; and Mo + cALL + LPA, monocytes cocultured with cALL tumor blasts followed by LPA stimulation. $^*P < .05$, versus Mo + LPA. (A-B) Data are mean ± SEM (n = 4). (C) LPA-induced expression of CXCL10 gene was assessed in monocytes cocultured with the cALL tumor blasts at different ratios as indicated in the figure. The procedure for coculturing and LPA stimulation remains the same as indicated for panel A. cALL + LPA indicates cALL blasts stimulated with 100 ng/mL LPA for 4 hours. Data are mean ± SEM (n = 3). $^*P < .05$, versus Mo (1:0). $^{**}P < .05$, versus Mo (1:0) + LPA. (D) Expression of CXCL10 protein by monocytes cocultured with cALL blasts, pre-B ALL blasts, or B cells from healthy donors (normal B), treated with or without 100 ng/mL LPA overnight. The procedure for coculturing remains the same as indicated for panel A. CXCL10 was measured by ELISA in the cell-free culture supernatant of the cells. Data are mean ± SEM (n = 3). $^*P < .03$, versus Mo + LPA. (E) Monocytes were cultured alone or cocultured with a panel of human BCP-ALL cell lines for 18 hours using the same protocol as described in panel A. Thereafter, cells were stimulated or not with LPA (100 ng/mL) for 4 hours and CXCL10 gene expression assessed. Data are mean ± SD from a representative experiment. $^*P < .05$, versus Mo. $^{**}P < .05$, versus Mo + LPA.

Results

Monocytes cocultured with BCP-ALL cells show a modulation in their inflammatory response with an up-regulation of CXCL10

Although studies with solid tumors have shown the ability of cancer cells to polarize monocytes and macrophages to favor tumor growth, such information is lacking for hematologic cancers.[26,27] Addressing this point in a human BCP-ALL, we first investigated the effect of BCP-ALL tumor blasts on monocyte activation and response. Among BCP-ALL, which is an heterogeneous subgroup of leukemia characterized by different maturation stage of B cells,[22] we focalized our attention on the most common subtypes (ie, cALL and pre-B ALL). Tumor blasts isolated from cALL patients were cocultured with human monocytes in a transwell system for 18 hours. Thereafter, the transwells containing the cALL cells were removed; the monocyte monolayers in the lower chamber were isolated and assessed for the expression of genes relating to cytokines, chemokines, and tumor promotion (tumor angiogenesis and invasion). To standardize the assay, we initially cocultured the monocytes with cALL blasts at different ratios: 1:0.01, 1:0.5, 1:1, and 1:5 (Mo/cALL). Because a Mo/cALL ratio of 1:5 showed a consistent trend, the results using this ratio were presented in the rest of the study.

Compared with monocytes cultured alone, monocytes cocultured with cALL blasts showed approximately 1.5- to 2-fold increase in the expression of inflammatory cytokine genes *TNFA*, *IL1B*, *IL6*, and *IL12p40* as well as genes related to tumor promotion, such as *CXCL8* and *MMP9* (Figure 1A). However, these changes were not significant. In contrast, the IFN-inducible proinflammatory chemokine gene, *CXCL10* was significantly up-regulated in monocytes cocultured with cALL blasts compared with monocytes alone (Figure 1A). No change was observed in the levels of the anti–inflammatory gene *IL10*.

Next, we studied whether coculturing monocytes with cALL blasts altered their ability to respond to classic activation stimuli, such as lipid A (LPA). On LPA stimulation, cALL cocultured monocytes showed significantly higher expression of *CXCL10* than the monocytes, which were cultured alone (Figure 1B). The expression of all other genes shown in Figure 1B remained comparable between the 2 experimental groups, after LPA stimulation. The specificity of the increased *CXCL10* expression by cALL cocultured monocytes was evident from its dose-dependent up-regulation corresponding to an increase in the ratio of cALL cells: monocytes in the cocultures, as well as the lack of CXCL10 production by the cALL cells itself (Figure 1C). Figure 1D provides protein validation for the increased CXCL10 production by monocytes cocultured with cALL cells. This observation holds true irrespective of the stage of the BCP-ALL because coculturing monocytes with pre-B as well as cALL cells showed the same results (Figure 1D). However, monocytes cocultured with nonmalignant B cells do not show any such increase in CXCL10 production (Figure 1D), suggesting this to be specific to the malignant B cells.

We also performed experiments where monocytes were cocultured with a panel of human BCP-ALL cell lines followed by stimulation with or without LPA (Figure 1E). The BCP-ALL cell lines used were RS4;11, NALM6, and REH, corresponding to the BCP-ALL subtypes, pro-B ALL, pre-B ALL, and cALL, respectively, and carried different chromosomal translocation, t(4;11), t(5;12), and t(12;21), respectively.[20-22] In all cases, the cocultured monocytes showed significantly increased *CXCL10* expression (Figure 1E). These results extend our observation obtained with primary blasts to other BCP-ALL cell lines, indicating that the increased CXCL10 expression is independent of different chromosomal rearrangements.

Together, the results indicate BCP-ALL cells to "condition" or polarize monocytes to an inflammatory phenotype characterized by the specific up-regulation of the chemokine CXCL10.

Monocytes from BCP-ALL patients show an inflammatory phenotype with increased CXCL10 production

Having demonstrated the effect of BCP-ALL cells in up-regulating the expression of CXCL10 in monocytes in vitro, we wanted to ascertain whether such an effect was also visible ex vivo for monocytes isolated from patients with BCP-ALL. A flow cytometric analysis of BM blasts from cALL patient revealed the presence of approximately 1% $CD14^+$ monocytes (ALL-Mo) and more than 90% $CD19^+$ cALL tumor cells (Figure 2A). These ALL-Mo were isolated by flow sorting and subsequently used for gene expression study. This study showed ALL-Mo to express significantly higher levels of *CXCL10* compared with the monocytes from healthy donors (Mo; Figure 2B). The possibility that the up-regulation in *CXCL10* was the result of a contamination from the tumor cells was ruled out because the $CD19^+$ cALL cells express very little *CXCL10* (Figure 2B right column). Correlating with the enhanced *CXCL10* expression by ALL-Mo, elevated plasma levels of this chemokine was also detectable in cALL patients (Figure 2C). Indeed, of the panel of 27 cytokines/chemokines studied in the plasma of cALL patients, none of the factors showed any significant change compared with their levels in healthy donors, except for CXCL10 (supplemental Figure 1).

To further characterize the ALL-Mo, we checked the expression of a few other inflammatory and protumoral genes in these cells, as described in the earlier figures. Figure 2D shows these cells to display increased expression of *IL12p40*, *TNFA*, and *IL10* than the Mo. In parallel, higher expression of protumoral genes *COX2* and *MMP9* was also noted in the ALL-Mo. These results were also mirrored in ALL-Mo isolated from the peripheral blood of the cALL patients (Figure 2E). Moreover, a similar experiment performed on sorted monocytes obtained from BM blasts from pre-B ALL patients also confirmed higher expression of *CXCL10* compared with the healthy subjects (Figure 2F). Taken together, these results not only demonstrate the elevated expression of *CXCL10* in ALL-Mo but also indicate an inflammatory as well as protumoral phenotype of these cells.

ALL cocultured monocytes promote migration of BCP-ALL cells via CXCL10

CXCR3, the receptor for CXCL10, is expressed highly on malignant B cells.[28,29] Thus, it was logical for us to investigate whether elevated CXCL10 expression by the ALL "conditioned" monocytes had any effect on BCP-ALL cells and tumor progression. We hypothesized whether elevated CXCL10 expression could directly influence the tumorigenicity of BCP-ALL cells by inducing increased migration, invasion, proliferation, and/or survival of these cells. For this purpose, we first tested the ability of cALL-cocultured monocytes to induce migration of cALL cells. Supernatants collected from monocytes alone or monocytes cocultured with patient cALL cells (treated with or without LPA) were placed in the lower wells of a transwell culture system. Patient cALL cells were then added to the upper transwell insert, and their migration to the lower well was assessed as described in "Tumor cell invasion and migration assay." Figure 3A left panel shows supernatants from cALL-cocultured monocytes induced significantly higher migration of patient cALL cells than the supernatant of monocytes alone (as well as those from patient cALL cells alone). Addition of anti–CXCL10 neutralizing antibody to the ALL-cocultured monocyte supernatant abrogated the elevated cell migration, demonstrating this effect to be mediated by CXCL10. Further, recombinant human CXCL10 induced increased migration of patient cALL cells, indicating a direct effect of this chemokine in this process (Figure 3A right panel). We performed additional migration assays with a panel of BCP-ALL cell lines (ie, REH, RS4;11, and NALM6). As shown in Figure 3B through D (left panels), supernatants from monocytes cocultured with BCP-ALL patient cells induced significant migration of all the 3 cell lines. This effect was mediated by CXCL10, as revealed by the blocking action with anti–CXCL10 neutralizing antibody and the enhanced migration of these cell lines in response to human recombinant CXCL10 (Figure 3B-D right panels). Collectively, our results indicate that ALL-conditioned monocytes through their elevated CXCL10 expression possibly induced the enhanced migration of BCP-ALL cells.

ALL cocultured monocytes promote invasive capacity of BCP-ALL cells via CXCL10

In a next set of experiments, we performed in vitro invasion assay to determine whether supernatants from ALL-cocultured monocytes could induce increased invasiveness of BCP-ALL cells. First, culture supernatants from monocytes alone or monocytes cocultured with patient cALL cells were placed in the lower wells of a transwell culture system. Then, patient cALL cells were placed in the upper well, and cell invasion through the matrigel-coated transmembrane was assessed as described in "Tumor cell invasion and migration assay." Compared with the supernatants from

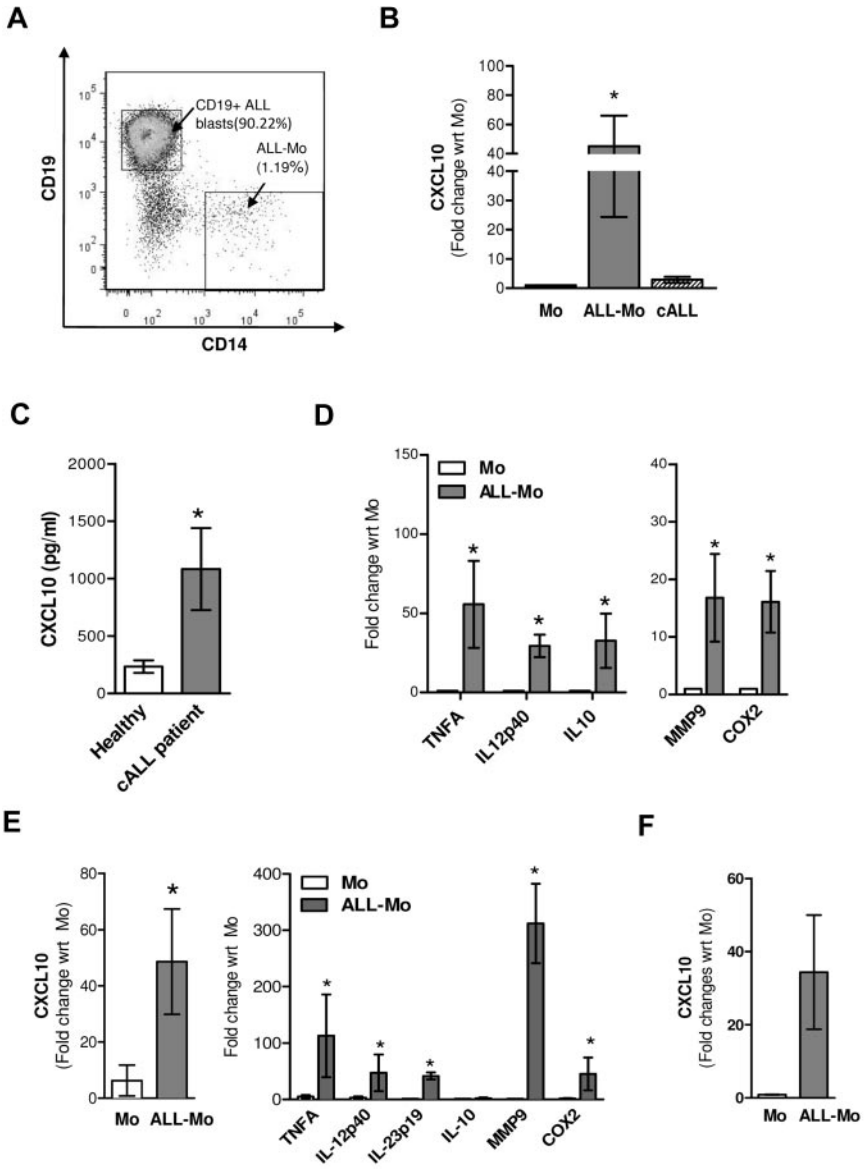

Figure 2. Up-regulated CXCL10 expression in monocytes isolated from BCP-ALL patients. (A) Representative flow cytometric analysis of the BM cALL blasts. cALL blasts are indicated by the $CD19^+/CD14^-$ cells, whereas tumor-associated monocytes (ALL-Mo) were indicated by the $CD19^-/CD14^+$ cells. Numbers in parentheses indicate relative percentage of cALL cells and ALL-Mo. (B) Monocytes (ALL-Mo) as well as $CD19^+$ cALL cells (cALL) were isolated from the blasts by FACS sorting and checked for the expression of CXCL10 by quantitative PCR. Monocytes from normal donors (Mo) were used as a control. (C) CXCL10 levels in the plasma of patients (n = 13) and healthy donors (n = 6) determined by Bioplex assay. Data are mean ± SEM. $^*P < .03$, versus healthy. (D) Expression of a panel of inflammatory and protumoral genes in ALL-Mo by quantitative PCR. Data represent fold change expression of genes with respect to their basal line expression in monocytes from healthy donors. (E) Expression of a panel of inflammatory and protumoral genes in monocytes (ALL-Mo) isolated from the peripheral blood of cALL patients by quantitative PCR. (F) Quantitative PCR analysis of the indicated genes in ALL-Mo isolated from pre-B ALL patient blasts, compared with monocytes from healthy donors. Data are mean ± SEM (n = 2). (B,D-E) Data are mean ± SEM (n = 4). $^*P < .05$, versus Mo.

monocytes or cALL cells cultured alone, supernatants from cALL-cocultured monocytes elicited a marked increase in invasion of the patient cALL cells (Figure 4A left panel). This effect could be down-regulated by a neutralizing antibody against CXCL10, indicating it is role in this process. Further, we showed that recombinant human CXCL10 directly induce a dose-dependent increase in the invasion of the patient cALL cells (Figure 4A right panel). Similar experiments were performed with 3 other BCP-ALL cell lines, namely, RS4;11, REH, and NALM6, all showing significantly increased invasion in response to the cocultured monocyte supernatants (Figure 4B-D). In each case, the increased invasion was dependent on CXCL10, as revealed by its inhibition in the presence of CXCL10-neutralizing antibody (Figure 4B-D left panels) and increase in invasion of these cell lines to recombinant CXCL10 (Figure 4B-D right panels).

To investigate the mechanistic basis of the increased invasion of the BCP-ALL cells, we checked the activity and expression of MMP9, a key molecule controlling cancer invasion and metastasis.[30] Indeed, zymography assays showed increased MMP9 activity in the supernatants of the recombinant CXCL10-treated patient cALL cells and same in the case of REH, RS4;11, and NALM6 cells (Figure 5A). This also correlated with significantly up-regulated MMP9 gene expression in all these cells, in response to recombinant CXCL10 (Figure 5B). Collectively, these findings demonstrate the role of monocyte-derived CXCL10 in promoting the invasion of BCP-ALL cells via MMP9. Meta-analysis on gene expression datasets of 3 separate cohorts of B-cell ALL patients (n = 108, 145, and 99 patients, respectively) also revealed a significant correlation between *MMP9* and *CXCL10*, providing further support to our observations (Figure 5C).

In addition to the role of the conditioned monocytes in driving the enhanced invasion of BCP-ALL cells via CXCL10-induced MMP9 production, monocytes themselves expressed elevated MMP9 activity and transcripts, on coculture with BCP-ALL cells (supplemental Figure 2), indicating a direct contribution to cell invasion, in addition to the CXCL10-mediated pathway.

BCP-ALL–cocultured monocytes have no effect on the proliferation and viability of primary blasts

We checked whether BCP-ALL–cocultured monocytes promoted the proliferation and survival of the BCP-ALL cells. Proliferation

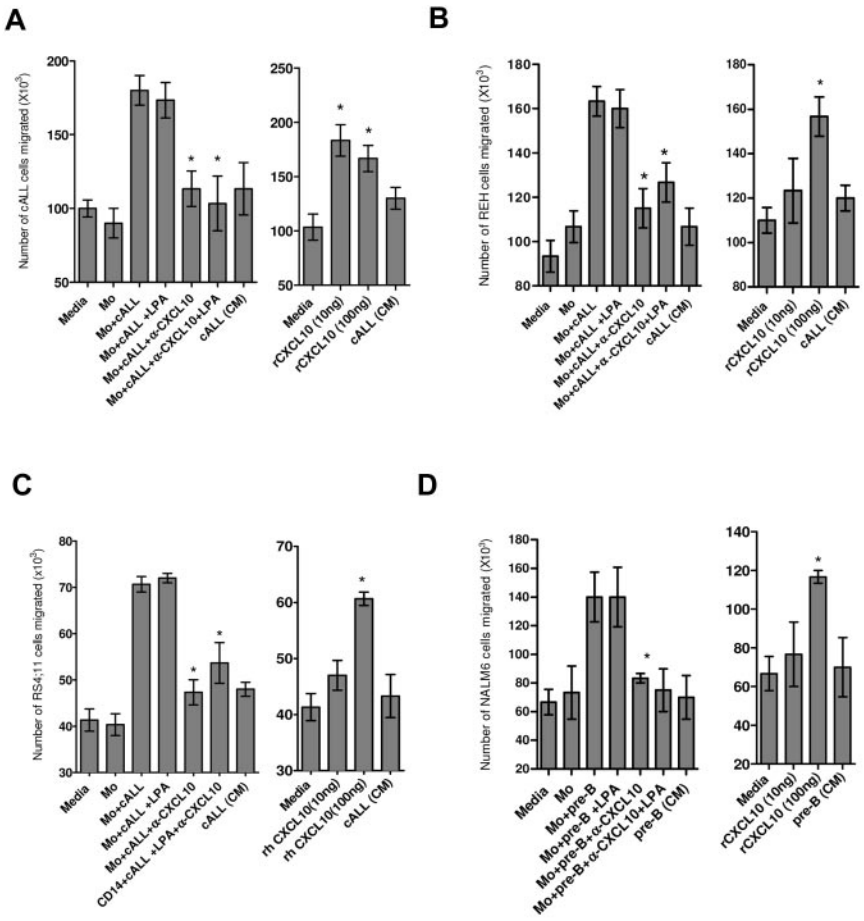

Figure 3. Tumor-cocultured monocytes promote the migration of BCP-ALL cells via CXCL10. (A) Migration assay for cALL cells. Left panel: Normal monocytes and patient (cALL or pre-B ALL) blasts were either cultured alone or together for 24 hours. Thereafter, their cell-free culture supernatants were used for an in vitro migration assay with (A) patient cALL, (B) REH, (C) RS4;11, and (D) NALM6 cells in the presence or absence of anti–CXCL10 (10 μg/mL). BCP-ALL cells migrating through the transwell membranes were counted as described in "Tumor cell invasion and migration assay." In some cases, supernatants from monocytes treated with LPA (100 ng/mL) for 24 hours, after coculture with patient blasts, were also assayed. CM indicates conditioned media. *$P < .05$, versus Mo + cALL or Mo + pre-B ALL (with or without LPA). Right panels of each figure: Migration of the BCP-ALL cells (ie, patient cALL, REH, RS4;11, or NALM6) in the presence of the indicated doses of recombinant human CXCL10. *$P < .04$, versus media. Data are mean ± SEM of a representative experiment.

of BCP-ALL blasts was measured by CFSE staining using flow cytometry. Supplemental Figure 3A shows comparable CFSE staining for cALL cells cultured with or without monocytes, indicating no change in proliferative ability. Further, addition of recombinant human CXCL10 did not show any modulation in the proliferative effect on the cALL cells (supplemental Figure 3A). Similarly, coculturing cALL cells with monocytes with or without CXCL10 treatment had no effect on the viability of cALL cultures (supplemental Figure 3B). The results were also true for patient pre-B ALL cells (data not shown). Collectively, these observations rule out any role for monocytes or their CXCL10 production on BCP-ALL cell proliferation and survival.

BCP-ALL–cocultured monocytes show no visible effects on angiogenesis

Monocytes and macrophages are known to promote tumor angiogenesis.[10] We investigated whether this was also true for monocytes in human BCP-ALL. To check this, supernatants from monocytes cocultured with either cALL or pre-B ALL primary blasts were assayed for HUVEC tube formation in vitro, a measure of angiogenesis. As shown in supplemental Figure 4, no significant increase in tube formation was seen with supernatant for the BCP-ALL "conditioned" monocytes compared with those from monocytes alone, the blast cells alone, or medium itself. Similarly, using supernatants from BCP-ALL–cocultured monocytes treated with anti–CXCL10 antibody or recombinant CXCL10 showed no further change in tube formation. In addition, there was no significant difference in the levels of angiogenic factors (such as VEGFA or bFGF) in the plasma of patients compared with healthy donors (supplemental Figure 1). Thus, these results do not support a role for monocytes in promoting angiogenesis in human BCP-ALL.

Monocyte cocultured with B-cell lymphoma cells also exert protumoral effects

We investigated whether monocytes also exerted protumoral effects on other B cell–related malignancies, such as B-cell lymphoma. Figure 6A shows monocytes cocultured with the B-cell (Burkitt) lymphoma cell line, DAUDI markedly up-regulated *CXCL10* expression (with or without LPA stimulation) compared with monocytes or DAUDI cells cultured alone. Moreover, the supernatants of the cocultured monocytes induced enhanced migration and invasion of DAUDI cells, which was dependent on CXCL10, as shown by its inhibition with neutralizing CXCL10 antibody or the direct enhancing effect of recombinant CXCL10 on DAUDI cells (Figure 6B-C). Enhanced invasion was induced through the CXCL10-induced up-regulation of MMP9 activity and expression in the DAUDI cells (Figure 6D-E). Together, these results indicate a similar protumoral effect of monocytes on B-cell lymphoma, as seen for BCP-ALL, via CXCL10. However, whether this is true for other classes of B-cell lymphoma remains to be investigated.

Discussion

In the present article, we demonstrate the crosstalk between human monocytes and BCP-ALL cells and how such an interaction

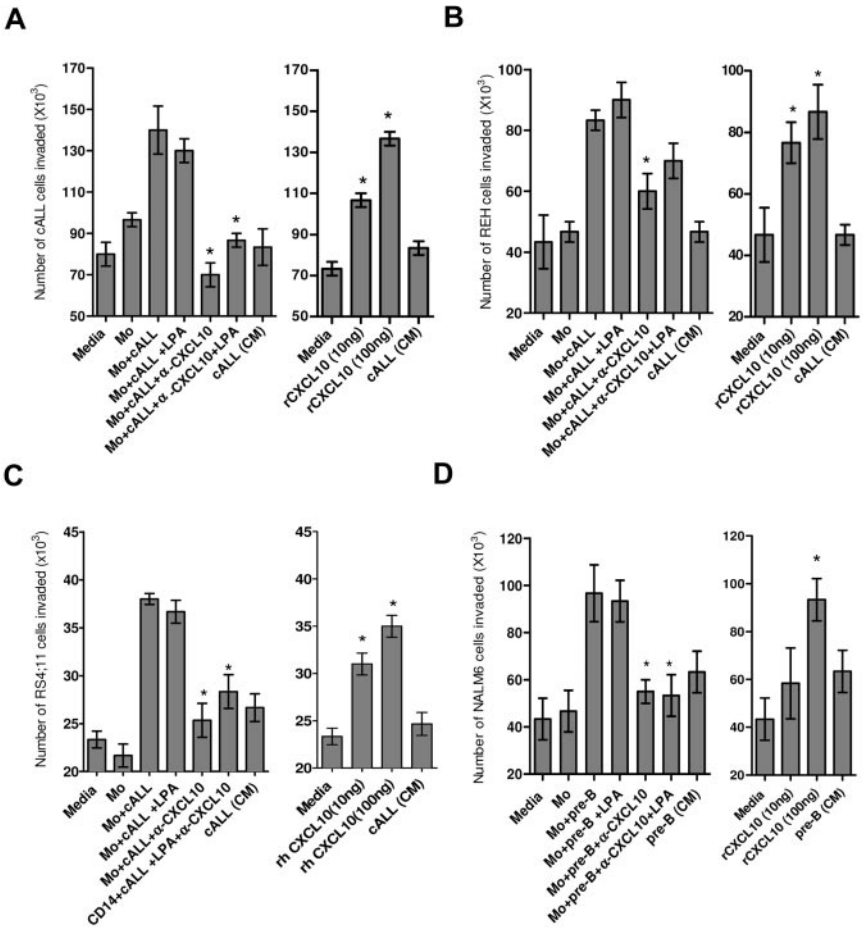

Figure 4. Tumor-cocultured monocytes promote invasion of BCP-ALL cells via CXCL10. Normal monocytes and patient (cALL or pre-B ALL) blasts were either cultured alone or together for 24 hours. Thereafter, the cell-free culture supernatants were used for an in vitro invasion assay with (A) patient cALL, (B) REH, (C) RS4;11, and (D) NALM6 cells in the presence or absence of anti-CXCL10 (10 μg/mL; left panels of the respective figure). Cells invading through the Matrigel-coated transwell membranes were counted as described in "Tumor cell invasion and migration assay." In some cases, supernatants from monocytes treated with LPA (100 ng/mL) for 24 hours, after coculture with patient blasts were also assayed. CM indicates conditioned media. *$P < .05$, versus Mo + cALL or Mo + pre-B ALL (with or without LPA). Right panels of each figure represent the invasion capacity of the BCP-ALL cells (ie, patient cALL, REH, RS4;11, or NALM6) in the presence of the indicated doses of recombinant human CXCL10. *$P < .05$ with respect to media. Data are mean ± SEM of a representative experiment.

"conditions" these monocytes to drive tumor-promoting activities. This is the first report to identify a protumoral role for monocytes in human BCP-ALL.

We demonstrated that human monocytes cocultured in the presence of primary BCP-ALL blasts or BCP-ALL cell lines significantly up-regulated the expression of the chemokine CXCL10 compared with monocytes cultured alone. The enhanced expression of CXCL10 by these monocytes was a specific effect because neither the leukemic cells alone nor monocytes cocultured with B cells from healthy donors showed such an effect. Moreover, this effect was independent of the maturation stage and chromosomal translocations of the BCP-ALL cells. Elevated CXCL10 was also noted in the ALL-associated monocytes isolated from the patients (as well as plasma of these patients). Importantly, we could show that the CXCL10 produced by the BCP-ALL "conditioned" monocytes promoted the migration and invasion of BCP-ALL cells (eg, primary blasts as well as RS4;11, REH, and NALM6 cell lines) in functional assays, suggesting a CXCL10-mediated protumoral activity for these monocytes.

Although earlier studies in ovarian carcinoma have shown mouse and human macrophages cocultured with cancers cells to cause increased tumor cell invasion,[14,27] evidence for such a tumor-promoting role for macrophages in hematologic cancers is lacking. Similarly, the contribution of CXCL10 in mediating tumor progression is also unprecedented. CXCL10 is an inflammatory chemokine that recruits naive as well as Th1-polarized cells.[31] In line with this idea, CXCL10 expression has been linked to M1-polarized macrophages, which orchestrate Th1 and anti–tumor responses.[3,32] Further, CXCL10 possesses strong anti–angiogenic effects both in vitro and in vivo.[33] These reports are in contrast to the tumor-promoting role for CXCL10 reported here. However, elevated CXCL10 expression was reported as a characteristic feature of protumoral TAMs in a murine fibrosarcoma model.[12] In our current study, we showed CXCL10 could directly promote the invasive capacity of BCP-ALL cells by inducing the expression and activity of matrix degrading protein MMP9 in these cells. It may be noted that CXCR3, the chemokine receptor for CXCL10, is abundantly expressed on ALL cells.[34] Therefore, it is envisaged that, under in vivo conditions, the interaction of BCP-ALL cells with monocytes/macrophages would induce CXCL10 expression, which in turn would act on the $CXCR3^+$ BCP-ALL cells (via a chemokine gradient) to promote their migration, invasion, and, possibly, metastatic spread (Figure 7). Thus, blood monocytes by amplifying the CXCR3/CXCL10 chemokine circuit may play a key role in BCP-ALL progression. In follicular lymphoma, TAMs express STAT1, a major transcription factor for CXCL10 expression, and their numbers correlate with poor disease prognosis.[35] Similar to the CXCR3/CXCL10 circuit, another chemokine/receptor couple CXCL12/CXCR4 has been shown to mediate crosstalk between BM stroma and tumor cells in a number of hematologic malignancies.[36]

We also hypothesized other means through which CXCL10-overexpressing monocytes might promote BCP-ALL progression (eg, the increased recruitment of naive or Th1 cells into the tumor microenvironment and their defective activation therein). In line with this idea, monocytes from non-Hodgkin lymphoma patients display a reduced HLA-DR expression and suppress T-cell proliferation.[37] In agreement, we also observed down-regulated HLA-DR

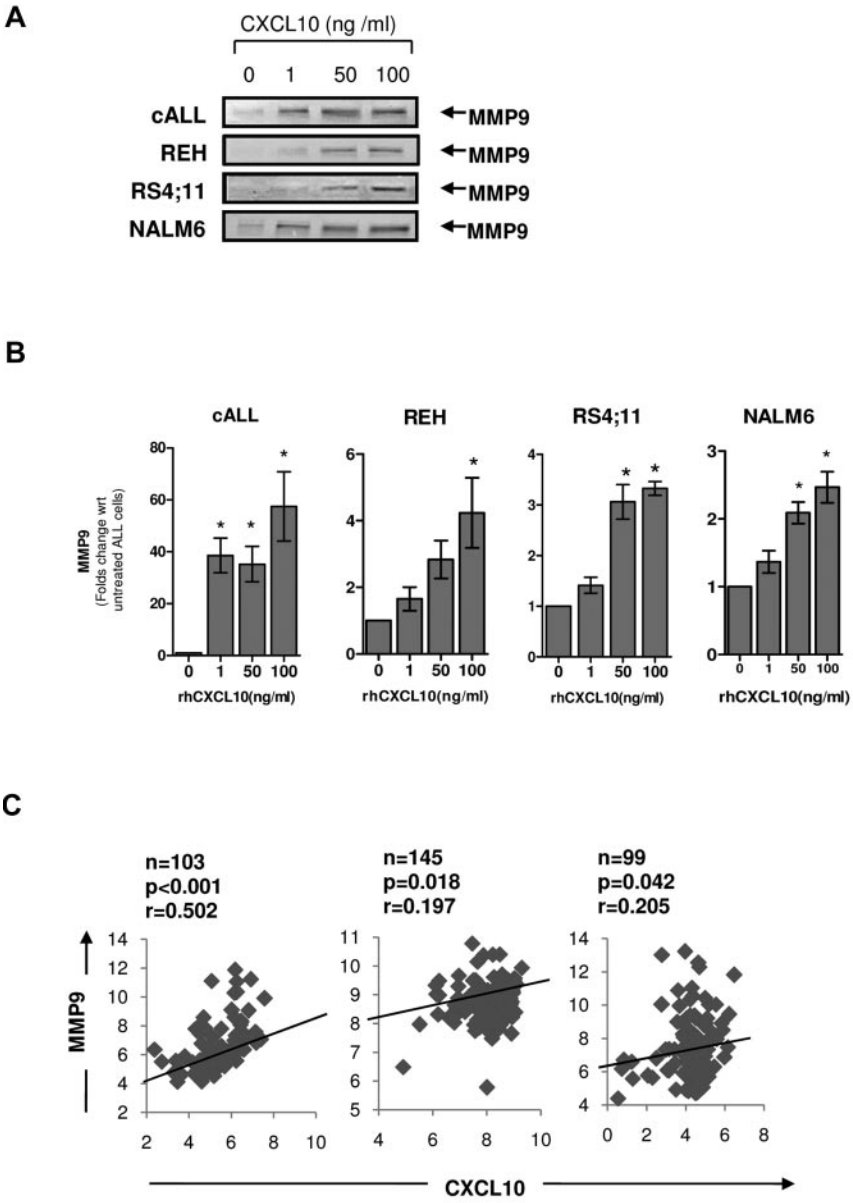

Figure 5. CXCL10 induces MMP9 expression and activity in BCP-ALL cells. (A) Representative zymography assay showing MMP9 activity in the supernatants of a panel of BCP-ALL cells treated with recombinant human CXCL10. (B) MMP9 gene expression in the indicated BCP-ALL cells after treatment with recombinant human CXCL10. Data are mean ± SEM (n = 3). *$P < .05$ with respect to untreated (0 ng/mL). (C) Correlation analysis of CXCL10 and MMP9 gene expression in human B-cell ALL patients. Correlation studies were performed on publicly available gene expression datasets of 3 cohorts of human B-cell ALL patients. Data normalization and correlation analysis are described in "Data analysis for correlation studies."

expression on BCP-ALL–cocultured monocytes as well as those isolated from the patients, but MLR assay revealed normal T-cell stimulatory capacity for these monocytes, ruling out their possible role in inducing a defective T-cell response (data not shown). Further, monocytes/macrophages may also have a tumor-promoting role independent of CXCL10 production. This seems possible because ALL-associated monocytes isolated from BCP-ALL patients showed up-regulated expression of protumoral genes, such as *MMP9* and *COX2*, associated with tumor invasion and metastasis.[30,38] However, a pro-angiogenic role for these cells in BCP-ALL is not supported by the results of the angiogenesis assay.

It may be noted that sera from BCP-ALL patients as well as other leukemias often contain elevated levels of IL-10,[39] which should polarize monocytes/macrophages to an IL-12^{low}/IL-10^{high} M2-like immunosuppressive phenotype, as reported for TAMs in many solid tumors.[7] However, such a phenotype was not observed for the ALL-associated monocytes isolated from BCP-ALL patients. Further, we also did not see any significant up-regulation of IL-10 in the patient plasma (supplemental Figure 1). In contrast, profiling of monocytes isolated from the BM as well as peripheral blood of BCP-ALL patients revealed up-regulated expression of typical M1 inflammatory genes, such as *IL12p40, TNFA*, and *CXCL10* as well as *IL10*. Further, LPA stimulation of the BCP-ALL–cocultured monocytes showed similar expression of M1 inflammatory genes (eg, IL-12p40, TNFA, IL-1B) compared with the monocytes from healthy subjects, suggesting no intrinsic defect in their inflammatory capacity.[40] In contrast to these results, some studies have reported decreased cytokine production in monocytes from ALL patients, but this was not because of their inability to produce inflammatory cytokines but the result of the severely reduced number of monocytes.[40] Although both cocultured monocytes as well as those isolated from the patients showed a significant up-regulation of CXCL10, the expression of other inflammatory genes, such as *IL12p40*, *TNFA* was significantly up-regulated only in the latter population. The reason for such a difference could be the complexity of the in vivo situation involving a plethora of microenvironmental stimuli, longer period (chronic) of conditioning to these stimuli, and interplay with other cell types compared with in vitro conditions.

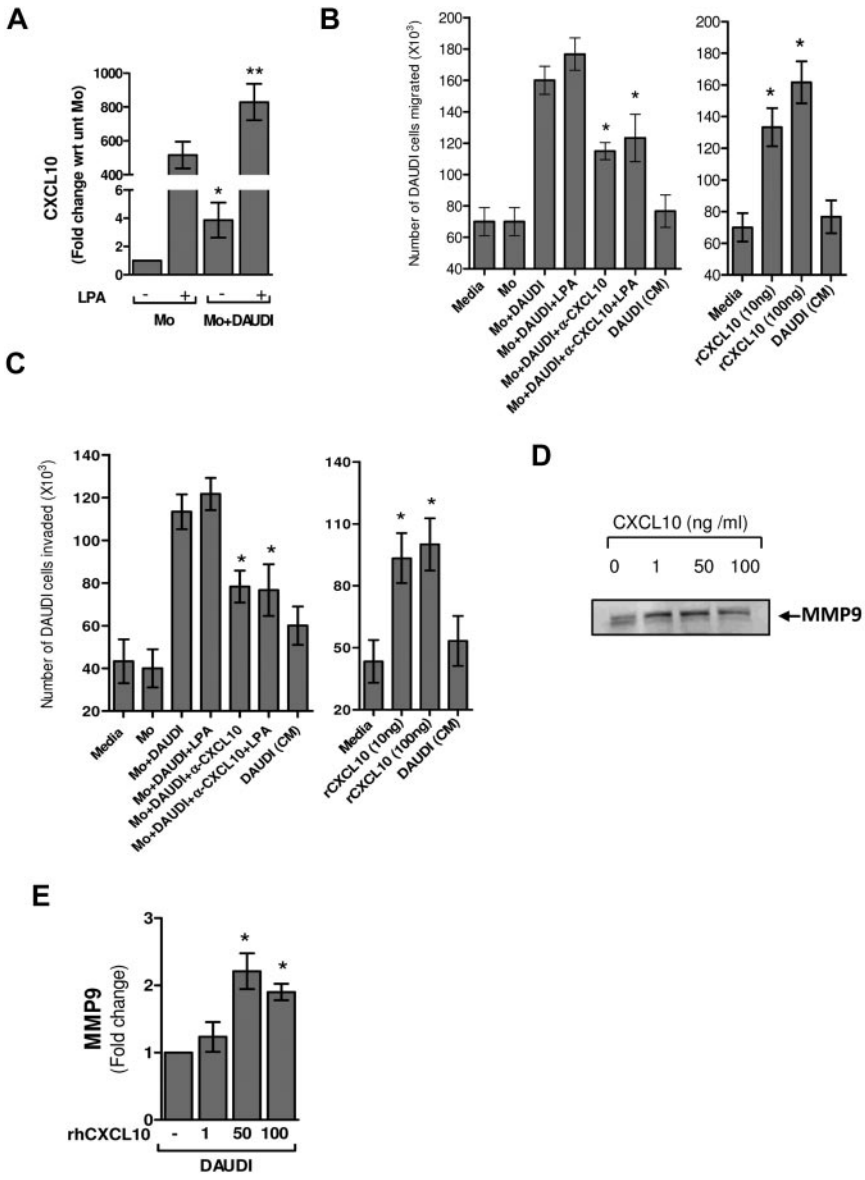

Figure 6. Monocytes cocultured with B-cell lymphoma cells show CXCL10 expression and protumoral effects. (A) CXCL10 expression by monocytes cocultured with or without DAUDI cells for 18 hours. Thereafter, the transwells containing DAUDI cells were removed; and the monolayers werre washed, incubated in fresh media for 1 hour, and stimulated with or without LPA (100 ng/mL) for 4 hours. Mo indicates monocytes alone; and Mo + DAUDI, monocytes cocultured with DAUDI cells. $*P < .05$, versus Mo. $**P < .02$, versus Mo + LPA. Migration (B) and invasion (C) of DAUDI cells in response to supernatants from DAUDI-cocultured monocytes, in the presence or absence of anti-CXCL10 (10μg/mL; left panels). $*P < .002$, versus Mo + DAUDI (with or without LPA). Right panels: Migration and invasion of DAUDI cells in response to recombinant human CXCL10. $*P < .002$, versus media. Data are mean ± SD (A) and mean ± SEM (B-C) of a representative experiment. (D) MMP9 activity by zymography. (E) MMP9 gene expression was assessed in DAUDI cells in response to recombinant human CXCL10. Data are mean ± SEM (n = 3). $*P < .001$, versus media.

While the inflammatory phenotype of monocytes associated with BCP-ALL is consistent with cancer-related inflammation,[9] the mechanism shaping this phenotype is not clear. A recent study reports expression of endogenous TLR4 ligands in the serum of acute leukemia patients,[41] which may be a possible stimuli for triggering inflammatory cytokines expression (especially CXCL10). In a murine B-cell lymphoma model, injection of lipopolysaccharide induced increased inflammatory response and the spread of the tumor to the liver.[42] In this scenario, it may be hypothesized that an elevated CXCL10 production induced by lipopolysaccharide in the monocytes/macrophages results in enhanced migration and spread of the tumor cells. Indeed, epidemiologic studies indicate that almost 32% of the children inflicted with leukemia are subject of bacteremia.[43] However, whether the incidence of such infection in ALL patients may lead to enhanced tumor progression remains to be investigated.

Our experiments with monocyte cocultures with DAUDI cells implicated a similar protumoral role of monocytes in B-cell lymphoma, as suggested for BCP-ALL. In support, a recent study shows the importance of $CD68^+$ macrophages in predicting prognosis in classic Hodgkin lymphoma patients.[18] Similarly, another study suggested B-cell lymphoma cells to polarize macrophages for enhanced capacity

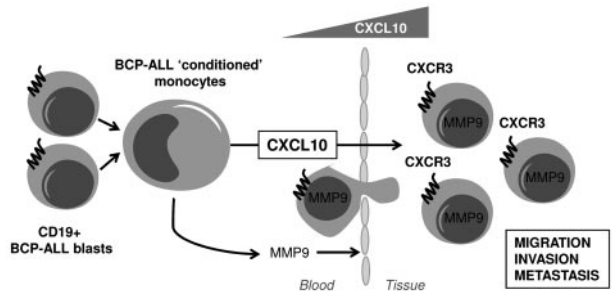

Figure 7. Schematic representation of the proposed tumor-promoting role of monocytes in BCP-ALL via the CXCL10/CXCR3 axis. The figure shows BCP-ALL cells to "condition" monocytes for elevated expression of the chemokine CXCL10. Increased CXCL10 release by these monocytes would possibly set up a chemokine gradient across which the $CXCR3^+$ BCP-ALL cells migrate (into the tissue), invade, and possibly lead to metastatic spread. It may be noted that CXCL10 induced MMP9 expression and activity in the BCP-ALL cells, which may explain its role in promoting their invasion. In addition, the "conditioned" monocytes also show elevated MMP9 expression and activity, which can as well contribute to the invasion and spread of BCP-ALL cells.

to phagocytose rituximab-opsonized leukemic cells with implication in tumor killing.[44] However, further study is needed to understand the role of monocytes/macrophages in driving B-cell lymphoma progression.

In conclusion, our study reveals a tumor-promoting role for human monocytes in the BCP-ALL. We identify a circuit involving CXCL10 and CXCR3 as a potential mechanism by which tumor-conditioned monocytes crosstalk with the malignant cells to promote their migration and invasion. Our observations, together with the recent evidence on TAMs in the prognosis of classic Hodgkin and follicular lymphomas, should trigger further investigation into the general role of monocytes/macrophages in hematologic malignancies.[18,35] Further, based on the targeting of CXCR4/CXCL12 circuit in preclinical lymphoma models,[45] it would be interesting to determine whether a similar approach could be used for the CXCL10/CXCR3 circuit to therapeutically target the progression of BCP-ALL in the near future.

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Authorship

Contribution: Y.L., M.C., V.A., and H.Z. performed experiments, analyzed results, and prepared the figures; and S.K.B., G.D., M.P., and A.B. supervised different aspects of research, designed the study, and participated in manuscript preparation.

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Protumoral role of monocytes in human B-cell precursor acute lymphoblastic leukemia: involvement of the chemokine CXCL10

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