Cellular & Molecular Immunology (2014) 11, 94–104

www.nature.com/cmi

RESEARCH ARTICLE

Rearrangement and expression of the immunoglobulin μ-chain gene in human myeloid cells

Jing Huang[1,2], Xiaoping Sun[3], Xiaoting Gong[1,2], Zhiqiao He[4], Lei Chen[5], Xiaoyan Qiu[1,2,4] and C Cameron Yin[4]

Immunoglobulin (Ig), a characteristic marker of B cells, has been reported to be expressed in epithelial cells, with a suggested role in their growth and survival. We have previously reported that IgG heavy chain is expressed in acute myeloid leukemia (AML), but not in the monocytes or neutrophils from patients with non-hematopoietic neoplasms or healthy controls. In the present study, we assessed IgM heavy chain expression and repertoire in human myeloid cells. We detected $V_{H\mu}DJ_{H\mu}$ rearrangement and expression in 7/7 AML cell lines, 7/14 primary myeloblasts from AML patients, and interestingly, 8/20 monocytes and 3/20 neutrophils from patients with non-hematopoietic neoplasms and healthy individuals. We also found evidence of somatic hypermutation of the variable (V) gene segments in AML-derived IgM gene rearrangements but not in IgM from monocytes or neutrophils from patients with non-hematopoietic neoplasms and healthy individuals. Furthermore, IgM $V_{H\mu}DJ_{H\mu}$ gene rearrangements in AML cell lines, primary myeloblasts, and monocytes and neutrophils from patients with non-hematopoietic neoplasms showed a restricted V usage and repertoire, whereas the $V_{H\mu}DJ_{H\mu}$ gene rearrangements in monocytes and neutrophils from healthy individuals displayed more diversity. Anti-human IgM inhibited cell proliferation, but did not induce apoptosis in AML cell lines. Our findings suggest that AML-derived IgM might be a novel AML-related molecule that is involved in leukemogenesis and AML progression and might serve as a useful molecular marker for designing targeted therapy and monitoring minimal residual disease.
Cellular & Molecular Immunology (2014) **11,** 94–104; doi:10.1038/cmi.2013.45; published online 21 October 2013

Keywords: acute myeloid leukemia; IgM; $V_{H\mu}DJ_{H\mu}$ gene rearrangements

INTRODUCTION

It is well known that immunoglobulin (Ig) plays an essential role as an antibody in blood and body fluids and is a protective protein produced by the immune system in response to foreign antigens. Although it was once presumed that B lymphocytes and plasma cells are the only sources of Ig, it has been recently reported that Ig is expressed in many types of neoplastic epithelial cells[1–6] and several types of non-hematopoietic cells from healthy individuals.[7–9] Moreover, non-hematopoietic cell-derived Ig shares some interesting features, including the restricted or biased usage of certain sequences,[9,10] a unique glycosylation profile[11,12] and novel regulatory mechanisms of gene expression.[13,14] Furthermore, non-hematopoietic cell-derived Ig has a role in cell growth and survival.[1,3,15,16] These findings suggest that Ig derived from non-hematopoietic cells might be a novel molecule with a unique structure and function and might play a role in carcinogenesis and/or tumor progression.

It has long been thought that myeloid cells and lymphocytes originate from different hematopoietic precursor cells in the bone marrow and that myeloid cells do not produce Ig. In an earlier study, we showed that IgG is rearranged, expressed and secreted in acute myeloid leukemia (AML) cells but not in monocytes or neutrophils.[15] Furthermore, AML-derived IgG variable (V) segments demonstrated somatic hypermutation and restricted or biased V region usage, and anti-human IgG inhibited cell proliferation and promoted apoptosis in AML cell lines. In the current study, we sought to assess the expression

[1]Center for Human Disease Genomics, Peking University, Beijing, China; [2]Department of Immunology, School of Basic Medical Sciences, Key laboratory of Immunology, Ministry of Health, Peking University Health Science Center, Beijing, China; [3]Department of Laboratory Medicine, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; [4]Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA and [5]Department of Pathology, The University of Texas-Medical School, Houston, TX, USA
Correspondence: Dr CC Yin, Department of Hematopathology, UT MD Anderson Cancer Center, Houston, TX 77030, USA.
E-mail: cyin@mdanderson.org
Or Dr XY Qiu, Department of Immunology, Peking University Health Science Center, Beijing 100191, China.
E-mail: qiuxy@bjmu.edu.cn
Received: 5 March 2013; Revised: 22 August 2013; Accepted: 23 August 2013

and repertoire of IgM heavy chain in human myeloid cells. We detected $V_{H\mu}DJ_{H\mu}$ rearrangement and expression in both AML cell lines and primary $CD33^+$ myeloblasts from AML patients. Interestingly, unlike IgG, IgM expression was also found in $CD33^+$ monocytes and $CD33^+$ neutrophils from patients with non-hematopoietic neoplasms and healthy individuals, with a similar frequency as that in primary myeloblasts.

In addition, IgM $V_{H\mu}DJ_{H\mu}$ gene rearrangements in AML cell lines, primary myeloblasts, and monocytes and neutrophils from patients with non-hematopoietic neoplasms exhibited a restricted V usage and repertoire, whereas the $V_{H\mu}DJ_{H\mu}$ gene rearrangements in monocytes and neutrophils from healthy individuals showed more diversity. Moreover, we found evidence of somatic hypermutation of the V gene segments in the AML-derived IgM gene rearrangements, but not in IgM derived from monocytes or neutrophils from patients with non-hematopoietic neoplasms and healthy individuals. Anti-human IgM inhibited cell proliferation, but did not induce apoptosis in AML cell lines.

These findings provide further evidence that Ig can be expressed by myeloid cells and that myeloid cell-derived $V_{H\mu}DJ_{H\mu}$ gene rearrangements may have a restricted usage pattern under certain selection pressure due to neoplastic conditions. All these results suggest that similar to AML-derived IgG, AML-derived IgM might be involved in AML pathogenesis and/or progression and might serve as a useful marker for the development of targeted therapy and the monitoring of minimal residual disease.

MATERIALS AND METHODS

Cell lines

The AML cell lines (HL-60, THP-1 and U937), the B-cell lymphoma cell line (Daudi), and the T-cell lymphoma cell line (MOLT-4) were provided by the Center for Human Disease Genomics, Peking University. The AML cell lines (KG-1, NB4, HEL and OCI-AML3) were provided by the Department of Hematopathology, MD Anderson Cancer Center. All cell lines were maintained as described previously.[15]

Patient samples

Peripheral blood samples were obtained from 14 AML patients for the assessment of membrane IgM expression on $CD33^+$ myeloblasts. In addition, peripheral blood samples were obtained from another 14 AML patients, 12 patients with non-hematopoietic neoplasms, and 8 healthy individuals to assess IgM $V_{H\mu}DJ_{H\mu}$ gene transcription in $CD33^+$ myeloblasts, monocytes and neutrophils. The clinical, laboratory and pathological data were collected by review of the medical records. The study was performed under an institutional review board-approved protocol.

Fluorescence-activated cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation, stained with monoclonal antibodies against CD19 (APC; BD Pharmingen, San Diego, CA, USA), CD33 (FITC; BD Pharmingen), and CD138 (PE; BD Pharmingen), and then sorted using a FACScan flow cytometry system (BD Pharmingen), as described previously.[15] The $CD33^+CD19^-CD138^-$ cells were selected and designated as myeloblasts. We also collected $CD33^-CD19^+$ cells and used them as a positive control.

The peripheral blood samples from patients with non-hematopoietic neoplasms and healthy individuals were stained with monoclonal antibodies against CD19 (APC), CD33 (FITC) and CD138 (PE) and sorted for monocytes and neutrophils after the lysis of red blood cells, as described previously.[15] The monocytes and neutrophils were gated using forward versus side-scatter dot plots. The $CD33^+CD19^-CD138^-$ cells in both gates were selected. We also collected $CD33^-CD19^+$ cells and used them as a positive control.

Flow cytometry immunophenotyping

To assess IgM expression on the cellular membrane of different cell lines, cultured cells were washed with PBS, blocked with 2% fetal bovine serum (FBS) at 4 °C for 30 min, and stained with monoclonal mouse anti-human IgM (μ-chain specific, 2 ng/ml; Mabworks Biotech Co. Ltd, Beijing, China) at 4 °C for 40 min. After two washes, the cells were stained with FITC-conjugated goat anti-mouse IgG (1 : 100, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4 °C for 30 min; the cells were then washed and analyzed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences, San Jose, CA, USA). To detect intracellular IgM, the cells were washed, fixed with 4% paraformaldehyde at room temperature for 30 min, washed, permeabilized with permeabilization buffer (eBioscience, San Diego, CA, USA), and centrifuged at 1500 r.p.m. at 4 °C for 5 min. The cells were then stained with the appropriate primary and secondary antibodies and analyzed as described above.

To assess IgM expression on the myeloblasts from AML patients, PBMCs were double stained with CD33-FITC and IgM-PE at 4 °C for 15 min after the lysis of red blood cells and analyzed using a FACSCalibur flow cytometer. The cells that were double stained with CD19-APC and IgM-PE were used as a positive control.

A complete immunophenotyping of the blasts was performed using multicolor flow cytometry on all patients who presented with new leukemia, as described previously.[17] The blast population was gated using right-angle side scatter and CD45 expression. The panel of monoclonal antibodies included reagents specific for CD2, CD3, cytoplasmic CD3, CD5, CD7, CD10, CD13, CD14, CD15, CD19, CD20, CD33, CD34, CD38, CD41, CD56, CD64, CD117, HLA-DR, TdT and myeloperoxidase (BD Pharmingen).

Immunocytochemistry

Cultured AML cells were centrifuged onto slides by cytospin, fixed, processed and stained with monoclonal mouse anti-human IgM (2 ng/ml) in a 37 °C humidified chamber for 1 h, as described previously.[15] The cells were then incubated with anti-mouse IgG-horseradish peroxidase (Dako, Carpinteria,

CA, USA), and the bound antibodies were detected using 3,3′-diaminobenzidine tetrahydrochloride (Dako).[15]

Immunoprecipitation and western blot analysis

To extract cytoplasmic proteins, AML cells were lysed with radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) on ice for 30 min. After centrifugation at 15 000 r.p.m. for 15 min, the lysate was incubated with monoclonal mouse anti-human IgM or mouse IgG1 (isotype control; Sigma, St Louis, MO, USA) and protein G/A beads (Abcam, Cambridge, MA, USA) for 30 min. The beads were washed, and the antigen–antibody complex was then eluted, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot analysis using goat anti-human IgM (1 : 2000; Sigma) as the primary antibody, and IRDye 800-conjugated, affinity-purified rabbit anti-goat IgG (LI-COR Biosciences, Lincoln, NE, USA) as the secondary antibody. The signals were detected using an Odyssey Infrared Imager (LI-COR Biosciences).

Analysis of IgM $V_{H\mu}DJ_{H\mu}$ transcripts by reverse transcription PCR and sequencing

Total RNA was isolated from AML cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) or from sorted $CD33^+CD19^-CD138^-$ or $CD33^-CD19^+CD138^-$ cells using the RNeasy Micro Kit (Qiagen Inc., Chatsworth, CA, USA). Reverse transcription (RT) was performed using the Sensiscript RT Kit (Qiagen Inc.). The IgM $V_{H\mu}DJ_{H\mu}$ transcripts were detected using nested PCR,[10,15,18] and the amplification products were gel-purified, cloned, and sequenced.[15] The rearranged IgM $V_{H\mu}DJ_{H\mu}$ sequences were analyzed for the V, D and J region usage and evidence of somatic hypermutation, as described previously.[15,19]

Cell proliferation assay

A cell proliferation assay was performed using the HL-60 and OCI-AML3 cell lines and a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cells were cultured in a 96-well plate at 5×10^3 cells/well in the presence of goat anti-human IgM (10 or 20 μg/ml; Sigma) or goat IgG (isotype control, 10 or 20 μg/ml; Sigma). At 24, 48, 72 and 96 h, the cell viability was assessed by adding 10 μl CCK8 solution in 100 μl RPMI-1640 media, incubating at 37 °C for 2 h, and measuring the absorbance with a 450-nm filter. This study was performed in triplicate, and the results were averaged.

Cell apoptosis assay

The HL-60 and OCI-AML3 cell lines were cultured in a 12-well plate at 1×10^5 cells/well in the presence of goat anti-human IgM (10 or 20 μg/ml; Sigma) or goat IgG (isotype control, 10 or 20 μg/ml; Sigma). At 24, 48 and 72 h, the cells were harvested, washed and resuspended in 200 μl binding buffer (10 mM HEPES (pH 7.4), 140 mM NaCl, 1 mM $MgCl_2$, 5 mM KCl and 2.5 mM $CaCl_2$). The cells were then incubated with FITC-conjugated annexin V (Beijing Biosea, Beijing, China) at room temperature for 30 min, followed by the addition of propidium iodide (1 μg/ml) and apoptosis analysis using the FACSCalibur flow cytometer.

Statistical analyses

Statistical analyses were performed using SAS 8.1 software (SAS Institute Inc., Cary, NC, USA). Differences between the groups were assessed using Student's *t*-test or the χ^2 test. A P value of <0.05 was regarded as statistically significant.

RESULTS

Patients' characteristics

We assessed membranous IgM expression on myeloblasts from 14 AML patients, the clinicopathological, cytogenetic and molecular features of whom have been previously described.[15] We analyzed IgM $V_{H\mu}DJ_{H\mu}$ gene transcription in sorted $CD33^+$ myeloblasts from another 14 AML patients, including acute myelomonocytic leukemia (AML-M4, five cases), AML with myelodysplasia-related changes (AML-MRC, three cases), AML without maturation (AML-M1, two cases), AML with maturation (AML-M2, two cases), acute promyelocytic leukemia (one case) and acute monocytic leukemia (AML-M5, one case) (Table 1). We also evaluated IgM $V_{H\mu}DJ_{H\mu}$ gene transcription in $CD33^+$ monocytes and neutrophils from 12 patients with non-hematopoietic neoplasms and 8 healthy individuals. The group of patients with non-hematopoietic neoplasms included those with colon adenocarcinoma (four cases) and one case each of stomach adenocarcinoma, hepatocellular carcinoma, pancreas adenocarcinoma, glioblastoma, high-grade sarcoma, thymoma, squamous carcinoma of the tongue and a benign thyroid nodule (Table 1).

IgM is expressed in AML cell lines

We first assessed IgM expression in AML cell lines by immunocytochemical studies and flow cytometric analyses using a mouse monoclonal antibody against human IgM (μ-chain specific). A B-cell lymphoma cell line (Daudi) was used as a positive control, and a T-cell lymphoma cell line (MOLT-4) was used as a negative control. Flow cytometric analyses revealed that IgM was expressed both on the plasma membrane and in the cytoplasm in all four AML cell lines assessed (THP-1, OCI-AML3, HL-60 and U937) and in the Daudi cell line but not in the MOLT-4 cell line (Figure 1a). Immunocytochemistry further confirmed that the IgM molecule was localized both on the plasma membrane and in the cytoplasm of these cell lines (Figure 1b). Moreover, we performed immunoprecipitation and western blot analyses and detected the presence of Ig μ-chain in these AML cell lines (Figure 1c).

IgM is expressed in primary AML cells

To demonstrate whether IgM is also produced in primary AML cells, we assessed IgM expression on PBMCs from 14 AML patients by two-color flow cytometry using monoclonal mouse anti-human IgM-PerCP/Cy5.5 and $CD33^+$-FITC antibodies. Our results revealed that IgM was expressed on the cell surface of $CD33^+$ myeloblasts in 7 of 14 AML patients, including

Table 1 Clinicopathological features of the patients with AML, non-hematopoietic neoplasms and healthy controls used in this study

	AML	*Non-hematopoietic neoplasms*	*Healthy controls*	*Reference range*
Case number	14	12	8	na
Age (years)[a]	59 (5–87)	59 (40–83)	47 (34–63)	na
Sex	6M/8F	8M/4F	2M/6F	na
WBCs ($\times 10^9$/l)[a]	23.7 (1.2–122.0)	6.2 (4.3–12.3)	7.5 (5.8–9.7)	4.0–11.0
Hgb (g/dl)[a]	10.2 (8.2–11.2) (M)	13.7 (8.5–15.2) (M)	6.2 (5.8–6.6) (M)	14.0–18.0 (M)
	9.4 (8.3–11.3) (F)	13.1 (11.4–14.0) (F)	12.7 (9.3–14.4) (F)	12.0–16.0 (F)
Platelets ($\times 10^9$/l)[a]	27 (10–87)	249 (112–356)	230 (194–368)	140–440
Blasts (%)[a]	82 (25–99)	na	na	na

Abbreviations: AML, acute myeloid leukemia; F, female; Hgb, hemoglobin; M, male; na, not available; WBCs, white blood cells.
[a] Data shown as the median (range).
These three groups of patients were assessed for the expression of IgM $V_{H\mu}DJ_{H\mu}$ transcripts after fluorescence-activated cell sorting by flow cytometry.

patients with AML-M4 (three cases), AML-M1 (two cases), AML-M5 (one case) and AML-MRC (one case; Table 2). Moreover, we observed that strong IgM staining was more frequently observed in cases of AML-M4 and AML-MRC compared to AML-M1 and AML-M2 (Figure 2).

We also performed complete immunophenotyping for these AML cases, and the blasts from all patients showed a myeloid immunophenotype. Among the seven AML cases with IgM expression, the blasts expressed CD13 (seven cases), CD33 (seven cases), CD38 (seven cases), myeloperoxidase (seven cases), HLA-DR (seven cases), CD34 (six cases), CD117 (six cases), CD14 (four cases), CD64 (four cases), CD15 (three cases), CD7 (three cases), CD56 (two cases) and CD19 (dim partial, two cases). With regard to the other seven AML cases, an immunophenotype was unavailable in two patients who were initially diagnosed with AML at outside institutions and consulted us upon relapse. The blasts were positive for CD13 (five cases), CD33 (five cases), CD38 (five cases), CD117 (five cases), myeloperoxidase (five cases), CD64 (four cases), CD34 (three cases), HLA-DR (three cases), CD14 (two cases) and CD15 (two cases).

IgM $V_{H\mu}DJ_{H\mu}$ transcripts are detected in AML cell lines, primary myeloblasts, and non-neoplastic monocytes and neutrophils

To detect rearrangements and Ig μ-chain transcription in AML cell lines, we performed RT-PCR using degenerate primers specific for the FR2 and JH regions of IgM $V_{H\mu}DJ_{H\mu}$ that can amplify nearly all IgM $V_{H\mu}DJ_{H\mu}$ rearrangements (Figure 3a). Rearranged IgM $V_{H\mu}DJ_{H\mu}$ transcripts were successfully amplified in all seven AML cell lines assessed (THP-1, OCI-AML3, HL-60, U937, HEL, KG-1, and NB4) and in the Daudi cell line (positive control), but not in the MOLT-4 cell line (negative control, Figure 3b). As expected, CD19 transcripts were only detected in the Daudi cell line and not in the AML cell lines and MOLT-4 cell line, indicating that no contamination of B cells was present in the AML cell lines utilized.

To identify rearrangements and Ig μ-chain transcription in primary AML cells, we conducted flow cytometry cell sorting of PBMCs (Figure 4a, P1) from 14 AML patients using monoclonal antibodies against CD19 (APC), CD33 (FITC) and CD138 (PE). The $CD33^+CD19^-CD138^-$ cells were isolated and were regarded as myeloblasts (Figure 4a, P4); we also isolated $CD19^+CD33^-$ cells (Figure 4a, P3) and used them as a positive control. We then conducted RT-PCR using the primers described above on the sorted $CD33^+CD19^-CD138^-$ myeloblasts. We detected IgM $V_{H\mu}DJ_{H\mu}$ transcripts in the $CD33^+CD19^-CD138^-$ myeloblasts from 7 of 14 AML patients, including AML-M4 (three cases), AML-M2 (two cases), AML-M5 (one case) and AML-MRC (one case; Table 2). Moreover, we performed immunoprecipitation and western blot analyses and detected the expression of Ig μ-chain in two cases of primary AML cells (Figure 1c).

To investigate whether IgM rearrangements and transcription also occur in adult monocytes and neutrophils, we sorted $CD33^+$ monocytes (Figure 4b, P5) and neutrophils (Figure 4b, P7) from PBMCs (Figure 4b, P1 and P2) from 12 patients with non-hematopoietic neoplasms and 8 healthy individuals. $CD19^+$ B cells were also collected and used as a positive control (Figure 4b, P6). We then conducted RT-PCR using the primers described above on the sorted $CD33^+$ adult monocytes and neutrophils. Surprisingly, IgM $V_{H\mu}DJ_{H\mu}$ transcripts were also detected in monocytes from 4 of 12 patients with non-hematopoietic neoplasms and 4 of 8 healthy individuals and in neutrophils from 1 of 12 patients with non-hematopoietic neoplasms and 2 of 8 healthy individuals (Tables 3 and 4).

IgM $V_{H\mu}DJ_{H\mu}$ rearrangements demonstrate differentially restricted or biased usage of V, D and J segments in AML cell lines, primary AML cells, and monocytes and neutrophils from patient with non-hematopoietic neoplasms, but show diversity in healthy individuals

We further analyzed the rearranged sequences of AML-derived IgM $V_{H\mu}DJ_{H\mu}$ and observed a clonal (HEL) or biclonal (THP-1, HL-60, U937, NB4 and KG-1) pattern of $V_{H\mu}DJ_{H\mu}$ rearrangements (Table 5). Interestingly, we found that the VH3 gene family, particularly VH3-23, was preferentially used in six of the seven cells lines assessed (THP-1, OCI-AML3, HL-60, U937, NB4 and KG-1). This was in contrast to the positive control cell line, Daudi, which showed a preference of the

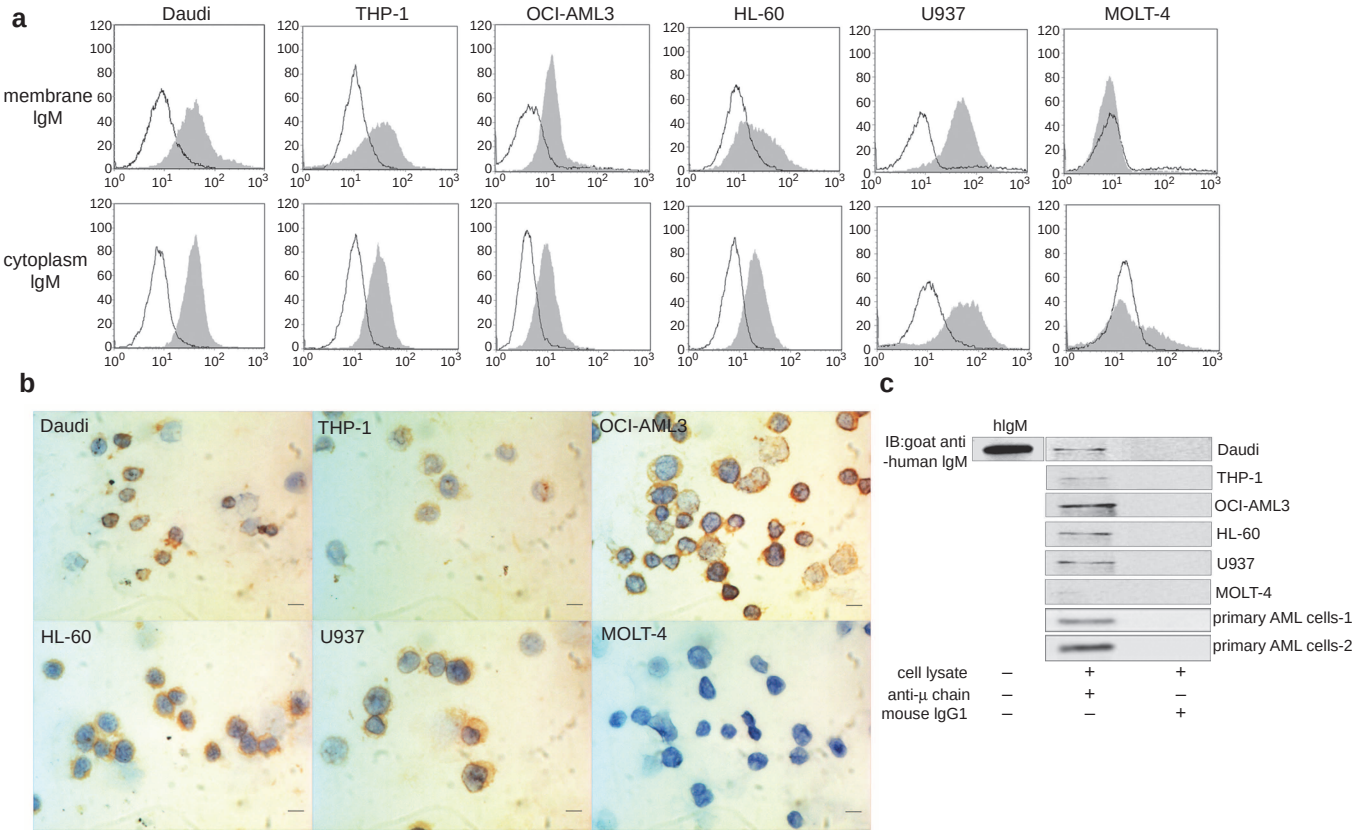

Figure 1 IgM is expressed in AML cell lines and primary AML cells. (**a**) Flow cytometry analysis showed that IgM is expressed both on the cell membrane and in the cytoplasm in all four cell lines assessed (THP-1, OCI-AML3, HL-60 and U937) and the Daudi cell line (positive control) but not in the MOLT-4 cell line (negative control). In all panels, the solid gray and white histograms represent IgM and isotype control IgG1, respectively. (**b**) An immunocytochemistry analysis demonstrates that IgM is expressed both on the cell membrane and in the cytoplasm in all four cell lines assessed (THP-1, OCI-AML3, HL-60 and U937) and the Daudi cell line (positive control), but not in the MOLT-4 cell line (negative control). (**c**) Immunoprecipitation and western blot analyses confirm that IgM is expressed in all four cell lines assessed (THP-1, OCI-AML3, HL-60 and U937), the myeloblasts from two AML patients and the Daudi cell line (positive control), but not in the MOLT-4 cell line (negative control). Monoclonal mouse anti-human Ig μ-chain was used to bind to AML-derived IgM; mouse IgG1 was used as an isotype control. Polyclonal goat anti-human Ig μ-chain was used for the western blot analysis. hIgM represents IgM purified from human serum. AML, acute myeloid leukemia; Ig, immunoglobulin.

VH7 gene family (Table 5). Furthermore, the D6 gene family, particularly D6-19 and D6-13, and the JH5 gene family were preferentially used in five of the seven cell lines assessed (THP-1, OCI-AML3, HL-60, NB4 and KG-1) (Table 5). Moreover, several identical patterns of $V_{H\mu}DJ_{H\mu}$ combinations were observed in these cell lines, with VH3-23/D6-19/JH5 being the most common, suggesting a restricted or biased usage of V, D and J segments (Table 5).

We also studied the rearranged sequences of IgM $V_{H\mu}DJ_{H\mu}$ in primary AML cells. We found the typical $V_{H\mu}DJ_{H\mu}$ recombination in the V region that usually occurs with B-cell-derived IgM, such as the inclusion of an N or P insert, and that JH gene recombination occurred primarily at the TG nucleotide sequence.[20] Similar to the occurrence in AML cell lines, the $V_{H\mu}DJ_{H\mu}$ sequences in primary AML cells demonstrated a unique monoclonal (six of seven) or oligoclonal (one of seven, with three different $V_{H\mu}DJ_{H\mu}$ rearrangements) $V_{H\mu}DJ_{H\mu}$ pattern (Table 2). However, unlike $V_{H\mu}DJ_{H\mu}$ rearrangements in AML cell lines, none of the seven patients showed an identical $V_{H\mu}DJ_{H\mu}$ pattern. When comparing these $V_{H\mu}DJ_{H\mu}$ sequences with the best-matching functional germline sequences, there was a preferential use of VH3-48 (three cases) and VH3-23 (two cases). Each of the other two cases exhibited the use of VH6-1 or VH4-61 (Table 2). Among 36 cloned $V_{H\mu}DJ_{H\mu}$ sequences from the seven AML samples, VH3-48 and VH3-23 accounted for 33.3% (12 clones) and 27.8% (10 clones) of all sequences, respectively, and VH6-1 (6 clones, 16.7%) and VH4-61 (5 clones, 13.9%) were also used at a higher frequency than was expected (Table 2). Furthermore, the D1 and D2 sequences were among the most frequently used D sequences, and the JH sequences showed preferential JH4 and JH5 usage (Table 2).

Similar to the observations in primary AML patients, the $V_{H\mu}DJ_{H\mu}$ rearrangements in the $CD33^+$ monocytes and neutrophils from patients with non-hematopoietic neoplasms exhibited a monoclonal (one of four) or biclonal (three of four) pattern. However, no identical $V_{H\mu}DJ_{H\mu}$ rearrangement was detected in the monocytes or neutrophils from different individuals (Table 3). Only one case of monocytes from a patient with gastric adenocarcinoma showed a monoclonal pattern. In

Table 2 Assignment of the best matching germline variable region genes to $V_{H\mu}DJ_{H\mu}$ recombination in sorted $CD33^+$ myeloblasts from 7 of 14 AML patients

Case no.	*AML subtype*	*$V_{H\mu}DJ_{H\mu}$ (number of clones)*
1	M1	VH3-33/D3-3/JH6 (2)
		VH4-39/D3-16/JH6 (1)
		VH3-48/D5-24/JH6 (1)
2	M2	na
3	M4	VH3-48/D1-26/JH4 (5)
4	MRC	na
5	M4	na
6	M4	na
7	MRC	VH4-61/D1-1/JH5 (5)
8	M4	VH3-23/D2-21/JH4 (5)
9	M1	VH6-1/D2-2/JH4 (6)
10	M4	VH3-48/D2-21/JH5 (6)
11	M3	na
12	M2	na
13	M5	VH3-23/D4-17/JH3 (5)
14	MRC	na

Abbreviations: AML, acute myeloid leukemia; M1, AML without maturation; M2, AML with maturation; M4, acute myelomonocytic leukemia; MRC, AML with myelodysplasia-related changes; M3, acute promyelocytic leukemia; M5, acute monocytic leukemia; na, no $V_{H\mu}DJ_{H\mu}$ rearrangement detected.

contrast, the $V_{H\mu}DJ_{H\mu}$ rearrangements from healthy individuals appear to display more diversity; again, no identical $V_{H\mu}DJ_{H\mu}$ rearrangement was detected in the monocytes or neutrophils from different individuals (Table 4). Moreover, the usage of V, D, and J segments in the healthy individuals was different from that of the primary $CD33^+$ myeloblasts from AML patients (Tables 3 and 4).

Furthermore, we assessed the mutational status of the IgM V genes from AML patients. Unexpectedly, somatic hypermutations were detected in the AML-derived IgM $V_{H\mu}DJ_{H\mu}$ genes but not in IgM derived from monocytes or neutrophils from patients with non-hematopoietic neoplasms or healthy individuals.

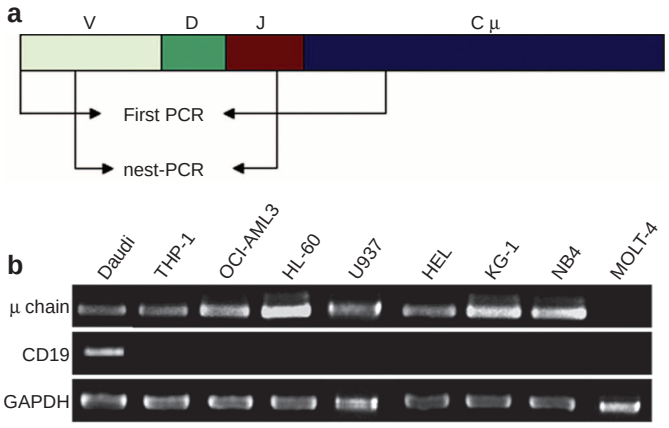

Figure 3 An RT-PCR analysis revealed that IgM is transcribed in AML cell lines. (**a**) A diagram (not to scale) illustrating the DNA segments that were analyzed in this study. The arrows indicate the locations of the primers used to amplify the V, D and J segments. (**b**) An RT-PCR analysis showing that $V_{H\mu}DJ_{H\mu}$ rearrangements were detected in all seven cell lines assessed (THP-1, OCI-AML3, HL-60, U937, HEL, KG-1 and NB4) and the Daudi cell line (positive control), but not in the MOLT-4 cell line (negative control). CD19 was co-amplified to confirm that there was no contamination of B cells in the AML cell lines. GAPDH was used as an internal control to demonstrate that the quality and quantity of mRNA in all cell lines were adequate. AML, acute myeloid leukemia; Ig, immunoglobulin; RT, reverse transcription.

Anti-human IgM inhibits cell proliferation, but does not induce apoptosis in AML cell lines

We next addressed whether AML-derived IgM was involved in cell proliferation and survival. The HL-60 and OCI-AML3 cell lines were incubated with goat anti-human IgM or goat IgG as an isotype control. We assessed cell proliferation at different time points using a CCK8 kit and apoptosis using flow cytometry after labeling with Annexin-V/propidium iodide. As shown in Figure 5, anti-human IgM slightly inhibited cell proliferation at 48, 72 and 96 h in a dose-dependent manner, but did not induce apoptosis in the HL-60 and OCI-AML3 cell

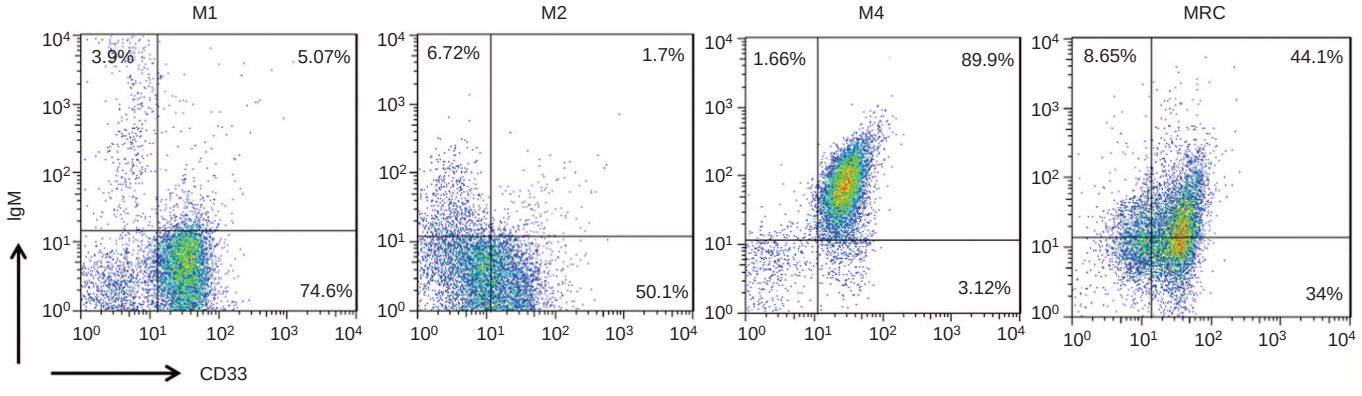

Figure 2 IgM is expressed in primary AML cells. Flow cytometry analysis of IgM expression in selected AML patients showing that IgM was more frequently expressed on the cell membrane of patients with acute myelomonocytic leukemia (M4) and AML with MRCs. Peripheral blood mononuclear cells from AML patients were isolated and stained with PerCP/Cy5.5-labeled anti-human IgM and FITC-labeled anti-human CD33. M1, AML without maturation; M2, AML with maturation. AML, acute myeloid leukemia; Ig, immunoglobulin; MRC, myelodysplasia-related change.

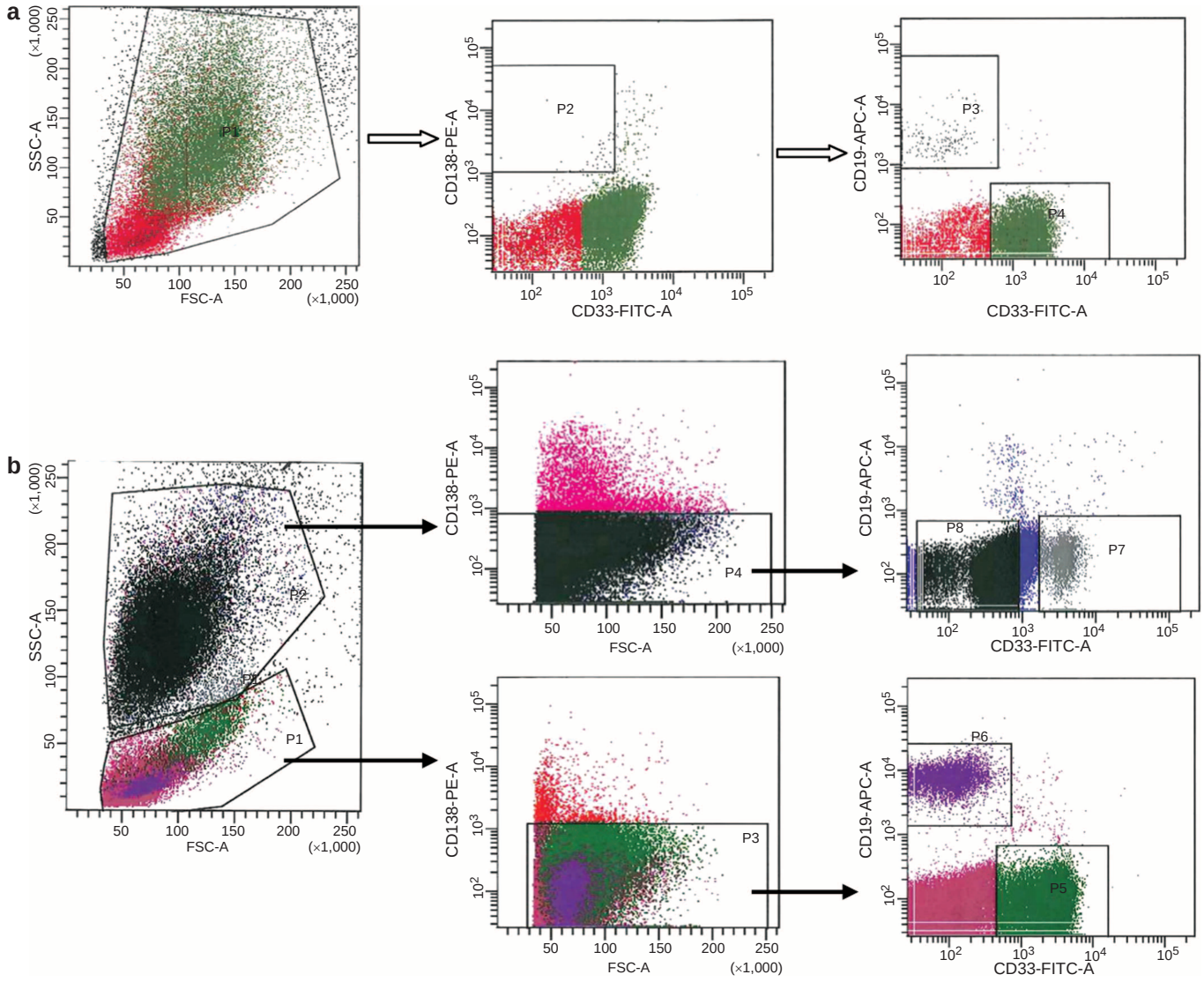

Figure 4 FACS successfully isolated $CD33^+CD19^-CD138^-$ myeloblasts from AML patients and $CD33^+$ monocytes and neutrophils from patients with non-hematopoietic neoplasms and healthy individuals. (**a**) $CD33^+CD19^-CD138^-$ myeloblasts from the peripheral blood of AML patients. P1, mononuclear cells; P2, $CD138^+$ cells; P3, $CD19^+$ cells; P4, $CD33^+CD19^-CD138^-$ myeloblasts. The cells in P4 were selected and defined as $CD33^+CD19^-CD138^-$ myeloblasts; the cells in P3 were collected and used as a positive control. (**b**) $CD33^+$ monocytes and neutrophils from the peripheral blood of patients with non-hematopoietic neoplasms or healthy controls. P1, mononuclear cells; P2, granulocytes; P3, $CD138^-$ mononuclear cells; P4, $CD138^-$ granulocytes; P5, $CD33^+CD19^-$ monocytes; P6, $CD19^+$ B cells; P7, $CD33^+$ neutrophils; P8, $CD33^-$ or dim^+ granulocytes. Cells in P5, P6 and P7 were selected. AML, acute myeloid leukemia; FACS, fluorescence-activated cell sorting.

lines, suggesting that AML-derived IgM is involved in cell proliferation but not survival.

DISCUSSION

It has been thought that Ig can only be produced by B lymphocytes and plasma cells. However, in the past decade, we and other groups have found that Ig can also be expressed in other types of cells, for instance, epithelial cells, germ cells and neurons, and in particular, many cancer cells.[1–9] More recently, we have shown that IgG heavy chain is transcribed and expressed in AML cells but not in non-neoplastic monocytes or neutrophils. Moreover, IgG V_HDJ_H rearrangements in AML cells display restricted or biased V segments and have experienced somatic hypermutation. In addition, anti-human IgG inhibits cell proliferation and induces apoptosis in AML cell lines.[15] These findings suggest a role of IgG gene expression in AML pathogenesis and progression.

In the present study, we extended our initial findings and demonstrated that IgM heavy chain is also transcribed and expressed in AML cell lines and primary myeloblasts. Surprisingly, unlike IgG, IgM was also transcribed and expressed in non-neoplastic monocytes and neutrophils from patients with non-hematopoietic neoplasms or healthy individuals. However, the observed myeloblast-derived $V_{H\mu}DJ_{H\mu}$ rearrangements displayed a unique monoclonal or oligoclonal pattern, which was different from the diversity of $V_{H\mu}DJ_{H\mu}$

Table 3 Assignment of the best matching germline variable region genes to $V_{H\mu}DJ_{H\mu}$ recombination in sorted $CD33^+$ monocytes and neutrophils from patients with non-hematopoietic disease

Caseno.	*Diagnosis*	*$V_{H\mu}DJ_{H\mu}$ (number of clones)*		
		$CD33^+$ monocytes	*$CD33^+$ neutrophils*	*$CD19^+$ B cells*
		(4/12)	*(1/12)*	*(3/3)*
1	Benign thyroid nodule	na	na	nd
2	Glioblastoma	na	na	nd
3	Hepatocellular carcinoma	na	na	VH3-21/D1-26/JH4(1) VH3-21/D3-22/JH4(3) VH3-13/D1-26/JH6(1)
4	Colon adenocarcinoma	VH3-7/D1-26/JH4(4) VH3-48/D1-26/JH4(1)	na	VH3-23/D1-26/JH4(3) VH3-30/D6-6/JH4(2)
5	Colon adenocarcinoma	na	na	nd
6	Colon adenocarcinoma	na	na	nd
7	Stomach adenocarcinoma	VH3-30/D6-13/JH4(6)	VH3-23/D6-13/JH4(4) VH3-11/D3-10/JH6(1) VH3-33/D6-19/JH4(1)	VH3-23/D3-16/JH4(2) VH3-23/D3-3/JH6(1) VH3-23/D4-17/JH6(1) VH4-59/D6-19/JH6(1) VH3-23/D6-6/JH4(1)
8	High-grade sarcoma	na	na	nd
9	Thymoma	VH3-48/D3-3/JH5(3) VH3-23/D3-3/JH6(2)	na	nd
10	Pancreatic adenocarcinoma	na	na	nd
11	Squamous cell carcinoma (tongue)	na	na	nd
12	Colon adenocarcinoma	VH4-61/D6-13/JH4(4) VH3-23/D6-13/JH4 (1)	na	nd

Abbreviations: na, no $V_{H\mu}DJ_{H\mu}$ rearrangement detected; nd, not done.

Table 4 Assignment of the best matching germline variable region genes to $V_{H\mu}DJ_{H\mu}$ recombination in sorted $CD33^+$ monocytes and neutrophils from healthy individuals

Case no.	*$V_{H\mu}DJ_{H\mu}$ (number of clones)*	
	$CD33^+$ monocytes	*$CD33^+$ neutrophils*
	(4/8)	*(2/8)*
1	na	na
2	VH4-31/D4-17/JH5 (3) VH4-39/-24/JH4 (1) VH3-23/D2-2/JH6 (1)	na
3	VH3-30/D5-5/JH5 (1) VH3-33/D5-5/JH5 (1) VH3-33/D6-13/JH6 (1) VH1-69/D6-13/JH6 (1) VH3-30/D6-13/JH6 (1)	na
4	VH3-7/D2-2/JH5 (3) VH3-23/D2-2/JH6 (2)	VH3-48/D3-16/JH4 (5)
5	VH3-21/D3-10/JH4 (2) VH3-7/D4-17/JH6 (2) VH3-7/D1-26/JH4 (1)	VH3-48/D6-6/JH4 (4) VH4-61/D6-13/JH6 (3) VH3-21/D1-26/JH4 (1)
6	na	na
7	na	na
8	na	na

Abbreviation: na, no $V_{H\mu}DJ_{H\mu}$ rearrangement detected.

rearrangements in the non-neoplastic myeloid cells from healthy individuals. Moreover, myeloblast-derived $V_{H\mu}DJ_{H\mu}$ revealed somatic hypermutation. Our preliminary functional assay showed that anti-human IgM inhibited AML cell proliferation in culture. These findings may indicate a role of IgM expression in AML pathogenesis and progression.

Table 5 Assignment of the best matching germline variable region genes to $V_{H\mu}DJ_{H\mu}$ recombination in AML cell lines

Cell line	*Total no. of clones*	*$V_{H\mu}DJ_{H\mu}$ (number of clones)*
THP-1	3	VH3-23/D6-19/JH5 (2) VH3-23/D5-24/JH4 (1)
OCI-AML3	2	VH3-23/D5-19/JH5 (1) VH3-23/D6-13/JH5 (1)
HL-60	5	VH3-23/D6-19/JH5 (4) VH3-23/D6-13/JH5 (1)
U937	5	VH3-7/D3-10/JH6 (4) VH3-23/D4-17/JH4 (1)
NB4	5	VH3-23/D6-19/JH5 (4) VH2-23/D6-25/JH5 (1)
HEL	2	VH4-61/D3-10/JH5 (2)
KG-1	3	VH3-23/D6-13/JH5 (2) VH3-23/D1-1/JH5 (1)
Daudi	5	VH7-4-4/D1-7/JH6 (5)
MOLT-4	0	na

Abbreviation: na, no $V_{H\mu}DJ_{H\mu}$ rearrangement detected.

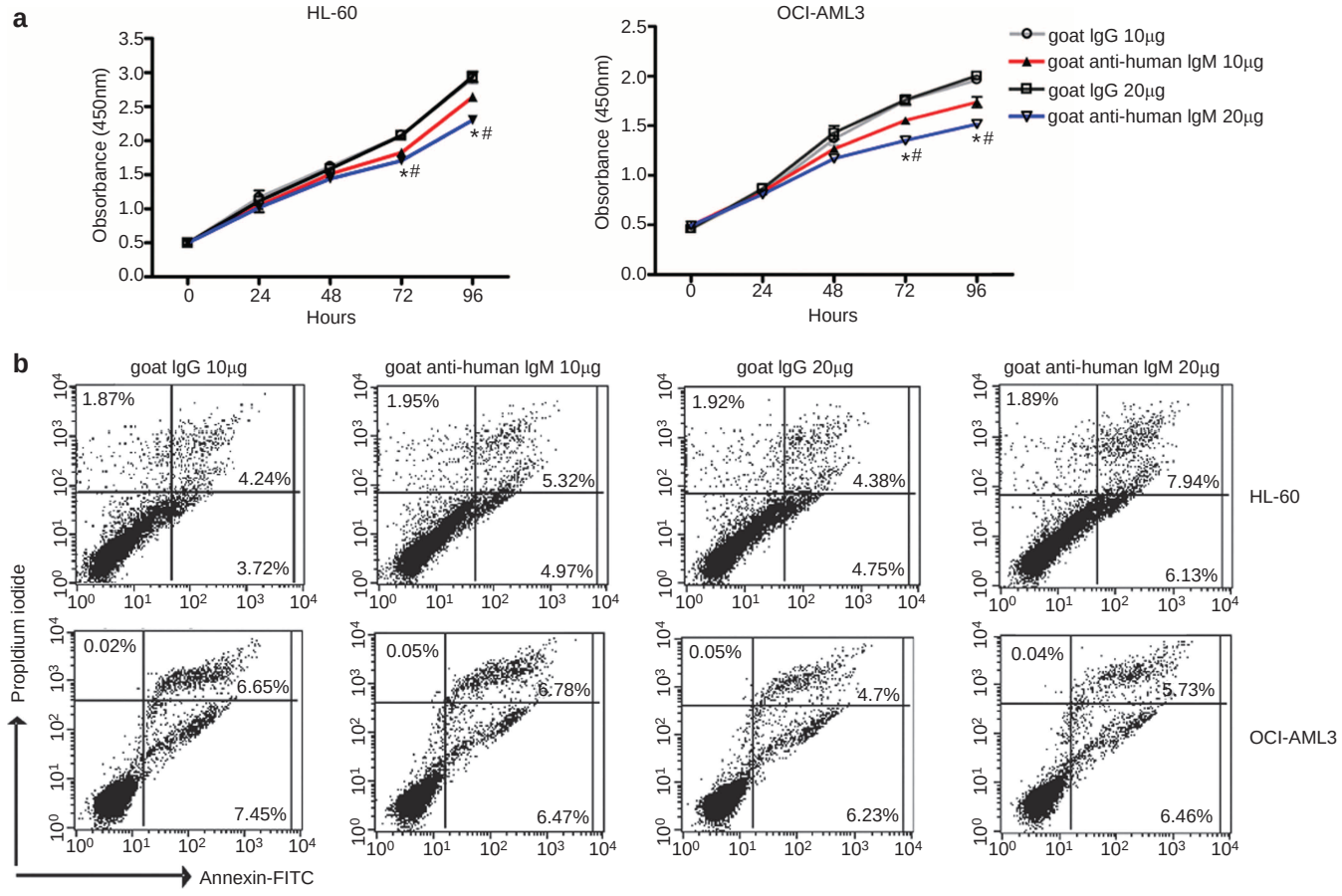

Figure 5 Anti-human IgM inhibited cell proliferation but did not induce apoptosis in AML cell lines. (**a**) Cell proliferation was assessed in the HL-60 and OCI-AML3 cell lines using a CCK-8 kit after the addition of goat anti-human IgM (10 or 20 μg/ml) or goat IgG (isotype control, 10 or 20 μg/ml) for 24, 48, 72 or 96 h. (**b**) Apoptosis was assessed in the HL-60 and OCI-AML3 cell lines using Annexin V/propidium iodide staining after the addition of goat anti-human IgM (10 or 20 μg/ml) or goat IgG (isotype control, 10 or 20 μg/ml) for 48 h. AML, acute myeloid leukemia; Ig, immunoglobulin.

Although it has been reported, albeit rarely, that the Ig gene is expressed in a small subset of AML patients,[21] it has remained unclear whether Ig is truly produced by myeloid cells or is secreted by residual B lymphocytes in these specimens. It is also unknown whether Ig is transcribed and translated or merely derived from residual B lymphocytes and simply bound non-specifically to myeloid cells. Moreover, it has not been investigated whether Ig is expressed in non-neoplastic myeloid cells, e.g., monocytes and neutrophils. To address these issues, we conducted flow cytometry cell sorting to isolate $CD33^+CD19^-CD138^-$ myeloblasts from AML patients and monocytes and neutrophils from patients with non-hematopoietic neoplasms and healthy individuals. We utilized flow cytometry immunophenotyping to confirm that there were no residual B lymphocytes and/or plasma cells present in the specimens. We then studied IgM gene rearrangement and transcription using RT-PCR with primers specific to the IgM $V_{H\mu}DJ_{H\mu}$ genes and successfully detected $V_{H\mu}DJ_{H\mu}$ transcripts in $CD33^+$ myeloblasts in 7 of 14 AML patients. Surprisingly, unlike our previous findings for IgG, IgM was also detected in $CD33^+$ monocytes and neutrophils from patients with non-hematopoietic neoplasms and healthy controls. These findings confirm that the expression of IgM in myeloid cells, as detected by flow cytometry and immunohistochemistry, is indeed produced by myeloid cells and is not due to the non-specific membranous binding of IgM that is produced and secreted by residual B lymphocytes in the specimens analyzed.

We compared the diagnostic classification between AML patients with and without $V_{H\mu}DJ_{H\mu}$ transcript expression and found that IgM was expressed at a higher frequency and level in AML with monocytic differentiation (AML-M4 or -M5). Consistently, IgM was expressed at a higher frequency in monocytes (4 of 12 patients with non-hematopoietic neoplasms, 4 of 8 healthy controls) than neutrophils (1 of 12 patients with non-hematopoietic neoplasms, 2 of 8 healthy controls). Although the number of cases in our study was small, these findings suggest that monocytes may be able to express IgM more frequently than neutrophils under certain physiological and pathological conditions. Moreover, unlike epithelial cancer cell-derived IgG, which tends to be expressed in cancer cells with higher frequency than that in normal cells,[10] we did not observe a significant difference in the frequency of IgM expression between primary AML cells (50%) and non-neoplastic monocytes (50%) in healthy individuals. It was

noted that two of the seven patients who exhibited IgM expression showed dim partial CD19 expression; however, a complete flow cytometry immunophenotyping proved that the blasts were of myeloid lineage rather than mixed myeloid and B-cell lineage. We also compared the clinical outcome between AML patients with and without IgM expression and did not observe any significant differences. Regardless, the number of cases in this study may be too small to reach any definitive conclusions.

We have previously reported that epithelial cancer cell-derived $V_{H\mu}DJ_{H\mu}$ sequences display high conservation among different tumor types and different individuals.[10] Several sets of $V_{H\mu}DJ_{H\mu}$ rearrangements, such as VH3-33 and VH6-1 (not VH3-23 and VH3-48, which were used more often in the AML cells in this study), have been reported to be frequently expressed among different types of epithelial cancer cells, sometimes with 100% homology.[10] These data suggest that epithelial cancer cell-derived IgM has a similar function among different types of cancer cells. However, although myeloid cell-derived $V_{H\mu}DJ_{H\mu}$ rearrangements showed an oligoclonal or monoclonal pattern in each AML patient, no conservative usage was observed among the AML-derived $V_{H\mu}DJ_{H\mu}$ rearrangements. These findings suggest that the structure and/or function of AML-derived IgM may be unique in each individual, a fact that may prove to be useful for implementing this molecule as a marker for monitoring minimal residual disease after chemotherapy.

With 22 functional genes, the VH3 gene family is the largest of the seven families (VH1 to VH7) and comprises approximately one-half of the expressed VH repertoires in adult peripheral B cells.[22] Similar to B cells, among a total of 115 $V_{H\mu}DJ_{H\mu}$ sequences derived from myeloid cells (25 in AML cell lines, 36 in AML patients, 27 in patients with non-hematopoietic neoplasms and 27 in healthy controls; Tables 2–5), the VH3 gene family was used most frequently (90 clones, 78.2%), particularly VH3-23 (43 clones, 37.4%) and VH3-48 (16 clones, 13.9%). It has been reported that VH3-23 is a promiscuous VH gene segment that contributes to many antigenic specificities in B cells. VH3-23 is also frequently expressed during the fetal development of antibody repertoires.[23] In a single-cell PCR analysis of peripheral blood lymphocytes from a normal adult, VH3-23 was found in approximately 17% of all functional rearrangements,[24] and VH3-48 has been reported as an unusual gene in circulating B-cell repertoires with a high homology to VH3-23.[25] Our results also revealed that VH3-23 accounted for 47.5% (29 of 61 clones; Tables 2 and 5) of all rearrangements in AML cell lines and primary AML cells, which was higher than that in patients with non-hematopoietic neoplasms (26.0%, 7 of 27 clones) and healthy individuals (26.0%, 7 of 27 clones). Furthermore, although our results showed an over-representation of VH3-23 and VH3-48 similar to that in B cells, it was surprising that VH4-61 and VH6-1 occurred at a much higher frequency than expected when compared to $CD19^+$ B-cell-derived VH. Furthermore, there was preferential usage of JH4 and JH5 that was also remarkably higher than expected compared to $CD19^+$ B cell-derived JH. Such preferential usage of certain V and J gene segments in myeloid cell-derived $V_{H\mu}DJ_{H\mu}$, combined with our findings that epithelial cell-derived IgM displays natural antibody activity (unpublished data), suggest that myeloid cell-derived IgM may have a unique function, such as involvement in innate immunity as a natural antibody.

In summary, we demonstrated that IgM is transcribed and expressed in AML cell lines and primary myeloblasts and in the monocytes and neutrophils from patients with non-hematopoietic neoplasms and healthy controls. Anti-human IgM inhibits AML cell proliferation in culture. Thus, AML-derived IgM might play a role in AML pathogenesis and progression and might serve as a useful marker for monitoring minimal residual AML and as a potential target for AML therapy.

AUTHOR CONTRIBUTIONS

JH and XQ designed and performed most of the experiments and wrote the manuscript. XS, XG, ZH and LC performed some of the experiments. CCY and XQ conceptualized the study and edited the manuscript.

ACKNOWLEDGEMENTS

This work was supported by a research fund awarded to C C Yin from The University of Texas MD Anderson Cancer Center (#18079170) and a research fund awarded to X Qiu from National Natural Sciences Foundation of China (#81320108020). We thank Markeda Wade from Scientific Publication at The University of Texas MD Anderson Cancer Center for editorial review of the manuscript.

1 Qiu X, Zhu X, Zhang L, Mao Y, Zhang J, Hao P *et al*. Human epithelial cancers secrete immunoglobulin G with unidentified specificity to promote growth and survival of tumor cells. *Cancer Res* 2003; **63**: 6488–6495.
2 Li M, Feng DY, Ren W, Zheng L, Zheng H, Tang M *et al*. Expression of immunoglobulin kappa light chain constant region in abnormal human cervical epithelial cells. *Int J Biochem Cell Biol* 2004; **36**: 2250–2257.
3 Babbage G, Ottensmeier CH, Blaydes J, Stevenson FK, Sahota SS. Immunoglobulin heavy chain locus events and expression of activation-induced cytidine deaminase in epithelial breast cancer cell lines. *Cancer Res* 2006; **66**: 3996–4000.
4 Zheng H, Li M, Ren W, Zeng L, Liu HD, Hu D *et al*. Expression and secretion of immunoglobulin alpha heavy chain with diverse VDJ recombinations by human epithelial cancer cells. *Mol Immunol* 2007; **44**: 2221–2227.
5 Chen Z, Gu J. Immunoglobulin G expression in carcinomas and cancer cell lines. *FASEB J* 2007; **21**: 2931–2938.
6 Zhu X, Li C, Sun X, Mao Y, Li G, Liu X *et al*. Immunoglobulin mRNA and protein expression in human oral epithelial tumor cells. *Appl Immunohistochem Mol Morphol* 2008; **16**: 232–238.
7 Huang J, Sun X, Mao Y, Zhu X, Zhang P, Zhang L *et al*. Expression of immunoglobulin gene with classical V–(D)–J rearrangement in mouse brain neurons. *Int J Biochem Cell Biol* 2008; **40**: 1604–1615.
8 Huang J, Zhang L, Ma T, Zhang P, Qiu X. Expression of immunoglobulin gene with classical V–(D)–J rearrangement in mouse testis and epididymis. *J Histochem Cytochem* 2009; **57**: 339–349.
9 Zhang S, Mao Y, Huang J, Ma T, Zhang L, Zhu X *et al*. Immunoglobulin gene locus events in epithelial cells of lactating mouse mammary glands. *Cell Mol Life Sci* 2010; **67**: 985–994.

10 Zheng J, Huang J, Mao Y, Liu S, Sun X, Zhu X *et al*. Immunoglobulin gene transcripts have distinct VHDJH recombination characteristics in human epithelial cancer cells. *J Biol Chem* 2009; **284**: 13610–13619.
11 Lee G, Cancer cell-expressed immunoglobulins: CA215 as a pan cancer marker and its diagnostic applications. *Cancer Biomark*2009; **5**: 137–142.
12 Lee G, Ge B. Cancer cell expressions of immunoglbulin heavy chain with unique carbohycrate-associated biomarker. *Cancer Biomark* 2009; **5**: 177–188.
13 Liu H, Zheng H, Duan Z, Hu D, Li M, Liu S *et al*. LMP1-augmented kappa intron enhancer activity contributes to upregulation expression of Ig kappa light chain *via* NF-kappa β and AP-1 pathways in nasopharyngeal carcinoma cells. *Mol Cancer* 2009; **27**: 92–108.
14 Zhu X, Wu L, Zhang L, Hao P, Zhang S, Huang J *et al*. Distinct regulatory mechanism of immunoglobulin gene transcription in epithelial cancer cells. *Cell Mol Immunol* 2010; **7**: 279–286.
15 Qiu X, Sun X, He Z, Huang J, Hu F, Chen L *et al*. Immunoglobulin gamma heavy chain gene with somatic hypermutation is frequently expressed in acute myeloid leukemia. *Leukemia* 2013; **27**: 92–99.
16 Li M, Zheng H, Duan Z, Liu U, Hu D, Bode A *et al*. Promotion of cell proliferation and inhibition of ADCC by cancerous immunoglobulin expression in cancer cell lines. *Cell Mol Immunol* 2012; **9**: 54–61.
17 Yin CC, Lin P, Carney DA, Handy BC, Rassidakis GZ, Admirand JH *et al*. Chronic lymphocytic leukemia/small lymphocytic lymphoma associated with IgM paraprotein. *Am J Clin Pathol* 2005; **123**: 594–602.
18 Yin CC, Medeiros LJ, Cromwell CC, Mehta AP, Lin P, Luthra R *et al*. Sequence analysis proves clonal identity in five patients with typical and blastoid mantle cell lymphoma. *Mod Pathol* 2007; **20**: 1–7.
19 Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL *et al*. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J Clin Invest* 1998; **102**: 1515–1525.
20 Dorner T, Brezinschek HP, Brezinschek RI, Foster SJ, Domiati-Saad R, Lipsky PE. Analysis of the frequency and pattern of somatic mutations within nonproductively rearranged human variable heavy chain genes. *J Immunol* 1997; **158**: 2779–2789.
21 Ha K, Minden M, Hozumi N, Gelfand EW. Immunoglobulin gene rearrangement in acute myelogenous leukemia. *Cancer Res* 1984; **44**: 4658–4660.
22 Matsuda F, Ishii K, Bourvagnet P, Kuma K, Hayashida H, Miyata T *et al*. The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. *J Exp Med* 1998; **188**: 2151–2162.
23 Schroeder HW, Hillson JL, Perlmutter RM. Early restriction of the human antibody repertoire. *Science* 1987; **238**: 791–793.
24 Brezinschek HP, Brezinschek RI, Lipsky PE. Analysis of the heavy chain repertoire of human peripheral B cells using single-cell polymerase chain reaction. *J Immunol* 1995; **155**: 190–202.
25 Glanville J, Zhai W, Berka J, Telman D, Huerta G, Mehta GR *et al*. Precise determination of the diversity of a combinatorial antibody library gives insight into the human immunoglobulin repertoire. *Proc Natl Acad Sci USA* 2009; **106**: 20216–20221.