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EpCAM Inhibition Sensitizes Chemoresistant Leukemia to Immune Surveillance

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Abstract

The lack of effective tumor-associated antigens restricts the development of targeted therapies against myeloid leukemia. In this study, we compared gene expression patterns of acute myeloid leukemia (AML) and normal bone marrow samples and found that epithelial cell adhesion molecule (EpCAM) is frequently overexpressed in patients with AML, with EpCAM$^+$ leukemic cells exhibiting enhanced chemoresistance and oncogenesis. The chemotherapeutic resistance of EpCAM-positive leukemic cells is a consequence of increased WNT5B signaling. Furthermore, we generated EpCAM antibodies that enabled phagocytosis or cytotoxicity of AML cells by macrophage or natural killer cells, respectively. Finally, EpCAM antibody treatment depleted AML in subcutaneous, disseminated, and intramedullary engrafted mice. In summary, EpCAM exhibits promise as a novel target for the treatment of leukemia. *Cancer Res; 77(2); 482–93. ©2016 AACR.*

Introduction

Over the past 30 years, antibody-based cancer therapeutics have become one of the most valuable approaches for the treatment of patients with hematologic malignancies (1, 2). This notion is most evident in the case of B- and T-cell leukemia, where CD19, CD20, and CD52 antibodies have revolutionized the standard of care (3, 4). However, cure rates for patients with myeloid leukemias, which are the most common adult hematological malignancies, have changed very little over the past decade mainly because very few broadly expressed markers are available for use as clinical therapeutics in these types of leukemia patients. Therefore, there is still a need to identify and expand novel targets for the treatment of myeloid leukemias.

The efficacy and safety of therapeutic monoclonal antibodies in tumor treatment vary depending on the original nature of the targeting antigen. Ideally, the therapeutic target should be expressed abundantly, exclusively, homogeneously and consistently on the cell membrane of tumors (1, 2, 5, 6). On the basis of these optimal conditions, we sought to identify a novel target in leukemia by comparing the transcriptional profiles of normal or leukemic bone marrow cells by microarray.

From this high-throughput screen, we observed that epithelial cell adhesion molecule (EpCAM) was overexpressed on abnormal cells from myeloid leukemia patients but not on normal bone marrow cells from healthy donors (7). The EPCAM gene encodes a membrane glycoprotein that has been reported as a carcinoma-associated antigen in solid tumors (8, 9). EpCAM is being used as an effective therapeutic target in phase II/III clinical trials in solid-tumor patients (10–12). Therefore, EpCAM is relatively safe as an antigen target for antibody-based therapies. Nevertheless, the utility of EpCAM as a potential therapeutic target in hematologic malignancies requires further verification.

Conventional chemotherapeutic drugs have proven successful in treating hematologic malignancies (13), yet many patients die from relapse after an initial therapy response due to the failure to eradicate chemotherapy-resistant leukemic stem cells (LSC; ref. 14). EpCAM was reported as a marker for cancer stem cells in hepatocellular carcinoma, colorectal cancer and breast cancer (15–17). In the present study, we found that EpCAM$^+$ cells are analogous to leukemia stem cells and display enhanced tumor-forming ability compared with EpCAM$^-$ cells. Moreover, EpCAM expression is associated with chemoresistance. Further elucidating the molecular mechanism of the EpCAM protein, we found that WNT5B is activated in EpCAM$^+$ leukemic cells. On the basis of these findings, we hypothesized that targeting EpCAM will clear LSCs and reverse the resistance to chemotherapy. Thus, we propose EpCAM as an effective antibody-therapeutic target for hematologic malignancies. We generated EpCAM therapeutic antibodies that demonstrated significant efficacy in multiple myeloid leukemia models. The EpCAM antibody eliminated myeloid leukemia cells through the action of innate immune cells.

Materials and Methods

Cell lines, transfection, and clinical samples

K562, HL60, Karpas, Namalwa, Jurkat, NS-1, P3×63Ag8.653, HCT-116 and A549 cells were obtained directly from the Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China) and passaged in our laboratory for fewer than 6 months after receipt or resuscitation. DNA-fingerprinting and isoenzyme

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testing were used to authenticate these cell lines by the Shanghai Cell Bank. The transfection and RNA interference were performed by nucleofection or lentiviruses infection (18). Newly diagnosed, relapsed or post-treatment patients with leukemia were included. The clinical characteristics of the patients in this study are presented in Supplementary Table S1. For details, see Supplementary Materials and Methods.

Reverse transcription PCR, immunofluorescence, immunohistochemistry, and apoptosis assay and flow cytometry

Reverse transcription PCR, immunofluorescence, immunohistochemistry, and flow cytometry were performed as previously described (19–23). The One-step terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Apoptosis Assay Kit was purchased from Beyotime (Cat. no. C1086, Shanghai, China) and performed according to the manufacturer's instructions. The details of bone section were shown in Supplementary Materials and Methods.

Expression analysis by microarray and RNA sequencing

The molecular signatures of bone marrow samples from acute myeloid leukemia (AML) patients and normal donors were analyzed using a whole human genome oligo microarray (Agilent, G4112F). Microarray image analysis and hierarchical clustering were performed as previously described (24). For RNA sequencing analysis, the integrity was established using the TruSeq RNA Sample Prep Kit (Illumina), Agilent 2100 bioanalyzer, and Agilent HiSeq 2500 Sequencing System (25). Target genes for selected pathways were predicted using online bioinformatics databases, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (http://www.kegg.jp/kegg/pathway.html) and STRING (http://string-db.org). The microarray data were deposited into the National Center for Biotechnology Information GEO repository under accession number GSE79605.

Colony formation and tumorigenicity

For colony formation assays, 2000 $EpCAM^{high}$ or $EpCAM^{low}$ K562/HL60 cells were cultured 10 days in 6-well plates and counted by microscopy. For tumorigenicity assays, 1×10^6 cells were suspended in 200 μL of RPMI-1640 medium and subcutaneously injected. For details, see Supplementary Materials and Methods.

Antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis

Antibody-dependent cellular cytotoxicity (ADCC) was performed by the cytotoxicity assay (^{51}Cr Release). Coculture of GFP-positive HL60 cells and macrophages was applied to antibody-dependent cellular phagocytosis (ADCP). For details, see Supplementary Materials and Methods.

Antibody production

The AE7 (mouse IgG2a-κ) and AE4 (mouse IgG2b-κ) EpCAM-specific hybridomas were raised against the His-tagged extracellular domain of EpCAM proteins and screened by enzyme-linked immunosorbent assay (ELISA) and flow cytometry. AE7 and AE4 monoclonal antibodies were purified by protein-A affinity chromatography (GE Healthcare Bio-Sciences AB) and labeled as follows: AE7-FITC (AE7 labeled with FITC). EpCAM-specific antibodies containing AE7/AE4 and the others from BD Biosciences and BioLegend were validated for anti-EpCAM extracellular epitope.

Determination of the therapeutic effects of the EpCAM antibody

Female NOD-SCID mice were used to establish subcutaneous, intravenous, and intramedullary leukemia xenograft models to assess the therapeutic effects of the EpCAM antibody. In the subcutaneous leukemia xenograft model, EpCAM antibody was treated once the tumors were palpable, and an MRI system was used to actively screen tumors *in vivo*. In the intravenous and intramedullary models, human AML engraftment was assessed using a luciferin imaging system. For details, see Supplementary Materials and Methods.

Statistical analysis

All data are presented as the mean ± SEM and statistical plots generated using GraphPad Prism. Differences between groups were determined by two-tailed *t* tests. Differences among three or more groups were determined by one-way ANOVA followed by two-tailed *t* tests. A *P* value of ≤ 0.05 was considered significant.

Results

Myeloid leukemia frequently contains EpCAM-expressing cancer subpopulations

To search for novel molecular targets in myeloid leukemia, we initially analyzed the gene expression profiles of primary bone marrow samples from human acute myelogenous leukemia (AML) patients or healthy donors using an oligonucleotide microarray. We identified 6,864 genes whose expression levels were at least 2-fold upregulated ($P < 0.01$) in AML samples compared with healthy tissues (Fig. 1A). To focus our studies on cell-surface proteins with the potential to serve as therapeutic targets, we evaluated membrane-related genes whose expression was increased by at least 12-fold (Fig. 1B). Notably, the expression of EPCAM was elevated by 12.59-fold in bone marrow samples form AML patients relative to normal donors (Fig. 1B). The abundance of the target antigen on the surface of tumor cells determines the efficacy of the targeting agents (1). Therefore, we quantified the levels of EpCAM and other previously reported leukemia targeting antigens, including CD47, CD52, Notch1, and CD44, in myeloid leukemia cells by Illumina RNA sequencing (Fig. 1C). We detected enhanced levels of EpCAM in myeloid leukemia cells compared with common tumor antigens (Fig. 1C). These data support EpCAM as a novel tumor antigen for myeloid leukemia.

Next, to investigate EpCAM overexpression in myeloid leukemia, we assessed the mRNA and protein levels in a series of cell lines and clinical hematologic malignancy specimens. Reverse transcription PCR analysis of bone marrow mononuclear cells from AML ($n = 16$), acute lymphoblastic leukemia (ALL, $n = 13$) or multiple myeloma (MM, $n = 6$) patients or from normal healthy donors ($n = 6$). EpCAM-positive cells were noted in leukemia patients (AML, 56.3%, 9/16; ALL, 30.7%, 4/13; MM, 83.3%, 5/6), but no expression was noted in healthy donors (healthy, 0%, 0/6; Fig. 1D). Flow cytometric analysis of identified leukemia cell fractions (markers shown in Supplementary Table S1) in bone marrow single-cell suspensions derived from clinical leukemia specimens ($n = 58$ patients) revealed frequent EpCAM expression in ALL (43.7%, 7/16), MM (63.6%, 7/11), and AML (70%, 21/30) patients. However, no expression was noted in

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multiple populations of bone marrow single-cell suspensions from healthy donors (0%, 0/14; Fig. 1E, Supplementary Fig. S1A). EpCAM showed more frequent overexpression in AML and MM than ALL patients. Immunofluorescence labeling of clinical bone marrow smears for EpCAM confirmed EpCAM protein expression in myeloid leukemia (57.1%, 4/7, Supplementary Fig. S1B).

We also analyzed EpCAM expression in peripheral blood mononuclear cells (PBMC) from clinical leukemia specimens using reverse transcription PCR and flow cytometry. EpCAM expression was detected in AML patients but not in normal donors (Fig. 1F and G, Supplementary Fig. S1C). The identified leukemia and abnormal cell fractions from these patients (markers shown in Supplementary Table S1) were gated for flow cytometric analysis, with EpCAM-positive specimen frequencies ranging from 33.3 to 87.5% (ALL, 40%, 2/5; MM, 33.3%, 1/3; CML, 87.5%, 7/8; AML, 73.3%, 22/30).

To further determine EpCAM overexpression in myeloid leukemia, we surveyed EpCAM expression in human and murine leukemia cell lines using immunofluorescence and flow-cytometry analysis. Low levels of EpCAM expression were detected in human Karpas, Namalwa, and Jurkat lymphocytic leukemia cells, whereas high levels of EpCAM expression were noted in human HL60 and K562 as well as murine NS-1 and P3×63Ag8.653 myeloid leukemia cells (Fig. 1H and I, Supplementary Fig. S1D).

These data confirmed the frequent expression of EpCAM in cells from myeloid leukemia and highlighted the potential of EpCAM as a novel target for myeloid leukemia therapeutics.

EpCAM promotes myeloid leukemia chemoresistance through WNT5B

Clinically, chemotherapy is typically used to treat leukemia patients. To investigate the effect of EpCAM expression in leukemic cells on clinical chemotherapeutics, we examined PBMCs collected before and after cytarabine (Ara-C) or etoposide (VP-16) chemotherapy in EpCAM-positive patients with myeloid or lymphoblastic leukemia. Flow cytometric analysis of gated leukemia cell fractions (markers shown in Supplementary Table S1) revealed the enrichment of EpCAM-positive leukemia subpopulations after chemotherapy (Fig. 2A), suggesting that EpCAM-positive leukemic cells may have lower chemotherapeutic sensitivity than EpCAM-negative cells.

To confirm that EpCAM expression is associated with chemoresistance, we selected two EpCAM-positive myeloid leukemia cell lines, K562 and HL60, to study chemotherapy-induced apoptosis. After a 24-hours treatment with increasing doses of Ara-C or VP-16, EpCAM silencing increased the percentage of apoptotic K562 and HL60 cells (Fig. 2B–D), indicating that EpCAM expression reduced the chemotherapeutic drug sensitivity of leukemic cells.

As shown in Fig. 2E, K562 and HL60 cells exhibited varying levels of EpCAM protein expression and were therefore divided into $EpCAM^{high}$ and $EpCAM^{low}$ subpopulations. We compared the sensitivity of $EpCAM^{high}$ and $EpCAM^{low}$ cells (Fig. 2F and G). We enriched $EpCAM^{high}$ and $EpCAM^{low}$ populations from K562 or HL60 cells by fluorescence activated cell sorting, with >95% purity noted in these two populations. After a 24-hours treatment, Ara-C and VP-16 both induced more enhanced apoptosis in $EpCAM^{low}$ K562 cells than in $EpCAM^{high}$ K562 cells (Fig. 2F and G). Similar results were also observed in $EpCAM^{low}$ and $EpCAM^{high}$ HL60 cell populations (Fig. 2F and G). These results further confirmed that $EpCAM^{high}$ leukemic cells resist drug treatment.

Next, to explore the molecular etiology of EpCAM-mediated chemoresistance in leukemic cells, we compared the transcriptional profiles of $EpCAM^{low}$ and $EpCAM^{high}$ HL60 or K562 cells by Illumina RNA sequencing. From this analysis, we identified fourteen mRNAs that were significantly increased in $EpCAM^{high}$ cells (Fig. 2H, Supplementary Fig. S2A). Notably, the expression of WNT5B increased in $EpCAM^{high}$ K562 cells (Fig. 2H). Similar results were also observed by comparing the transcriptional profiles of $EpCAM^{low}$ and $EpCAM^{high}$ HL60 cells (Fig. 2I). Reverse transcription PCR analysis of WNT5B detected markedly upregulated expression in $EpCAM^{high}$ K562/HL60 cells (Fig. 2J).

Wnt family members participate in physiological and pathological signaling events that span stem cell functions, developmental biology, and neoplasia (26, 27). Acting cell nonautonomously, WNT16B promotes the survival of prostate tumor cells after cytotoxic treatment. Resistance to bromodomain and extra terminal protein inhibitors in myeloid leukemia therapy is a consequence of increased Wnt/β-catenin signaling (25). We confirmed that WNT5B associates with the resistance of $EpCAM^{high}$ myeloid leukemia cells to cytotoxic chemotherapy (Fig. 2H and I). Transcripts encoding other Wnt family members were not substantially increased or decreased between $EpCAM^{low}$ and $EpCAM^{high}$ myeloid leukemia cells (Supplementary Fig. S2B). These data suggested that the chemotherapeutic resistance of EpCAM-positive leukemic patients is partially a consequence of increased WNT5B signaling.

The above data indicated that the chemoresistance of leukemic cells mainly arise from a subpopulation of EpCAM-positive cells and suggested that EpCAM-positive cells may be the chief culprit in the relapse of leukemia patients after chemotherapy via WNT5B signaling. Hence, targeting EpCAM may reverse chemoresistance and eradicate leukemia.

$EpCAM^+$ cells are analogous to leukemia stem cells

EpCAM is abundantly expressed on solid tumors and is a marker for cancer-initiating cells in colon and hepatocellular carcinoma (15, 16). Clinically, tumor-initiating cells are thought to the main cell subpopulation for resistance to chemotherapy. Therefore, $EpCAM^+$ cells may act as leukemia stem-like cells with the ability to resist drug treatment. To test this hypothesis, we first evaluated the expression patterns of several leukemia stem cell markers in clinical primary EpCAM-positive leukemic cells. Flow cytometric analysis of gated $EpCAM^+$ leukemia cell fractions in bone marrow single-cell suspensions derived from clinical leukemia specimens ($n = 19$) revealed a great percentage of $EpCAM^+$ cells that presented $CD34^+CD38^-$ or $CD34^+CD38^+$ (Fig. 3A). Multiple studies have confirmed that leukemic stem cells are rare and confined to the $CD34^+CD38^-$ subset. In the past few years, several studies demonstrated that this $CD34^+CD38^+$ subset contained leukemia-initiating cells in approximately 50% of cases (14, 28–30). We also found that EpCAM-positive cells expressed several reported leukemic stem cell markers, such as CD33, CD123, CD20, CD44 or HLA-DR (Fig. 3B; refs. 4, 31–34). We successfully enriched $EpCAM^{high}$ and $EpCAM^{low}$ populations from K562 and HL60 cells by fluorescence-activated cell sorting (Fig. 2E) and detected the increased levels of CD33 and CD44

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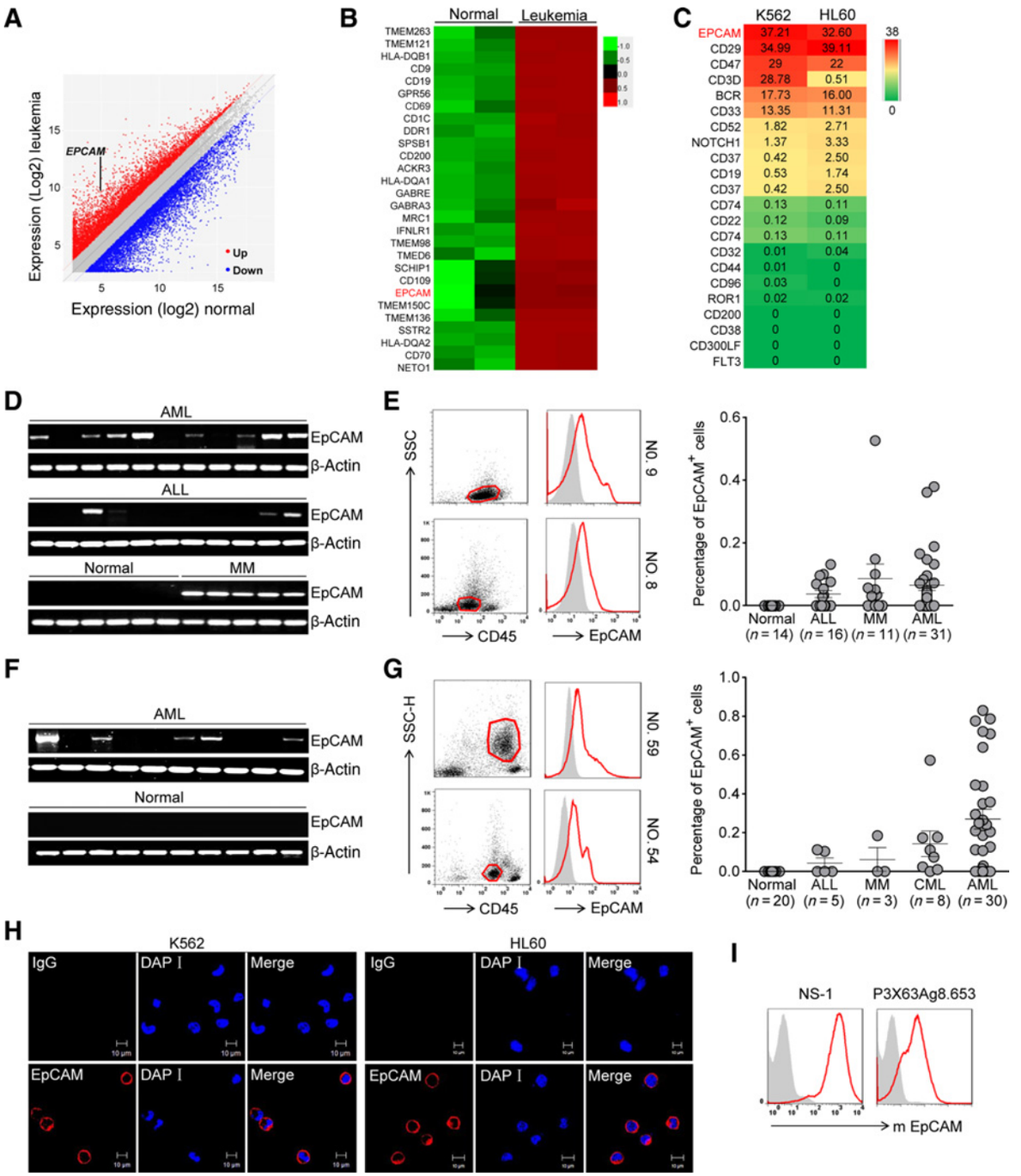

Figure 1.

EpCAM overexpression by myeloid leukemia cells. **A** and **B,** Analysis of gene expression changes in primary bone marrow samples from human AML patients or normal donors based on whole human gene oligo microarray. AML patients compared with normal donors. **A,** Here, 2-fold upregulated genes are noted in red, whereas 2-fold downregulated genes are indicated in blue ($P < 0.05$). Each symbol represents the mean of two samples per genotype. **B,** The heatmap depicts cell-surface molecules upregulated by at least 12-fold in AML patients relative to normal donors ($P < 0.01$). **C,** Heatmap of the mRNA levels of EpCAM and other previously reported leukemia-targeting antigens in myeloid leukemia cells by Illumina RNA sequencing. **D** and **E,** EpCAM expression in human bone marrow samples from leukemia patients or normal donors was determined by reverse transcription PCR and flow cytometry. **D,** Each band represents a different patient sample. **E,** Statistical analysis of the percentage of $EpCAM^+$ cells among abnormal cells of leukemia patients and the percentage of $EpCAM^+$ cells among $CD45^+$ cells of normal donors. Each point represents a different patient sample. Gray, mouse IgG2b κ staining. **F** and **G,** EpCAM expression on human PBMCs of AML, ALL, MM, chronic myeloid leukemia (CML) patients and normal donors was determined by RT-PCR and flow cytometry. **G,** Statistical analysis of the percentage of $EpCAM^+$ cells among abnormal cells from leukemia patients and the percentage of $EpCAM^+$ cells among $CD45^+$ cells from normal donors. Gray, mouse IgG2b κ staining. **H,** Immunofluorescence staining for EpCAM in human myeloid leukemia cell lines (K562 and HL60). The nucleus was stained with DAPI; scale bar, 10 μm. **I,** Flow cytometry staining for murine EpCAM in murine myeloma cell lines (NS-1, P3 × 63Ag8.653). Gray, rat IgG2a κ staining (**I**).

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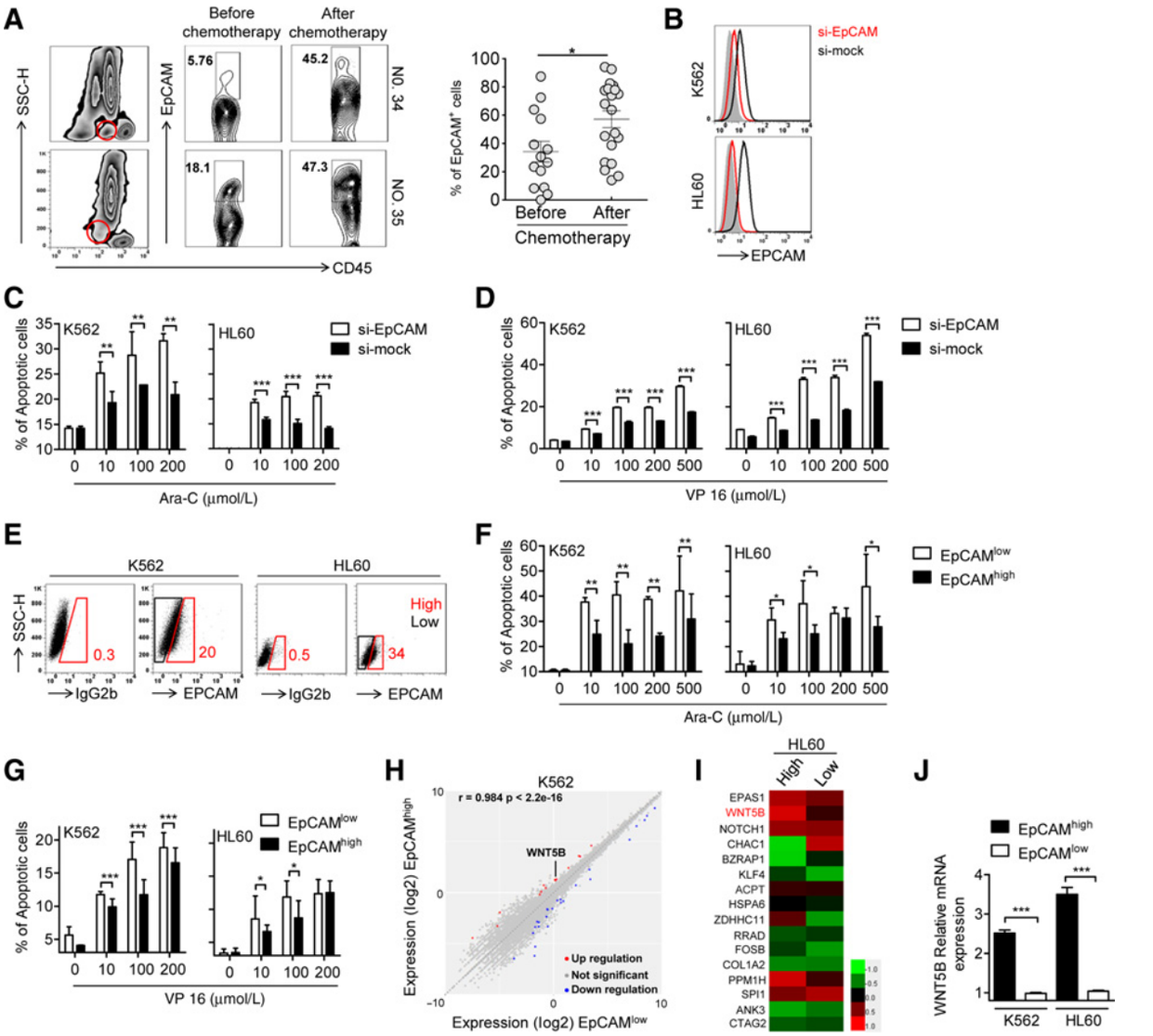

Figure 2.

EpCAM resists chemotherapeutic of myeloid leukemia through WNT5B. **A,** Flow cytometry of cells from PBMCs of AML patients before and after chemotherapy. Frequency of EpCAM^{+} cells among abnormal cells. **B,** Flow-cytometry analysis of EpCAM expression in K562 and HL60 cells transfected with EpCAM siRNA or si-mock. Gray, mouse IgG2b κ staining. **C–D** and **F–G,** The percentage of apoptotic cells after a 24-hours treatment with VP-16 or Ara-C, as determined by Annexin V/7AAD staining. **E,** Sorting of EpCAM^{high} and EpCAM^{low} cells by flow cytometry. **H–J,** Analysis of gene expression changes in EpCAM^{high} and EpCAM^{low} K562/HL60 cells based on whole human gene oligo microarray. EpCAM^{high} cells compared with EpCAM^{low} cells. Upregulated genes are shown in red at the top, whereas downregulated genes are noted at the top in blue ($P < 0.05$). Each symbol represents a genotype (**H**). Heatmap of the mRNA levels of markedly upregulated genes (**I**) in EpCAM^{high} cells relative to EpCAM^{low} cells. **J,** Reverse transcription PCR analysis of WNT5B expression in EpCAM^{high} or EpCAM^{low} K562/HL60 cells; **, $P < 0.01$; ***, $P < 0.001$. The data are presented as the mean ± SEM.

expression in EpCAM^{high} cells compared with EpCAM^{low} cells (Fig. 3C).

To further evaluate whether EpCAM^{high} cells were analogous to leukemia stem cells, we studied the colony formation and tumorigenesis of EpCAM^{high} and EpCAM^{low} populations from K562/HL60 cells. The isolated EpCAM^{high} cells formed more colonies than EpCAM^{low} cells (Fig. 3D). EpCAM^{high} K562 cells, but not EpCAM^{low} K562 cells, efficiently initiated tumors in NOD/SCID mice. EpCAM^{high} cells initiated tumors in 4 of 4 injected mice, whereas EpCAM^{low} cells did not produce tumors in 4 injected mice at 4 weeks after transplantation (Fig. 3E). In HL60 cells, both EpCAM^{high} and EpCAM^{low} subpopulations initiated tumors, but the tumor sizes were increased for the EpCAM^{high} cells compared with EpCAM^{low} cells (Fig. 3E). The EpCAM^{low} HL60 cell fraction produced small tumors, possibly due to the low expression of EpCAM in this subpopulation. We compared whole-gene transcriptional profiles of EpCAM^{low} and EpCAM^{high} HL60 or K562 cells by Illumina RNA sequencing and detected that EpCAM^{high} leukemia cells exhibit reduced levels of whole-gene expression profile and metabolic signaling (Fig. 3F and G). Hierarchical

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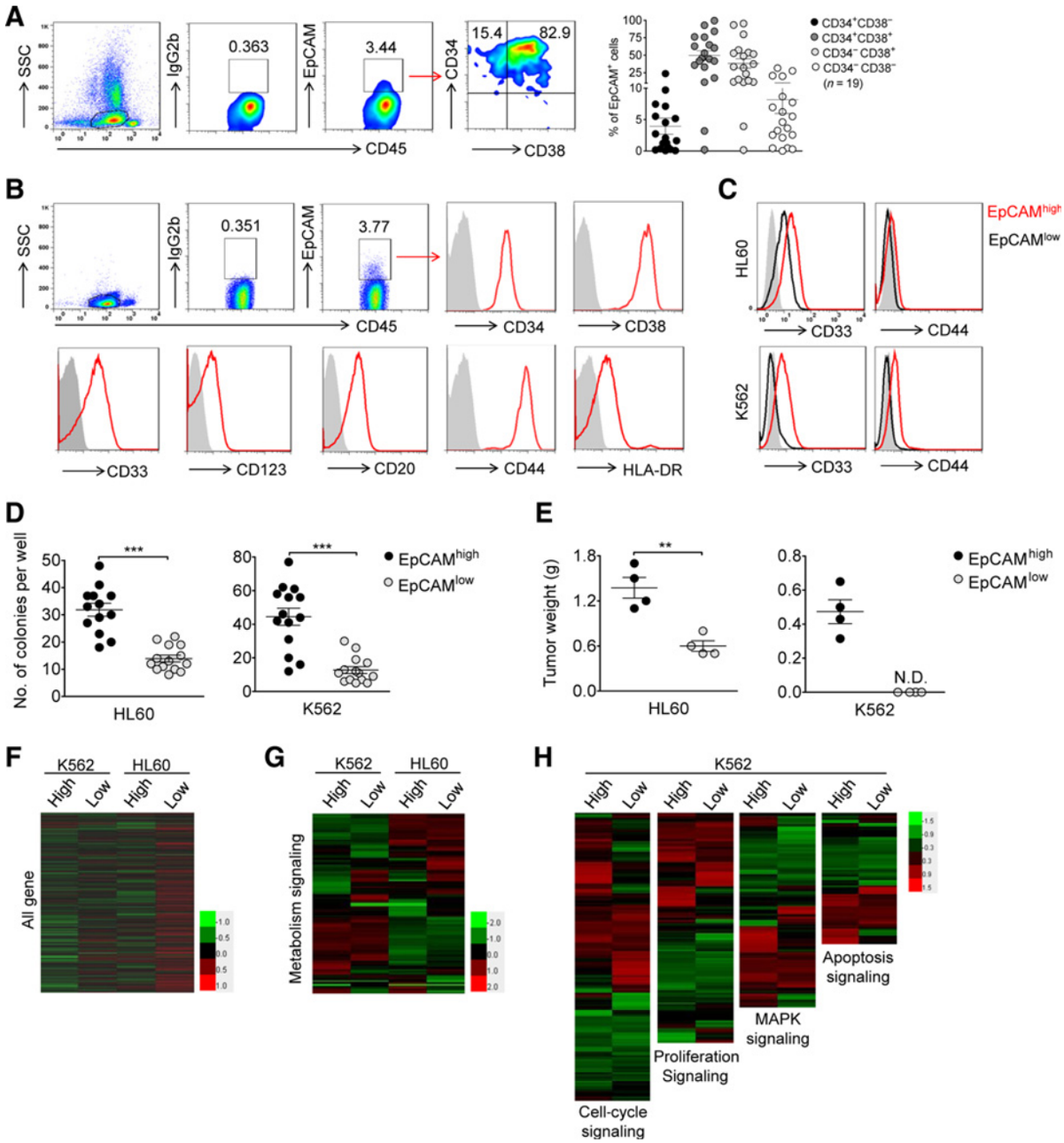

Figure 3.

EpCAM$^+$ cells are analogous to leukemia stem cells. **A,** Flow-cytometry analysis of cells from bone marrow of AML patients. The frequency of CD34$^+$CD38$^-$ cells or CD34$^+$CD38$^+$ cells among EpCAM$^+$ myeloid leukemia cells. **B** and **C,** Flow-cytometry analysis of cells from bone marrow of AML patients to assess stem cell marker expression on EpCAM$^+$ leukemia cells. Gray, IgG isotype control. **D,** Colony formation by EpCAM^{high} and EpCAM^{low} K562/HL60 cells; total numbers of spheroids from 2,000 sorted cells. **E,** Tumorigenic potential of EpCAM^{high} and EpCAM^{low} K562/HL60 cells *in vivo*. Tumor weight is shown. N.D., not detected. **F–H,** Heatmap of transcript expression of whole genes (**F**), metabolism signaling, cell cycle and proliferation signaling (**G**), and MAPK and apoptosis signaling (**H**) based on RNA sequencing; **, $P < 0.01$; ***, $P < 0.001$. The data are presented as the mean ± SEM.

clustering of pathways revealed the enriched cell cycle and proliferation signaling genes in EpCAM^{high} leukemia cells (Fig. 3H). Mitogen-activated protein kinase (MAPK) and apoptosis signaling exhibited no significant differences between the two types of cells (Fig. 3H). These dataset analytics further supported the similarity between EpCAM^{high} leukemia cells are and tumor-initiating cells. We also detected an increased percentage of isolated EpCAM^{low} K562 cells that underwent shrinkage and apoptosis upon resuscitation of frozen cells compared with EpCAM^{high} cells (Supplementary Fig. S3A). These data suggested

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that EpCAM^{high} cells are an essential subpopulation for maintaining the survival of K562 cells. The EpCAM^{high} fraction decreased with time in sorted EpCAM^{high} K562 cells from >90 to 41%, suggesting that EpCAM^{high} cells differentiate into EpCAM^{low} cells, eventually allowing the enriched EpCAM^{high} fraction to revert back to parental cells after 14 days in culture (Supplementary Fig. S3B). These phenomena were similar to those observed for EpCAM as a tumor stem cell marker in hepatoma (16).

These data indicated that EpCAM^{+} cells act as leukemia stem-like cells to accelerate leukemic progression and further highlighted the potential for EpCAM-targeted treatment to eradicate leukemia.

EpCAM antibody AE7 inhibits myeloid leukemia in localized and disseminated xenotransplant mouse models

EpCAM has the potential to serve as an effective therapeutic target in leukemia based on its absence in normal hematopoietic system cells, its expression in leukemic cells, its similarity to tumor stem cells, its ability to resist drug treatment and its proven safety in solid tumor patients. Therefore, we screened monoclonal antibodies for the ability to recognize EpCAM extracellular domain and inhibit tumor growth *in vivo*. We selected two antibodies, AE7 and AE4. These antibodies are mouse IgG2 clones that specifically bind to the extracellular domain of EpCAM. Flow cytometric analysis of gated leukemia cell fractions in bone marrow single-cell suspensions derived from clinical leukemia specimens and K562/HL60 cells revealed five clones of anti-EpCAM monoclonal antibodies (AE4; AE7; 9C4, Biolegend; EBA-1, BD Biosciences; Moc-31, Abcam) that detected cell-surface EpCAM protein (Supplementary Fig. S4A and S4B). To assess established antibody specificity, cell-surface EpCAM proteins were staining by AE7 antibody and Moc-31 antibody in bone marrow biopsies of leukemia patients (Supplementary Fig. S4C). Similarly, EpCAM staining by AE7 antibody show a clear membranous staining in colon tissues and colon cancer cell lines (Supplementary Fig. S4D and S4E).

To evaluate the therapeutic effect of the selected monoclonal antibody *in vivo*, we subcutaneously implanted K562 or HL60 tumors into NOD-SCID mice, treated the mice with IgG or AE7. Treatment with AE7 significantly reduced HL60 tumor growth. Increasing the dose of AE7 revealed that a dose of 80 mg/kg caused HL60 tumor regression. After 18 days of treatment, all mice ($n = 5/5$) treated with IgG exhibited tumorigenesis, whereas 60% of the mice ($n = 3/5$) treated with 80 mg/kg AE7 were tumor free (Fig. 4A). Similar results were observed after 18 days of treatment in K562 tumors (Fig. 4C). All mice ($n = 5/5$) treated with IgG exhibited tumorigenesis, whereas 60% ($n = 3/5$) or 80% ($n = 4/5$) of the mice treated with 40 mg/kg or 80 mg/kg AE7, respectively, were tumor free. To observe whether tumor lesions were eradicated after treatment *in vivo*, we performed ultra-high field MRI and found that treatment with AE7 significantly reduced the size of primary tumor lesions and that increased AE7 administration resulted in tumor regression (Fig. 4B).

We also evaluated whether AE4 and AE7 inhibited EpCAM-overexpressing solid tumor growth and observed that AE4 and AE7 significantly reduced the growth of subcutaneously transplanted A549 tumors (Supplementary Fig. S5A). These antibodies did not induce apoptosis or inhibit cell proliferation in K562 and HL60 cells *in vitro* (Supplementary Fig. S5B and S5C).

To better assess the therapeutic effect of the selected monoclonal antibody AE7 *in vivo*, we implanted K562 or HL60 tumors into NOD-SCID mice by tail-vein injection to establish disseminated xenotransplant mouse models. One to 2 weeks later, engrafted mice were treated with IgG or AE7 and then monitored for tumorigenesis via D-luciferin-based bioluminescence. After 3 and 4 weeks of treatment, AE7 significantly inhibited HL60 micrometastasis formation and colonization in cervical lymph node, bone marrow, and peripheral blood (Fig. 4D). With increasing doses of AE7, 60% of the mice ($n = 3/5$) that were treated with 80 mg/kg AE7 exhibited no micrometastases or colonization throughout the entire body (Fig. 4D). Using the same protocol, improved mouse survival was observed following treatment with AE7 (Fig. 4E and G), and human leukemic cells (hCD45^{+}) were not detected among the PBMCs of xenotransplant mice after 4 weeks of antibody treatment (Fig. 4F and H).

These data suggested that EpCAM antibody AE7 inhibits myeloid leukemia, highlighting EpCAM and its antibody AE7 as an effective therapeutic target and potential therapeutic drug for myeloid leukemia.

EpCAM antibody AE7 eliminated myeloid leukemia cells in bone marrow xenotransplant mouse models

The bone marrow microenvironment represents the principal barrier toward the effective eradication of leukemia (4). To explore the therapeutic effect of AE7 on myeloid leukemia cells in bone marrow *in vivo*, we established intramedullary leukemia xenograft models by implanting K562 or HL60 tumors into the tibias of NOD-SCID mice. After one to two weeks of treatment, AE7 significantly suppressed tumor growth in bone marrow. Bioluminescence was quantified in K562 and HL60 engrafted mice, and significantly reduced tumors were observed in AE7-treated mice (Fig. 5A and B). To perform a more intuitive detection of myeloma lesions, we imaged muscle-stripped tibias and found that AE7 treatment inhibited myeloid leukemia progression, with 40% to 60% of antibody-treated mice being tumor free (Fig. 5C). We observed similar results by quantifying tibia bioluminescence in K562- and HL60-engrafted mice (Fig. 5C). Histologic and flow cytometric analyses of myeloid leukemia cells (hCD45^{+}) from bone marrow of representative mice from each treatment group revealed that EpCAM antibody treatment significantly cleared leukemic blasts (Fig. 5D and E).

These data suggested that EpCAM antibody AE7 disrupts the tumor-protective bone marrow microenvironment and kills intramedullary myeloid leukemia cells *in vivo*, highlighting EpCAM antibody AE7 as have the potential to clear tumor cells in bone marrow to eradicate leukemia.

EpCAM antibody AE7 exerts antileukemic effects through innate immune cells

We next examined the effector mechanism underlying leukemia cell clearance. Notably, the response to AE7 treatment was not due to direct growth inhibition or apoptosis induction in leukemia cells (Supplementary Fig. S5B and S5C). We next asked whether the cytotoxic effect of AE7 *in vivo* required immunoreaction. We first assessed whether the antitumor activity of AE7 is mediated by the recruitment of effector cells that express Fc receptors. Macrophages are key FcR-bearing

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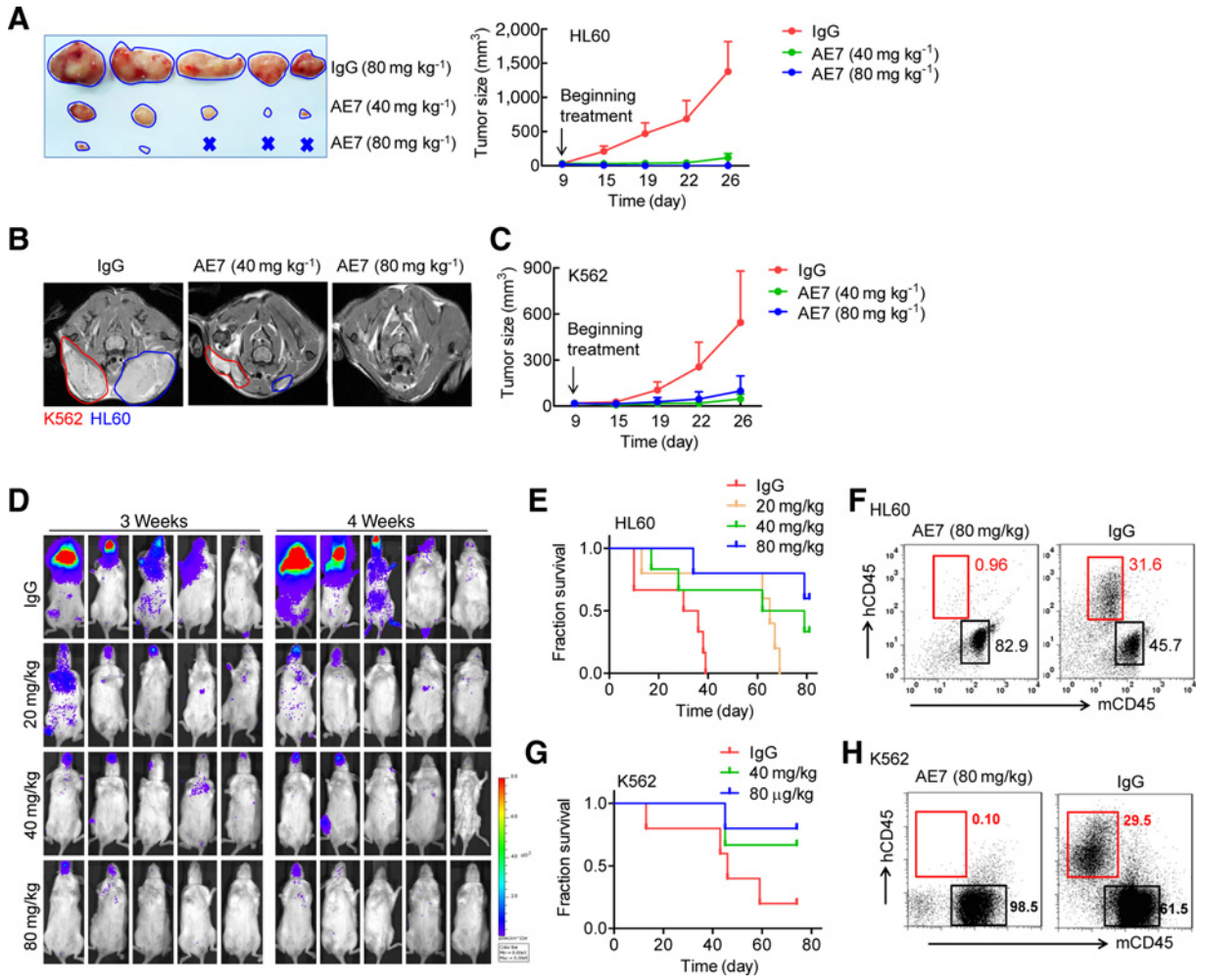

Figure 4.

EpCAM antibody AE7 eradicates myeloid leukemia in both localized and disseminated xenotransplant mouse models. **A–C,** HL60 or K562 cells were injected subcutaneously into NOD-SCID mice. When palpable tumors formed, treatment began with IgG or AE7. Isolated tumors and tumor sizes are presented (**A** and **C**). MRI scans of representative mice from each treatment group during treatment (day 24) are presented (**B**). **D–H,** NOD-SCID mice were transplanted intravenously with luciferase-expressing HL60/K5652 cells. When detectable tumors formed, treatment began with IgG or AE7. Luciferase imaging of mice 3 and 4 weeks posttreatment is presented (**D,** HL60 graft). Kaplan–Meier curves were generated to analyze survival (**E** and **G**). Flow cytometry of human myeloid leukemia cells in the PBMCs of xenotransplant mice is presented 4 weeks post-antibody treatment (**F** and **H**). The data are presented as the mean ± SEM.

effector cells that mediate antibody-directed processes *in vivo* (35, 36). Immunofluorescence analysis of F4/80-positive macrophages in NOD-SCID mice with subcutaneously implanted HL60 tumors revealed that AE7 treatment induced abundant macrophage infiltration into the tumor tissue (Fig. 6A). Observing macrophage distribution in tumor tissues from different treated mice revealed that macrophages mainly localized to the tumor edge in IgG treated mice but infiltrated intratumorally in AE7 treated mice (Fig. 6A). Similar results were also obtained in intramedullary leukemia xenograft models. After 2weeks of treatment, AE7 significantly promoted macrophage infiltration into tumors in bone marrow (Fig. 6B). Furthermore, immunofluorescence analysis showed that TUNEL staining (apoptotic cells) was increased in tumors treated with AE7 (Fig. 6C).

Here, we specifically focused on the phenotype and quantity of macrophages as these cells may represent the main effector cells for anti-EpCAM antibody treatment *in vivo*. First, flow cytometric analysis of bone marrow macrophage content was quantified, with AE7 treatment promoting a progressive increase in the concentration of $F4/80^{+}/Gr\text{-}1^{lo}$ tumor macrophages (Fig. 6D and E). Interestingly, $F4/80^{+}/Gr\text{-}1^{lo}$ spleen macrophages clearly decreased after AE7 therapy (Fig. 6E). These data suggested that EpCAM antibody treatment induced macrophage relocalization from the spleen to the tumor. To confirm macrophages as the main effector cells of anti-EpCAM antibody treatment in K562- and HL60-engrafted mice, we depleted macrophages by clodronate liposome injection prior and subsequent to EpCAM antibody AE7 treatment and found that AE7 did not inhibit the tumor growth in the macrophage-depleted group (Supplementary Fig. S6, Fig. 6F).

Macrophages employ various strategies to clear targets, including the generation of nitric oxide and reactive oxygen species (ROS) and the direct phagocytosis of targeted cells. To explore the

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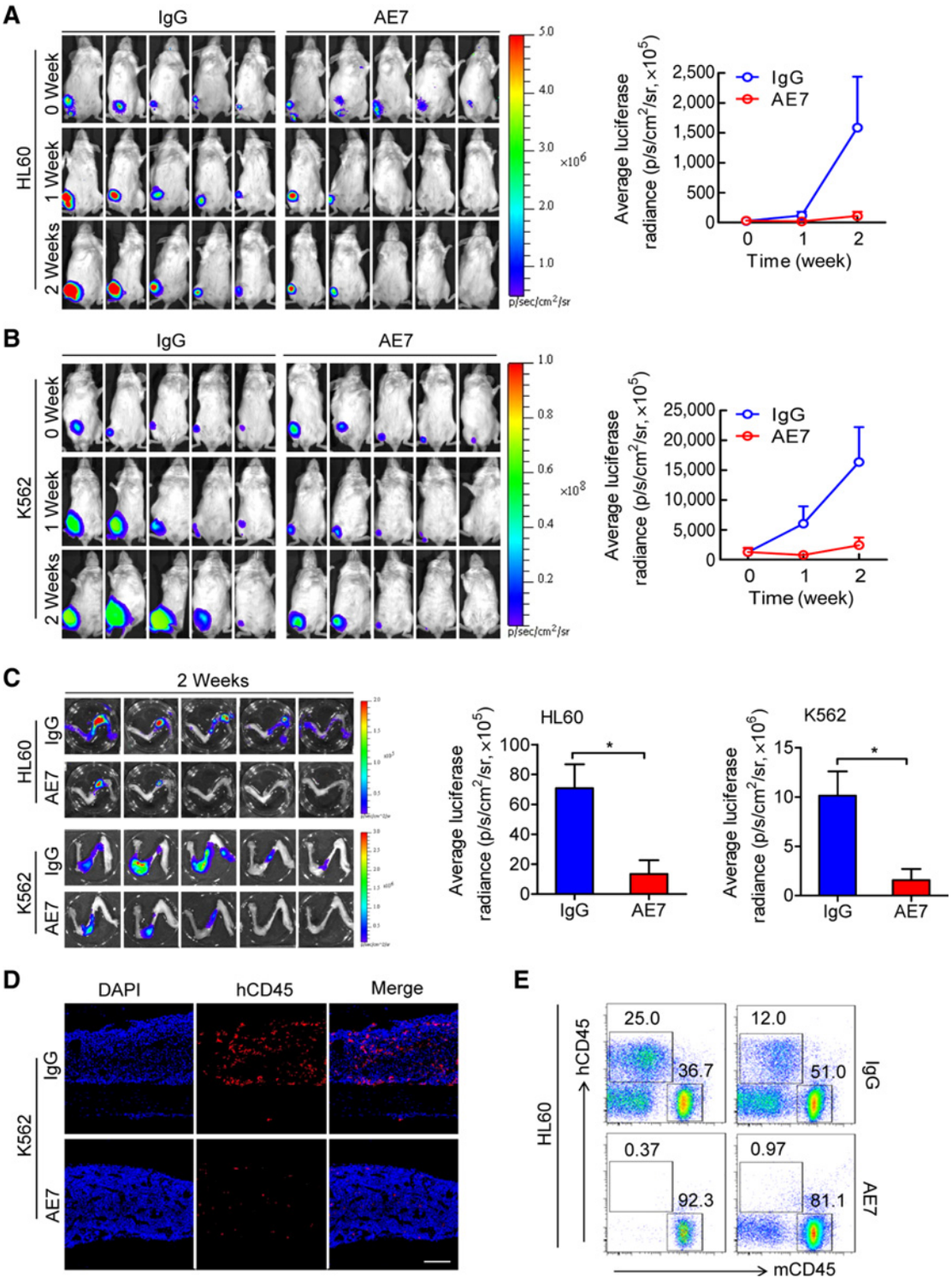

Figure 5.

EpCAM antibody AE7 eliminates myeloid leukemia cells in bone marrow xenotransplant mouse models. **A–E,** HL60 or K562 cells were injected intramedullary into NOD-SCID mice. The mice were treated with the indicated antibodies. Luciferase imaging of mice from pre- and 1 and 2 weeks posttreatment are presented and averaged for all mice in each treatment group (**A** and **B**). Luciferase imaging of isolated hind bones of injected myeloid leukemia cells are shown and averaged for all mice in each treatment group (**C**). Immunofluorescence staining for human myeloid leukemia cells (K562, anti-hCD45 antibody) in the bone marrow of representative mice from each treatment group (**D**). The cell nuclei were stained with DAPI; scale bar, 100 μm. Flow-cytometry analysis of human myeloid leukemia cells (HL60 cells, anti-hCD45 antibody) in the bone marrow of xenotransplant mice was presented 2 weeks post-antibody treatment (**E**). The data are presented as the mean ± SEM; *, $P < 0.01$.

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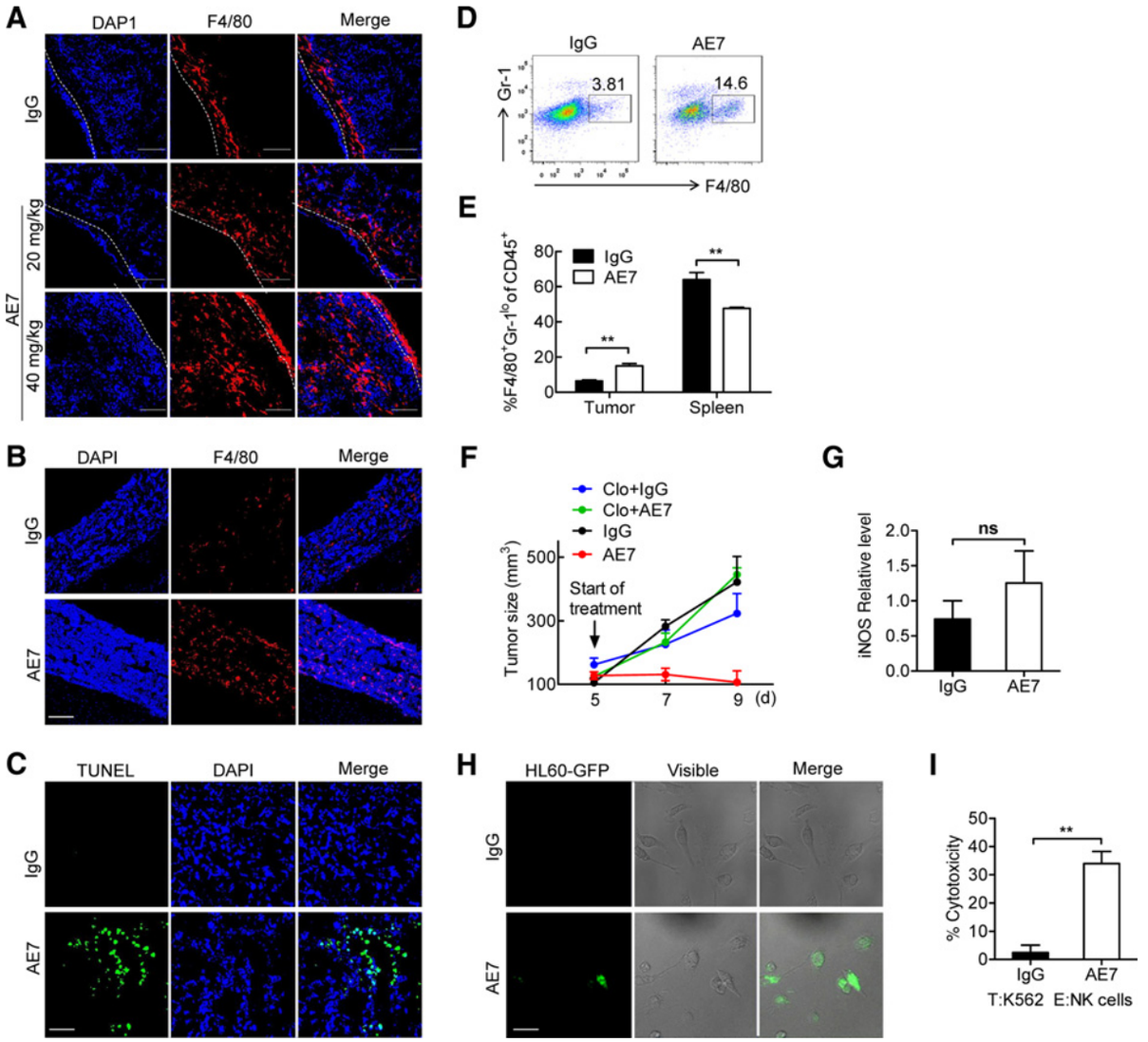

Figure 6.

EpCAM antibody AE7 exerts antileukemia effects through innate immune cells. **A, C–E,** and **G,** HL60 cells were injected subcutaneously into NOD-SCID mice. The mice were treated with IgG or AE7 for 3 weeks. Immunofluorescence staining for intratumor F4/80$^+$ macrophages (**A**) and apoptotic leukemia cells (**C**) in representative mice from each treatment group is shown. Nuclei were stained with DAPI; scale bar, 50 μm (**A** and **C**). Flow cytometry of intratumor macrophage (F4/80$^+$Gr-1^{lo}) in representative mice from each treatment group are shown (**D**) along with the percentage of F4/80$^+$Gr-1^{lo} cells among mCD45$^+$ cells from all mice (**E**). Real-time PCR of relative iNOS levels in the tumors of all mice (**G**). **B,** HL60 cells were injected intramedullary into NOD-SCID mice. The mice were treated with IgG or AE7 for 2 weeks. Immunofluorescence staining for bone marrow macrophages (F4/80$^+$) in representative mice from each treatment group is presented. Nuclei were stained with DAPI; scale bar, 100 μm. **F,** HL60 cells were injected subcutaneously into NOD-SCID mice. The mice were treated with clodronate liposomes to deplete macrophages or with PBS liposomes as a control before 1 day of IgG or AE7 therapy. Tumor size is presented. **H,** GFP-transfect HL60 cells were incubated with murine macrophages and the indicated antibodies and examined by immunofluorescence microscopy to detect phagocytosis. Non-phagocytosed HL60 cells were washed out; scale bar, 5 μm. **I,** Cytotoxicity assays of murine splenic NK cells showing K562 and AE7 antibody-dependent ADCC. ns, nonsignificant.

main mechanism of EpCAM antibody-mediated tumor cell killing by macrophages, we first tested whether AE7 treatment led to increased ROS release in tumors. We detected no significant difference between the IgG and AE7 treatment groups in leukemia xenograft mice (Fig. 6G). To confirm whether target cells were killed via direct macrophage cytophagy, we incubated leukemia cells (GFP$^+$ HL60) with adherent macrophages in the presence of AE7 or IgG. AE7–cocultured macrophages exhibited enhanced engulfment of tumor cells (Fig. 6H).

Natural killer (NK) cells are also key FcR-bearing effector cells that mediate antibody-directed processes. Given that NK cells are compromised in NOD-SCID mice, these results implicated macrophages as the main effector cells in the antitumor effect of EpCAM antibody AE7 in this model. To confirm the antitumor activity of NK cell-mediated antibody-dependent cell-mediated cytotoxicity (ADCC), we performed cytotoxicity assays and observed that AE7 treatment significantly increased the percentage of leukemia cell death (Fig. 6I).

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These data suggested that macrophages and NK cells are the effector cells that mediate the antitumor effect of EpCAM antibody AE7.

Discussion

Our study provided several insights into EpCAM as a novel therapeutic target in hematologic malignancies. First, we had identified the frequent expression of EpCAM in leukemic cells, especially from myeloid leukemia and multiple myeloma patients, but not in normal bone marrow and PBMCs. In hematologic system, exclusive expression of EpCAM in leukemic cells contributes to the safety of *in vivo* EpCAM-based potential therapeutic drugs. The abundance of tumor antigen also determined the potential of the targeting agents. Here, we confirmed the enhanced abundance of EpCAM in myeloid leukemia cells compared with previous reported tumor antigens, including CD47, CD52, CD33, NOTCH1, and CD19.

Second, we detected that EpCAM-positive cells are analogous to leukemia stem cells to promote leukemic progression. Some group confirmed EpCAM-positive hepatocellular carcinoma cells act as tumor-initiating cells with stem cell features (16, 37). According to the cancer stem cell model, AML was initiated and maintained by a small subset of self-renewing LSCs that must be eradicated so as to eliminate the tumors (38, 39). Therefore, targeting EpCAM may eliminate tumors and was beneficial to decrease relapse in human leukemia patients.

Third, we detected that EpCAM-positive leukemic cells have the enhanced chemoresistance comparing with EpCAM-negative cells, and knockdown of EpCAM promoted chemotherapeutic drug induced leukemic cells apoptosis. EpCAM-positive cells similar to tumor stem cells were the probable mechanism of which to resist chemotherapeutic agent. Furthermore, by genetic screening, we revealed the activation of WNT5B pathway was the molecular etiology by which EpCAM promotes leukemic cell drug resistance. It was well known that the Wnt/β-catenin signaling pathway contributed to self-renew of leukemia stem cells and resistance to targeted therapies in tumors (25, 40, 41). Clinically, chemotherapy was a preferred strategy for treating leukemic patients. However, Relapse and resistance were the most common reasons related to the failure of chemotherapy for treatment. As shown in Fig. 2A, we revealed the enrichment of $EpCAM^+$ leukemia subpopulations after chemotherapy. Therefore, targeting EpCAM made for the elimination of $EpCAM^+$ chemical-resistant cells and may reduce the recurrence rate of leukemic patients.

Fourth, EpCAM antibody eliminated myeloid leukemia cells in bone marrow and disseminated xenotransplant mouse models. Protective tumor microenvironment was a major resistance to therapy in malignancies, especially in leukemia (4). The bone marrow supplied a resistant niche that could block leukemia cells from therapeutic drug killing. Here, we detected that EpCAM antibody break therapy-resistant microenvironment in bone marrow, and killed tumors by raising macrophages infiltration *in vivo*.

Toxic side effects are an important factor that determines a drug's clinical application. In EpCAM antibody clinical phase, some patients suffer side effect by systemic administration, but the sever problem recently was obviously improved on the basis of the development of moderate binding affinity or high-affinity antibodies (42). In our study, no cases of sudden death, tissue lesion or adverse weight loss were observed upon treatment of NOD-SCID mice with AE7. However, to confirm the safety of the approach, further preclinical and clinical tests are necessary.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Zheng, X. Fan, Z. Tian, H. Wei
Development of methodology: X. Zheng, R. Sun
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Fan, M. Zheng, A. Zhang, K. Zhong, J. Yan, R. Sun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zheng, B. Fu, M. Zheng, K. Zhong
Writing, review, and/or revision of the manuscript: X. Zheng, B. Fu, Z. Tian, H. Wei
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zheng, X. Fan, B. Fu, R. Sun, H. Wei
Study supervision: Z. Tian, H. Wei

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EpCAM Inhibition Sensitizes Chemoresistant Leukemia to Immune Surveillance

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