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Early Hematopoietic Zinc Finger Protein Prevents Tumor Cell Recognition by Natural Killer Cells[1]

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Early hematopoietic zinc finger/zinc finger protein 521 (EHZF/ZNF521) is a novel zinc finger protein expressed in hematopoietic stem and progenitor cells and is down-regulated during their differentiation. Its transcript is also abundant in some hematopoietic malignancies. Analysis of the changes in the antigenic profile of cells transfected with EHZF cDNA revealed up-regulation of HLA class I cell surface expression. This phenotypic change was associated with an increased level of HLA class I H chain, in absence of detectable changes in the expression of other Ag-processing machinery components. Enhanced resistance of target cells to NK cell-mediated cytotoxicity was induced by enforced expression of EHZF in the cervical carcinoma cell line HeLa and in the B lymphoblastoid cell line IM9. Preincubation of transfected cells with HLA class I Ag-specific mAb restored target cell susceptibility to NK cell-mediated lysis, indicating a specific role for HLA class I Ag up-regulation in the NK resistance induced by EHZF. A potential clinical significance of these findings is further suggested by the inverse correlation between EHZF and MHC class I expression levels, and autologous NK susceptibility of freshly explanted multiple myeloma cells. ***The Journal of Immunology,*** **2009, 182: 4529–4537.**

The early hematopoietic zinc finger (EHZF)[4] zinc finger protein 521 (EHZF/ZNF521) was identified in a comparative analysis of the transcriptional profile of human CD34$^+$ hematopoietic progenitors and mature peripheral blood leukocytes (1). EHZF is highly expressed in human stem and progenitors cells and is down-regulated during their differentiation (see references in Refs. 1, 2). EHZF inhibits the activity of early B cell factor, a transcription factor essential for specification of the B cell lineage. EHZF is likely to play a relevant role in the control of human hematopoiesis (1) and is frequently expressed in hematopoietic malignant cells. Interestingly high levels of EHZF transcripts have been found in over 50% of acute myelogenous leukemia cases, but in only 2–5% of the B cell acute lymphoblastic leukemia (ALL) cases analyzed (1, 2). The deregulation of EHZF expression or function in leukemic cells may play an important role in their in vivo growth or survival because Mullighan et al. (3) have recently described a translocation resulting in the fusion of the *PAX5* gene with *EHZF/ZNF521* gene in one case of B cell-progenitor ALL.

NK cells recognize hematological tumors, e.g., acute myeloid leukemic and multiple myeloma (MM) cells (4–7) as well as normal B cells of which they have been reported to regulate activation and differentiation (8). Other hematopoietic-derived cells like dendritic cells can stimulate NK cells (9). The B cell membrane-associated proteins CD40 and CD1 regulate NK cell cytotoxicity (10–12). Furthermore, NK cells are specifically activated after bone marrow grafting but not after grafting of other tissues (13). NK cells localize in lymph nodes and spleen, mainly in B cell follicles and in the marginal zone (14). Blood, spleen, and bone marrow are the anatomical districts where the highest number and activity of NK cells are present.

NK cells are cytotoxic and cytokine-producing lymphocytes, which play a role in the immune defense against viral infections and tumors (15). Their homeostasis is regulated by cytokines and membrane associated receptors able to inhibit or activate cellular programs (16, 17). The MHC class I recognizing inhibitory receptors are well characterized, as extensively reviewed elsewhere (18). Triggering of NK cells depends largely on NKG2D, the NK cell receptor group 2 member D of the lectin like receptor family, and natural cytotoxicity receptors NKp30, NKp44, and NKp46. Natural cytotoxicity receptors are involved in the recognition of cells, although their ligands remain elusive (19, 20). NKG2D recognizes the MHC class I chain-related (MIC) protein A (MICA) and MICB; both are nonclassical MHC class I molecules (21, 22).

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[4] Abbreviations used in this paper: EHZF, early hematopoietic zinc finger; ALL, acute lymphoblastic leukemia; APM, Ag-processing machinery; MM, multiple myeloma; MICA, MHC class I chain-related protein A; MICB, MHC class I chain-related protein B; ULBP, UL16-binding protein; shRNA, short hairpin RNA; β_2M, β_2-microglobulin; MFI, mean fluorescence intensity; EGFP, enhanced GFP.

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MIC proteins are expressed during virus infection or cell transformation. The UL16-binding proteins (ULBP)1–3 (or RAE-1 proteins) are the second group of NKG2D ligands in humans (23); DNAM-1 is a recently defined main NK cell-activating receptor recognizing molecules involved in cell adhesion (24).

In preliminary studies aimed at identifying changes in cell surface antigenic profile induced by EHZF/ZNF521, we observed a significant up-regulation of HLA class I expression. Because HLA class I Ags are known to inhibit NK cell activation, in this study we have investigated whether NK cell-tumor cell interactions could be affected by EHZF expression. Our results demonstrate that enforced expression of EHZF results in inhibition of NK recognition in hematopoietic and nonhematopoietic cell lines, and that expression of EHZF in primary MM cells is associated to high HLA class I expression and low susceptibility to NK-mediated lysis.

Materials and Methods

Cells

The cervical carcinoma cell line HeLa, the human embryonic kidney cell line 293T, and the monkey kidney cell line COS-7 were maintained in DMEM (Invitrogen). The B lymphoblastoid cell line IM9 derived from a MM patient was maintained in RPMI 1640 medium. All cell lines were grown at 37°C in a humidified 5% CO_2 atmosphere, in medium containing 10% FBS, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Invitrogen).

NK cells were isolated from PBMC, obtained from healthy donors' buffy coats by Ficoll-Paque (Biochrom) density gradient centrifugation, using the NK Cell Isolation kit and VarioMACS for the depletion of non-NK cells (Miltenyi Biotec) according to the manufacturer's recommendations. NK cell purity was at least 95%. NK cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, 3% human serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Patient recruitment

Six patients with cytologic and histologic diagnosis of MM were investigated in this study. The patients were treated at the Hematology Clinic of the University of Naples "Federico II" Medical School. All patients gave informed consent, according to the ethical regulations of our institutions and the Declaration of Helsinki. Bone marrow-derived $CD38^+CD138^+$ plasma cells were detected by direct immunofluorescence and FACS analysis using the anti-CD38 FITC or anti-CD138 PE directly conjugated mAbs (clones HB-7 and BB-4; BD Biosciences) gating on $CD38^{bright}$ or on $CD38^+/CD138^+$ cells. The immunophenotype of myeloma cells was obtained by double staining of either $CD38^{bright}$ or $CD38^+/CD138^+$ cells with the appropriate mAbs. To normalize the number of myeloma cells among the different patients, mononuclear cells were cultured for 48 h in the presence of human recombinant IL-6 (10 ng/ml; PeproTech). The IL-6 treatment yielded a specific $CD38^+CD138^+$ tumor cell proliferation; therefore, in each ex vivo cytotoxicity experiment $CD38^+CD138^+$ myeloma target cells were always 95–100% of the labeled target cells. Myeloma cells were purified with immunomagnetic beads using the CD138 purification kit (Miltenyi Biotec).

Plasmid construction

The EHZF/ZNF521 full-length sequence, contained in the p3xFlag-CMV7.1 expression vector (1), was subcloned by restriction and ligation in frame with expression of GFP (EGFP) in the pEGFPN1 vector to obtain the fusion protein Flag-EHZF-EGFP. The lentiviral vector FUGW, where EGFP is driven by the ubiquitin C promoter, was provided by Dr. D. Baltimore (CIT, Pasadena, CA). The vector was modified by replacing the cDNA encoding EGFP with a fragment comprising the multiple cloning sites, the internal ribosomal entry sequence, and the cDNA encoding EGFP was excised from the plasmid pIRES2-EGFP (BD Biosciences). In the resulting vector, termed FUIGW, the ubiquitin C promoter directs the transcription of a bicistronic mRNA thereby allowing the simultaneous expression of the transgene and of EGFP. The cDNA encoding 3xFLAG-EHZF was subcloned in the *Xba*I and *Bam*HI restriction sites of this plasmid, to generate the lentiviral vector designated FUIGW-EHZF.

To silence the endogenous *EHZF* gene, small interfering RNAs complementary to diverse target sequences within the EHZF mRNA were designed and validated in cotransfection assays (data not shown). Among the small interfering RNAs tested, one (H11: GCUAAAGAUAGUAAUG CATT) was selected to design an oligonucleotide encoding the corresponding short hairpin RNA (shRNA) that was inserted in the pSUPER shRNA expression vector (OligoEngine) according to the manufacturer's instructions. The RNA interference cassette including the H1 promoter and the shRNA sequence was subsequently excised from the resulting vector, designated pSuper-H11, and inserted in the FG-12 lentiviral vector (25), provided by Dr. D. Baltimore (CIT, Pasadena, CA), that carries the EGFP cDNA under the transcriptional control of the ubiquitin C promoter. The vector thus obtained, FG-12-H11, was used as an alternative to pSuper to knockdown *EHZF* expression in some of the experiments described below.

Transfection and transduction of cells

Transfection of HeLa cells (1.0×10^7 cells) was conducted using the calcium phosphate precipitation method; the total amount of transfected DNA was 10 μg per 100-mm plate, and an empty plasmid was cotransfected to achieve this amount. 293T cells ($3–5 \times 10^6$ cells) were transfected with a pEGFP-C1 plasmid (BD Biosciences) as an internal control and either void vector pSUPER or pSUPER-H11 were used.

For transductions, lentiviruses were generated by transient cotransfection of the packaging cell line 293T with 13 μg of Flag-EHZF-FUIGW or FG-12-H11, 12 μg of pCMV-VSVG, and 18 μg of pCMV-deltaR8–91 vectors, by calcium phosphate precipitation. Following 48 h of incubation at 37°C, the 293T cell supernatant was collected, filtered through a 0.45-μm filter, and applied to the target cells (5×10^5 cells) in the presence of polybrene (8 μg/ml) at 12-h intervals for three consecutive transduction rounds. The efficiency of the transduction was estimated by flow cytometry based on the expression of the GFP, using a FACSCalibur (BD Biosciences). The expression of Flag-EHZF was shown by Western blotting using the M2 anti-Flag-HRP mAb (Sigma-Aldrich). Expression of GFP-positive single clones was isolated by methylcellulose cloning.

Flow cytometry

In the indirect staining experiments, cells were incubated with the appropriate mAb followed by PE-conjugated goat anti-mouse IgG Abs (Jackson ImmunoResearch Laboratories). In all experiments, as a first step, cells were preincubated with human serum for 15 min, and isotype-matched controls were used to set up the negative values. Samples were analyzed by FACSCalibur flow cytometer (BD Biosciences). For the indirect immunofluorescence assays, the following Abs were used for surface expression staining of glycoproteins: the mouse mAbs W6/32, directed against β_2-microglobulin (β_2M)-associated HLA-A, HLA-B, HLA-C, HLA-E, and HLA-G H chains (Cymbus Biotechnology); mAb HCA-2, which recognizes β_2M-free HLA-A (excluding H-A24), HLA-B7301, and HLA-G H chains, the ICAM-1-specific mAb VF 27-516.1, the MICA-specific mAb BAM 195 (26), the MICA/B-specific mAb 6D4, and the ULBP-specific mAbs are ULBP1 mAb M295, ULBP2 mAb M310, ULBP3 mAb M550, and ULBP4 mAb M478, which was a gift from D. Cosman (Amgen). The PVR-specific mAb L95 and Nectin-2-specific mAb L14 were developed and characterized (27). As isotypic control in masking experiments mAb TIB200 specific for CD57 was used. R-PE- and FITC-conjugated goat anti-mouse IgG Abs were purchased from Jackson ImmunoResearch Laboratories and Sigma-Aldrich, respectively.

For intracellular staining cells were fixed in Cytofix and permeabilized by Cytoperm (BD Biosciences). They were then stained with the specific mAb followed by PE-conjugated goat anti-mouse IgG Abs (Jackson ImmunoResearch Laboratories) and analyzed by FACS. The acquired cytofluorographic data were analyzed using CellQuest software (BD Biosciences). The following Abs were used for intracytoplasmic staining and were developed and characterized (28): NAMB-1 (β_2M) (29), HC-10 (HLA class I H chain) (30, 31), NOB1 (TAP1) and NOB2 (TAP2) (32), TO-2 (ERp57) (33), TO-3 (tapasin) (33), TO-5 (calnexin) (33), TO-11 (calreticulin) (33), SY-1 (LMP2) (34), HB2 (LMP7) (34), and TO-6 (LMP10) (34). The acquired cytofluorographic data were analyzed using CellQuest software (BD Biosciences).

Cytotoxicity assay

Cytotoxicity assays were performed either using the fluorescent CFDA (carboxyfluorescein diacetate; Molecular Probes) NK assay or the classical ^{51}Cr release assay. In CFDA assay, cytotoxicity was analyzed by flow cytometry using the protocol described by McGinnes et al. (35). Briefly, target cells were labeled with CFDA. Target cells were mixed with effector cells at different E:T ratios in 96-well plates. Following 3 h of incubation at 37°C in a humidified 5% CO_2, cells were analyzed by flow cytometry using FACSCalibur (BD Biosciences). The percentage of specific lysis of target cells was calculated using the formula $(CT - TE)/(CT) \times 100$, where *CT* is mean number of fluorescent target cells in control tubes and *TE* is mean number of fluorescent cells in target plus effector tubes.

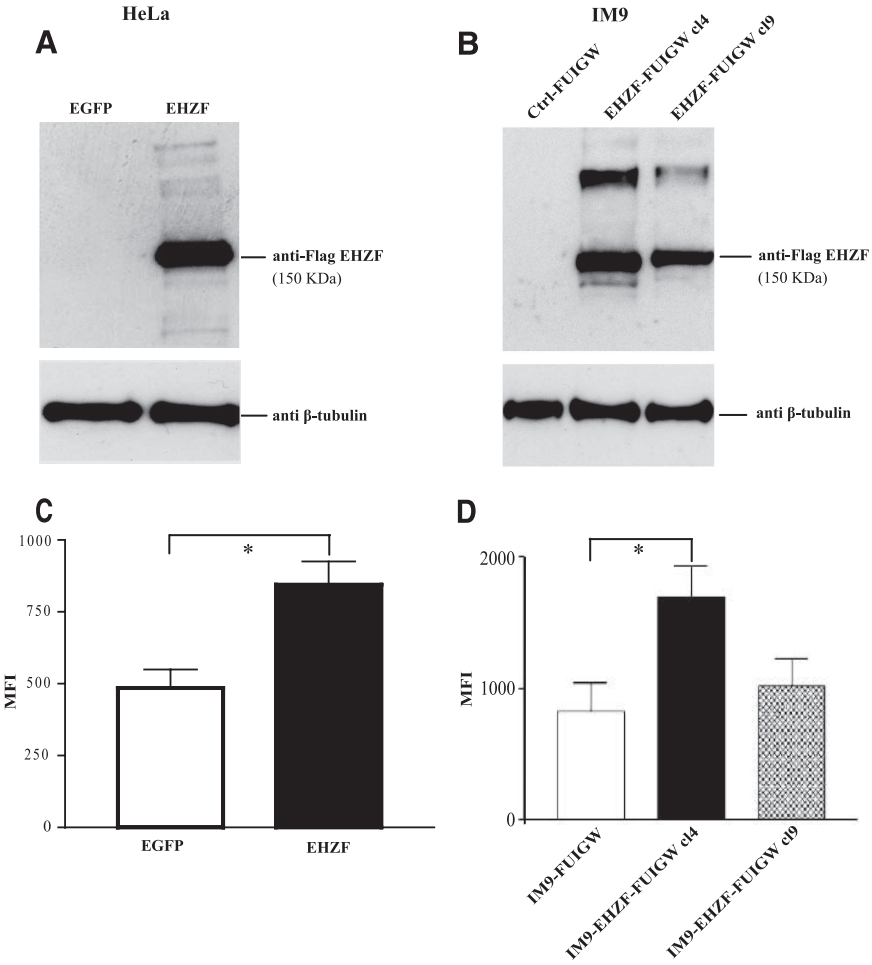

FIGURE 1. HLA class I expression on HeLa and IM9 EHZF-expressing cells. *A*, HeLa cells were transfected with either control EGFP vector (pEGFPN1) or with the vector carrying the cDNA encoding the fusion protein Flag-EHZF-EGFP. The efficiency of the transfections was over 80% as determined by FACS analysis expression of GFP. Expression of Flag-EHZF was determined by Western blotting analysis with anti-flag M2-HRP Ab. The normalization of the protein input was confirmed by detection of β-tubulin. *B*, IM9-EHZF cl4 and IM9-EHZF cl9 clones were isolated from IM9 cells transduced with EHZF-FUIGW. The levels of EHZF were monitored by Western blotting analysis of cell lysates from the two clones. The normalization of the protein input was confirmed by detection of the β-tubulin. *C*, Flow cytometry analysis of HeLa cells transfected with either control vector (pEGFP-N1) or EHZF-EGFP. Cell were stained with HLA class I Ag-specific mAb W6–32 and mouse PE Ab and gating specifically on the transfected cells expressing GFP. MFI values for HLA class I expression on EHZF-expressing cells (EHZF) (■) and controls (□). The level of HLA class I Ags expression on EHZF-transfected cells was significantly ($p < 0.05$) higher that on control cells. Results are mean of four independent experiments. Error bar represents SD. *C* and *D*, IM9-FUIG, IM9-EHZF-FUIG cl4, and IM9-EHZF-FUIG cl9 cells were stained with the HLA class I Ag-specific W6/32 mAb and secondary anti-mouse-PE Ab. Stained cells were analyzed with a flow cytometer after gating on the cells expressing EGFP. MFI of HLA class I in control cells and EHZF-expressing clones is indicated.

In blocking experiments with mAbs, target cells were preincubated for 30 min at room temperature with the HLA class I Ag-specific IgM mAb A6-136. The CD57-specific IgM mAb TIB200 was used as a control. The mAbs at the concentration of 10 μg/ml were present in the culture medium during the whole assay period.

Western blotting

Total extracts were prepared from transfected cells and 20 μg were electrophoresed on 4–12% NuPAGE Novex *bis*-Tris gradient polyacrylaminde gels under reducing conditions (Invitrogen) and electroblotted onto nitrocellulose. The M2 anti-flag mAb directly conjugated to HRP (Sigma-Aldrich) was used at 1/10000 dilution. A mouse anti β-tubulin antiserum (Santa Cruz Biotechnology) was used at 1/1000 dilution and detected with anti-mouse HRP-conjugated Ab. HRP Abs were detected by chemiluminescence reagent (Santa Cruz Biotechnology).

RT-PCR

cDNA was synthesized from 1 μg of total cellular RNA using SuperScript II reverse transcriptase (Invitrogen) and 2.5 μM random hexamers (Boehringer-Mannheim. cDNA aliquots were amplified using 0.2 U *Taq* (Eppendorf Scientific) using the Mastercycler 5331 for up to 40 cycles. The specific oligonucleotide primers used were the following: GAPDH (forward) CACCATCTTCCAGGAGCGAG, (reverse) TCACGCCACAGTTTCCCGGA; EHZF (forward) GGTGAAACTTGATATCAATGGCC, (reverse) GGAGTTTGGCAGGAGAGTCA; GAPDH was amplified at 58°C for 24 cycles and EHZF was amplified at 58°C for 40 cycles. Products were analyzed on 1.2% agarose gels and visualized with ethidium bromide. Quantitative real-time PCR was performed using Platinum Syber Green *Taq* (Invitrogen) and the I Q5 real-time PCR I Cycler (Bio-Rad).

Statistical analyses

Results were reported as mean ± SD. Significance level was determined by Student *t* test analysis. A value $p \leq 0.05$ was considered statistically significant. All the reported *p* values are two sided.

Results

Modulation of cell surface HLA class I Ag levels by EHZF *gene expression*

To determine whether *EHZF* was able to modulate HLA class I molecules, we analyzed their expression on HeLa cells transfected with *EHZF*. In Fig. 1*A* the EHZF protein expression levels were measured in HeLa cell lines. HLA class I Ag expression was increased by 25–55% compared with cells transfected with control vector (Fig. 1*C*). Similar results were obtained using COS-7 cells (data not shown).

The increased expression of HLA class I Ag upon EHZF was selective because no changes were detected in the expression of the membrane-associated activating ligands for NK cell receptor tested. In our analysis we included the activating NK cell receptor ligands MICA, MICB, ULBP1–4, Nectin-2, PVR, and ICAM-1. The mean ± SD of mean fluorescent intensity (MFI) values obtained from six flow cytometry analysis are shown in Table I. MHC class I, MICA, MICB, ULBP1–4, ICAM-1, PVR, and Nectin-2 were expressed at high levels. However, their expression did not change significantly in response to EHZF.

To determine whether EHZF up-regulates HLA class I Ag expression also on B lymphoid cells, the IM9 lymphoid cell line was stably transduced with the lentiviral vector FUIGW-EHZF that carries the flag-EHZF cDNA. Two EHZF$^+$ IM9 cell clones, cl4 and cl9, expressing different levels of EHZF mRNA, were selected and analyzed for flag-EHZF protein expression by Western blotting (Fig. 1*B*). On both cell clones, HLA class I expression was up-regulated and the extent of this increase correlated with the

Table I. *EHZF regulation of NK cell-activating receptor ligands in HeLa cells*

	MFI ± SD[a]	
	GFP[b]	EHZF[c]
MHC class I	485 ± 64.6	850 ± 87.4
MICA	41 ± 12	35 ± 6
MICA/B	56 ± 9	70 ± 15
ULBP1	4 ± 0.8	5 ± 0.5
ULBP2	7 ± 0.5	8 ± 0.6
ULBP3	4 ± 0.3	4 ± 0.8
ULBP4	3 ± 1	4 ± 0.2
ICAM-1	90 ± 9	127 ± 29
PVR	185 ± 71	205 ± 76
Nectin-2	72 ± 7.7	81 ± 15

[a] Mean of MFI values obtained from six flow cytometry analyses.
[b] GFP indicates HeLa cells transfected with empty vector.
[c] EHZF indicates cells transfected with *EHZF* gene.

level of EHZF expression. Consistently the IM9 cl4 cell clone that produced higher amounts of EHZF also displayed higher expression of HLA class I molecules than the IM9 cl9 cell clone (Fig. 1*D*). Based on this observation, we asked whether NK cell recognition of *EHZF* expressing tumors was affected.

EHZF transfection inhibits tumor cell recognition by NK cells

To test whether EHZF modulates NK cell-target cell interactions, we transiently transfected the *EHZF-EGFP* cDNA in HeLa cells, which do not express EHZF (1) and analyzed their susceptibility to NK cell-mediated lysis. The transfected cells were over 80% positive for EGFP by FACS analysis and expressed high levels of EHZF as detected by Western blotting analysis with anti-flag Ab (Fig. 1*A*). Using purified NK cells as effectors in cytotoxicity assays, we observed that EHZF inhibited significantly ($p < 0.05$) the lysis of HeLa cells (Fig. 2*A*); the extent of protection ranged between 30% and 60% of the control lysis. Similar results were obtained when the experiments were performed using the COS-7 cell line (data not shown). The correlation among EHZF, MHC class I expression and decreased NK cell recognition was functionally confirmed by the differential susceptibility to NK cell-mediated cytotoxicity of the clones IM9 cl4 and IM9 cl9 (Fig. 2*B*). Purified NK cells were used as effectors in these assays. With both target cells, a reduction in NK-mediated cytotoxicity was observed at low E:T ratios, but IM9 cl4 cells were less susceptible to lysis compared with IM9 cl9 at all E:T ratios. These results indicate that the level of EHZF and HLA class I Ags correlates inversely with target cell susceptibility to NK cell-mediated lysis.

To gain direct evidence that the modulation of NK cell-target cell interactions by EHZF was mediated by up-regulation of HLA class I molecules, we asked whether the inhibition of NK-mediated lysis was abolished by masking HLA class I Ags with a specific mAb. As shown in Fig. 2*C*, incubation with the HLA class I masking mAb A6-136 restored almost completely the susceptibility of EHZF transfected HeLa cells to NK cell-mediated lysis. To further strengthen the results, a CTL assay was performed using as effector CTL clone (36–38) and as target cells either the IM9 transduced with the empty vector or with EHZF gene. The results showed that EHZF expressing targets better were recognized by the CTL clone (data not shown).

Molecular mechanisms underlying HLA class I Ag up-regulation by EHZF

The cause-effect relationship between EHZF and HLA class I expression was further investigated by silencing the *EHZF* gene using the RNA interference technology. To this end, a hairpin loop shRNA (H11) specific for EHZF was designed and inserted in the FG-12 lentiviral vector. When cotransfected into 293T cells together with the 3xFlag-EHZF expression vector, FG12-H11 consistently interfered with the expression Flag-EHZF (Fig. 3*A*) compared with void vector. As an additional control, the EHZF shRNA was cotransfected with a plasmid carrying a truncated EHZF cDNA encoding exclusively the C-terminal region of EHZF/

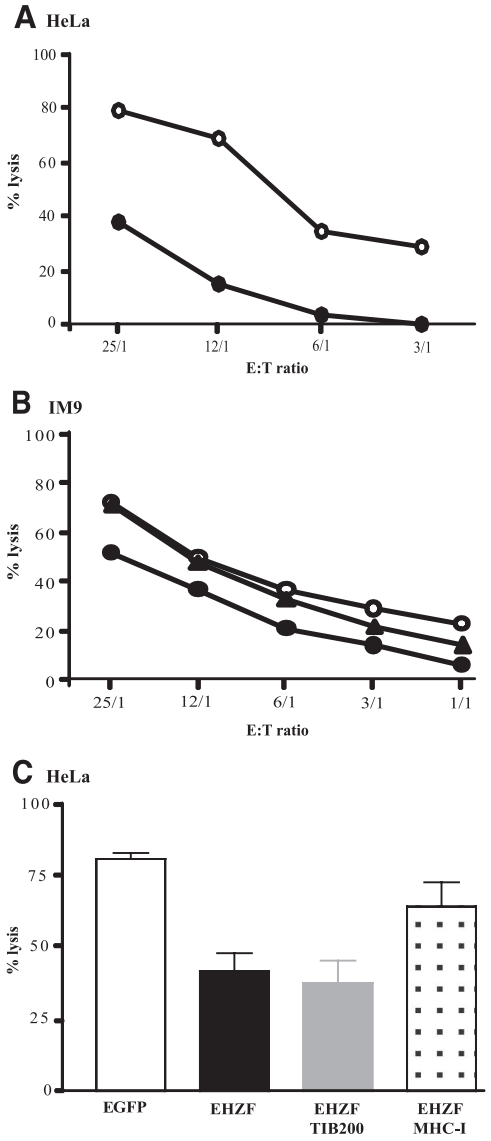

FIGURE 2. Reduced susceptibility to NK cell-mediated cytotoxicity of the HeLa cells transiently transduced with EHZF. *A*, Resting NK cell lysis of control (●) and EHZF-expressing (○) HeLa cells. *B*, NK recognition efficiency of the EHZF-transduced IM9 cell clones in a functional cytotoxicity assay. Purified resting NK cells were used against the three cell populations. A significant reduction in NK cell cytotoxic recognition was observed with IM9 cl4 showing inverse correlation between EHZF, HLA class I levels and NK susceptibility. *C*, MHC class I masking restores NK susceptibility in EHZF-expressing HeLa cells. A6-136 (IgM) is the anti-HLA-masking Ab. As a control the anti-CD57 clone TIB200 (IgM; American Type Culture Collection) was used. Mean percentage NK specific lysis of HeLa-EGFP and HeLa-EHZF-EGFP is reported. The E:T ratio used was 25:1. Error bar represents SD. Untreated HeLa transfected with EGFP or EHZF-EGFP, and the latter cells treated with TIB200 (isotype control mAb) and with MHC class I (masking Ab) are indicated.

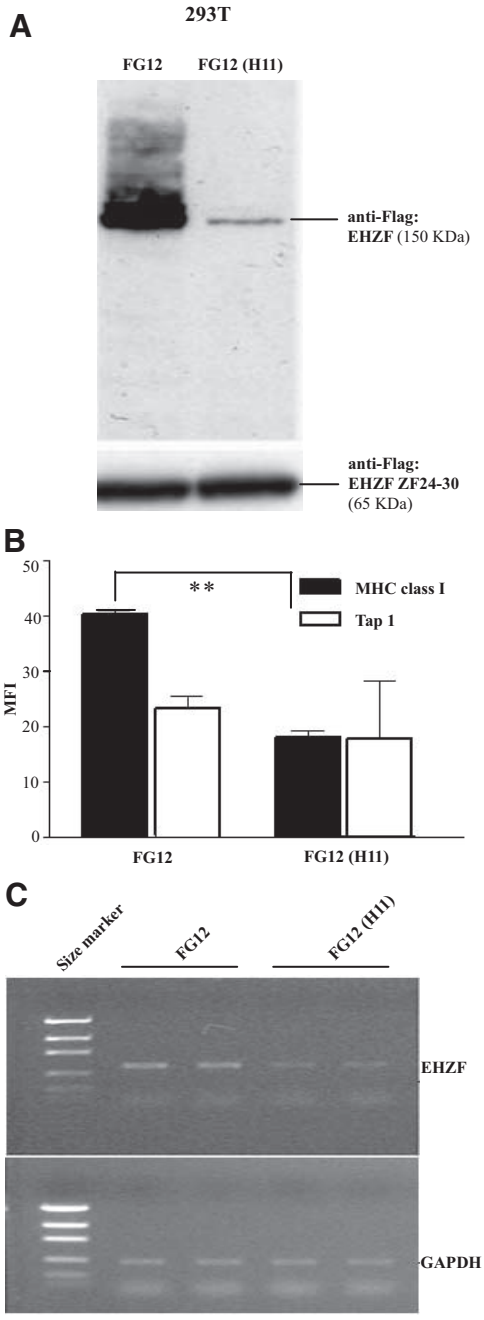

FIGURE 3. Down-regulation of HLA class I expression in 293T cells by shRNA mediated EHZF silencing. *A*, The 293T cells were cotransfected with an expression plasmid carrying the 3xFlag-EHZF cDNA and either the pSuper void vector or pSuper-H11, containing the EHZF-specific H11 shRNA. A vector containing a truncated cDNA encoding only the C-terminal portion of EHZF (EHZF-ZF24–30) that does not contain the corresponding shRNA target sequence was used as an additional control. The levels of EHZF were assayed by Western blotting analysis with the anti-Flag M2-HRP Ab on nuclear extracts prepared 72 h following transfection. *B*, 293T cells transduced with FG12 or FG12-H11 were stained with the HLA class I Ag-specific mAb W6/32 or with anti TAP1 and with secondary anti-mouse-PE Ab. Stained cells were analyzed by flow cytometry gating specifically on the transfected cells expressing EGFP. Error bar indicates SD. MFI is shown. *C*, RNA was isolated from 293T cells 72 h following transfection with FG12 or FG12-H11. cDNA was synthesized and amplified in duplicate with specific primers for EHZF (35 cycles) and for GAPDH (24 cycles). PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Molecular weight markers of multiples of 500 bp are in each panel (*first lane*).

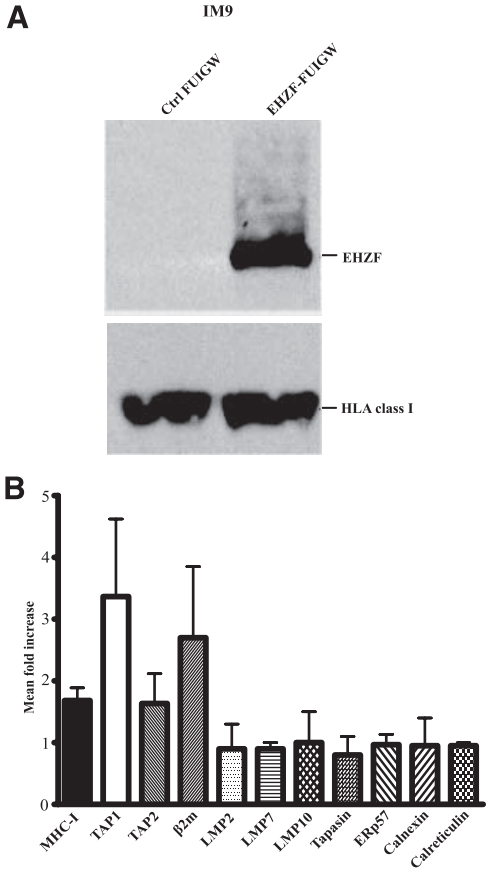

FIGURE 4. Analysis of HLA class I expression and APM components in IM9 cells. APM components were analyzed comparatively in IM9 transduced with FUIGW control virus and IM9 cl4. *A*, Expression of transducted EHZF and HLA class I monoclonal (TP25) is analyzed by Western blotting. Results show one representative Western blot of three performed. *B*, Mean fold increase calculated from the MFI values of three distinct experiments performed, obtained from permeabilized Flag-EHZF-EGFP transduced IM9 cl4 stained with HLA class I mAb and APM component-specific mAbs.

ZNF521 (EHZF zinc finger 24–30), which lacks the shRNA target sequence. In this case, no effect was observed.

The 293T cells display detectable expression of *EHZF* (1). When these cells were transfected with FG12-H11, a strong concomitant reduction of the HLA class I Ag surface levels (Fig. 3*B*) and of the EHZF mRNA levels (Fig. 3*C*) was observed. Moreover, the shRNA H11-induced modulation of the MHC class I H chain was highly reproducible and statistically significant, while the down-regulation of other components of the Ag-processing machinery (APM): β_2M and ERp57 (data not shown) was not statistically significant. The results obtained with TAP1 are shown in Fig. 3*B*.

To determine whether EHZF has a differential effect on the expression of HLA class I gene loci products, their expression was analyzed by staining EHZF-transduced HeLa and IM9 cl4 cells with pan HLA-A, pan HLA-B, HLA-A2, A28-specific mAb and with HLA-Bw6 cross-reacting group-specific mAb. All the HLA class I allele gene products were up-regulated to a similar extent (data not shown). Additional experiments investigated whether the up-regulation of HLA class I Ags by EHZF was associated with changes in the expression of APM components in EHZF-expressing cells. The results of one representative Western blotting experiment, of three which compared APM component expression in IM9 cl4 and in IM9 mock-transduced cells, are illustrated in

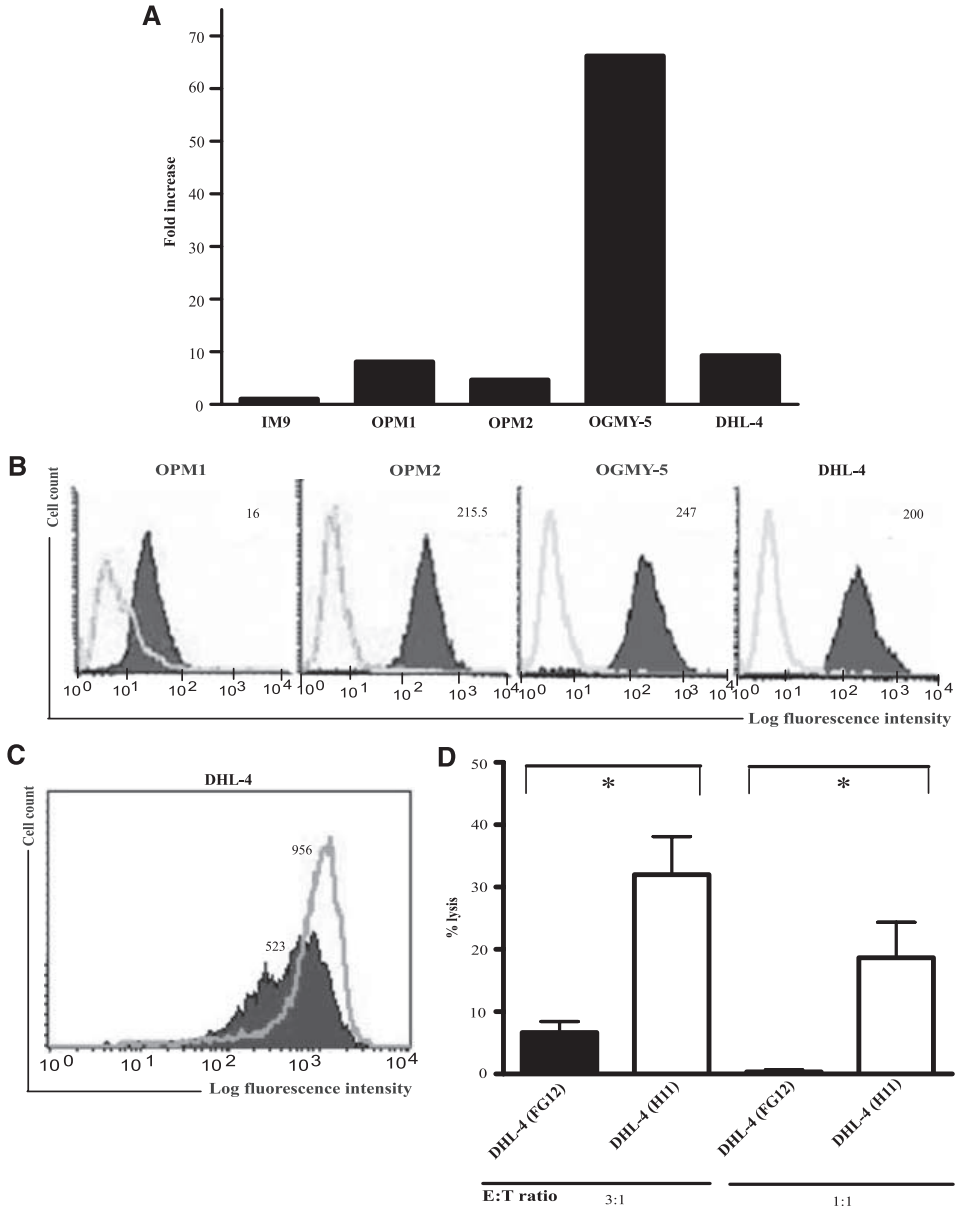

FIGURE 5. EHZF gene transcript detection in MM tumor cell lines. *A*, Quantitative PCR analysis of *EHZF* expression was performed in triplicate in IM9 and three MM cell lines (OPM-1, OPM-2, and OGMY-5) and in the DHL-4 B cell line from diffuse lymphoma as described in *Materials and Methods*. The expression of EHZF threshold cycle (CT) is calculated relative to IM9 ΔCT normalized for GAPDH. *B*, Flow cytometric analysis of HLA class I expression in each panel. Cell surface levels of HLA class I were assessed by indirect staining for HLA class I using W6/32 mAb followed by secondary staining with FITC-conjugated anti-mouse Ig (filled histogram). Controls were incubated only with FITC-conjugated anti-mouse Ig xenoantibodies (open histogram). Data were analyzed with the CellQuest software (BD Biosciences). MFI values are indicated. *C*, Flow cytometric analysis of HLA class I expression on DHL-4 transduced with control FG12 lentivirus (open histogram) and shRNA specific for EHZF, FG12-H11 (filled histogram). Gating on cells expressing GFP the MHC class I molecules expression was measured by W6/32 mAb staining and by secondary anti-mouse PE conjugate incubation. *D*, Inverse correlation between EHZF levels and NK cell lysis. The susceptibility of the DHL-4 cell line to NK-mediated cytotoxicity assays was tested in duplicate by using healthy donor NK cells as effectors against cells transduced with control FG12 lentivirus (filled histogram) and FG12-H11 (open histogram). The percentage of lysis ± SD was calculated using the results from three experiments. The statistical significance of the difference was calculated (∗) using unpaired *t* test analysis; $p < 0.05$ was considered significant.

Fig. 4*A*. The mean fold increase of the MFI values of three distinct experiments is reported in Fig. 4*B*. Significant and reproducible changes in the HLA class I H chain protein levels were found in EHZF-expressing IM9 cl4 cells whereas the levels of TAP1, TAP2, and β_2M showed a higher interexperimental variability (see related SD). Finally the expression of LMP2, LMP7, LMP10, calnexin, calreticulin, ERp57, and tapasin did not show any significant change (Fig. 4*B*).

EHZF expression correlates with poor NK susceptibility in lymphoid malignancies

We have previously shown that *EHZF* is highly expressed in immature hematopoietic progenitors and drastically down-regulated during hematopoietic cell differentiation (1, 2). Abundant levels of its transcript are also detected in many acute myelogenous leukemias and in T cell ALLs, suggesting that deregulated expression of EHZF might play a role in malignant cell growth, survival, or protection against host's immune defense. Because ectopic expression of EHZF results in modulation of HLA class I Ag expression and of target cell susceptibility to NK cell recognition, we investigated whether the expression of EHZF is expressed in several cell lines derived from hematopoietic malignancies. As shown in Fig. 5*A*, RT-PCR analysis detected EHZF transcripts in three MM cell lines and one B cell line derived from a diffuse histiocytic lymphoma. A direct correlation between EHZF transcript levels and surface HLA class I expression was observed in three of four cell lines (Fig. 5, *A* and *B*). To further prove the role of EHZF in regulating MHC class I expression, the EHZF$^+$ DHL-4 cells were transduced either with control lentivirus (DHL-4-FG12) or with the FG12-H11 (DHL-4-FG12-H11). The measurement of HLA class I expression showed that DHL-4-FG12 control cells had readily detectable HLA class I membrane expression while decreased levels were observed in DHL-4-FG12-H11 which expressed the shRNA directed against EHZF (Fig. 5*C*). FG12-H11 did not modulate NKG2D, DNAM-I and LFA-1 ligands (data not shown). An inverse correlation between EHZF expression and NK cell-mediated lysis was observed when healthy donor purified NK cells were used as effectors against DHL-4-FG12 control and DHL-4-H11 (shRNA) cell targets: EHZF silencing by shRNA in the DHL-4-FG12-H11 cells resulted in significantly higher

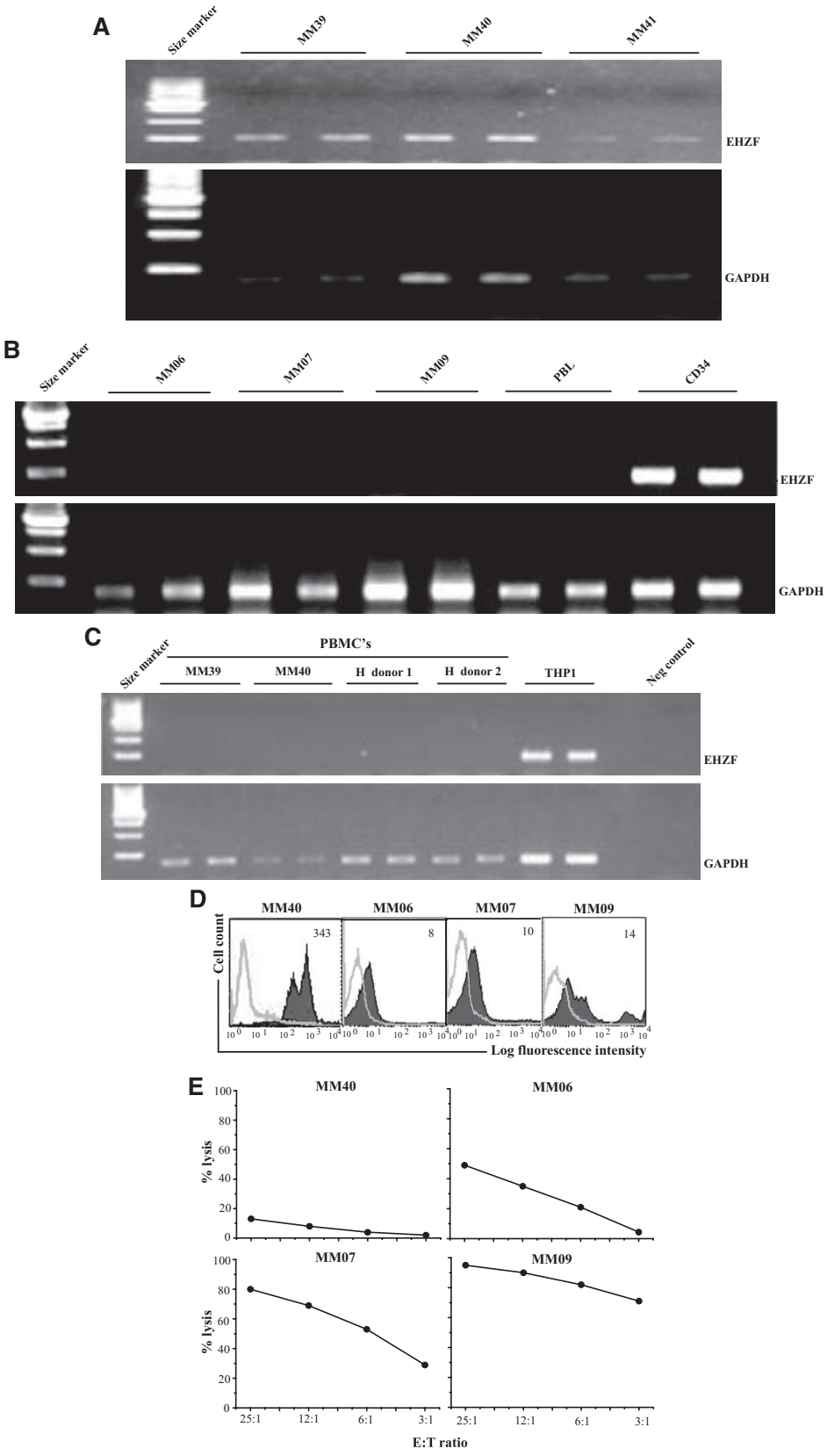

FIGURE 6. Detection of EHZF gene transcripts in MM cells obtained from patient bone marrow aspirates. *A* and *B*, RT-PCR analyses of *EHZF* expression performed in duplicate on freshly explanted MM cells from six patients. *B*, PBMC (PBL) from a healthy donor and purified $CD34^+$ cells were used as controls. The expression of GAPDH was analyzed as an internal control. Molecular weight markers of multiples of 500 bp are in each panel (*first lane*). PBMC from the patients in *C* and other patients (data not shown) were also analyzed and did not show detectable *EHZF* expression. *D*, Cell surface levels of HLA class I in patients' MM cells were assessed by indirect staining for HLA class I using W6/32 mAb followed by secondary staining with FITC-conjugated anti-mouse Ab (filled histogram). Controls were incubated only with FITC-conjugated secondary Ab (open histogram). As it can be observed, MFI indicated for each sample correlates with the levels of *EHZF* expression assessed by RT-PCR (*A* and *B*). *E*, Autologous NK recognition of primary MM cells expressing different levels of EHZF. E:T ratios were 25:1, 12:1, 6:1, 3:1 reported, respectively, for patients MM40, MM06, MM07, MM09.

susceptibility to NK cell lysis compared with that of the control cell line (Fig. 5*D*). Similar results were obtained using ^{51}Cr cytotoxicity release assays (data not shown).

NK cells play an important role in controlling disease progression in MM (4). Late disease stage MM cells express high levels of HLA class I Ag on their surface and become resistant to NK cell-mediated lysis. To ascertain whether the expression of EHZF correlated with high HLA class I levels and NK cell resistance, freshly explanted MM cells were purified from patient bone marrow aspirates and analyzed for *EHZF* expression. EHZF mRNA was undetectable in PBMC cell preparations from all patients (Fig. 6*C*), consistently with our previous findings (1). In contrast, three

of six MM primary cells (patients MM39, MM40, and MM09) displayed detectable levels of *EHZF* mRNA. These data were validated using quantitative PCR analysis for patients MM40, MM06, MM07, and MM09 (data not shown). Representative results are shown in Fig. 6, *A* and *B*. It must be emphasized that in parallel experiments using 18 melanoma cell lines, we found no detectable expression of *EHZF* (data not shown) The correlation among EHZF transcript levels, MHC class I expression (Fig. 6*D*) and autologous NK recognition in myeloma cells was analyzed in four patients (Fig. 6*E*). Strikingly, the patient MM40 showing the highest expression of *EHZF* and MHC class I molecules was also resistant to autologous NK recognition. Conversely, myeloma cells from three patients with no (MM06 and MM07) or low (MM09) expression of *EHZF* showed low or heterogenous MHC class I expression and were highly susceptible to autologous NK cell recognition and lysis.

Discussion

It is well recognized that abnormalities in HLA class I Ag expression or function frequently occur in malignant cells, and that MHC class I defects could potentially affect tumor susceptibility to NK cell recognition. A number of studies have dissected the defects leading to HLA class I loss and consequent CTL escape from tumor cells (28, 39, 40). In specific disease localizations or disease stages, NK cells could represent a key cytotoxic lymphocyte population, and tumor cell variants expressing high levels of HLA class I could be selected. In a preliminary functional study we found that enforced expression of EHZF/ZNF521, a zinc finger transcription cofactor with a potential regulatory role in the hematopoietic system, could induce increased levels of MHC class I molecules on the cell surface. Therefore we tested whether EHZF could affect the susceptibility of target cells to NK-mediated cells. In transient transfection and stable transduction experiments, we found a consistent reduction in NK cell recognition of EHZF-expressing targets and a concomitant increase in cell surface expression of MHC class I molecules.

In recent years, there has been increasing understanding of the role of the APM in generating peptides to be presented by HLA class I Ags. This understanding has stimulated interest in the analysis of APM component expression in malignant lesions (41, 42). To address the possibility that the HLA class I increase induced by EHZF could be due to alterations in APM component expression, intracellular staining experiments were conducted using specific Abs (33). Our data indicate that the EHZF enhancement effect on the APM is evident for MHC class I H chain, and an up-regulation tendency is present for TAP1, TAP2, and β_2M. However, for the latter three APM components a larger interexperimental variation was observed. At this stage of our study, the interpretation that we prefer is that EHZF has a prevalent effect of the MHC class I H chain and does not reduce the other APM components. It is conceivable to assume that the increased availability of H chain can result in increased amount of membrane associated MHC class I molecules. Our data demonstrate that EHZF increases the expression of HLA class I H chain proteins, whereas other surface glycoproteins such as the HLA class I-like molecule CD1 (data not shown), and the activating ligands for natural cytotoxicity receptors, NKG2D, and DNAM-1 were not affected. EHZF expression was originally found in hematopoietic stem and progenitor cells (1) and in acute myelogenous leukemias and T cell ALLs (1, 2) (L. Bullinger, et al., manuscript in preparation). Therefore we asked whether EHZF could be detected in additional hematopoietic malignancies such as MM, lymphoma, and melanoma. We analyzed the expression of EHZF by RT-PCR in four different hematological tumor cell lines (three MM and one lymphoma), in 18 melanoma cell lines, and in six freshly explanted MM cell preparations obtained from patient's bone marrow aspirates. We found that all hematological tumor cell lines tested had detectable levels of EHZF, whereas none of the melanoma cell lines analyzed express transcripts for EHZF. A direct correlation emerged between the amounts of EHZF mRNA and MHC class I expression levels in these tumor cell lines (Fig. 5). Using MM primary cell culture, we confirmed the association between EHZF, HLA class I expression levels, and autologous NK susceptibility (Fig. 6). In our previous study on MM, we demonstrated that disease progression is correlated with HLA class I increase, MICA decrease, and NK cell resistance (4). Data reported suggest that EHZF may be involved in a novel tumor NK immunosurveillance escape strategy based on MHC class I up-regulation. A potential role for EHZF in myeloma prognosis needs to be further addressed by tracking EHZF expression in PBLs obtained from MM patient's cohort with intramedullary and extramedullary disease diagnosis or comparing its presence between monoclonal gammopathy of undetermined significance and MM patient bone marrow cells. In contrast, the absence of EHZF expression in melanoma cell lines (data not shown), a tumor widely reported to have several defects in MHC class I expression (43) and to be highly susceptible to NK-mediated lysis (44), further complements the MM findings and suggest that EHZF may indeed be a relevant factor in determining NK resistance in neoplastic cells by stimulating HLA class I expression.

Although the molecular mechanisms leading to HLA class I down-regulation are well known (44, 45), in this study we have described a new tumor-associated gene able to positively regulate HLA class I expression. The T cell recognition of EHZF expressing hematological malignancies needs further investigative study.

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Disclosures

The authors have no financial conflict of interest.

References

1. Bond, H. M., M. Mesuraca, E. Carbone, P. Bonelli, V. Agosti, N. Amodio, G. De Rosa, M. Di Nicola, A. M. Gianni, M. A. Moore, et al. 2004. Early hematopoietic zinc finger protein (EHZF), the human homolog to mouse Evi3, is highly expressed in primitive human hematopoietic cells. *Blood* 103: 2062–2070.
2. Bond, H. M., M. Mesuraca, N. Amodio, T. Mega, D. Pelaggi, V. Agosti, D. Fanello, L. Bullinger, M. Grieco, M. A. S. Moore, et al. 2008. Early hematopoietic zinc finger protein/zinc finger protein 521 (EHZF/ZNF521): a candidate regulator of diverse immature cells. *Int. J. Biochem. Cell Biol.* 40: 848–854.
3. Mullighan, C. G., S. Goorha, I. Radtke, C. B. Miller, E. Coustan-Smith, J. D. Dalton, K. Girtman, S. Mathew, J. Ma, S. B. Pounds, et al. 2007. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 446: 758–764.
4. Carbone, E., P. Neri, M. Mesuraca, M. T. Fulciniti, T. Otsuki, D. Pende, V. Groh, T. Spies, G. Pollio, D. Cosman, et al. 2005. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* 105: 251–258.
5. Siegler, U., C. P. Kalberer, P. Nowbakht, S. Sendelov, S. Meyer-Monard, and A. Wodnar-Filipowicz. 2005. Activated natural killer cells from patients with acute myeloid leukemia are cytotoxic against autologous leukemic blasts in NOD/SCID mice. *Leukemia* 19: 2215–2222.
6. Frohn, C., M. Höppner, P. Schlenke, H. Kirchner, P. Koritke, and J. Luhm. 2002. Antimyeloma activity of natural killer lymphocytes. *Br. J. Haematol.* 119: 660–664.
7. Costello, R. T., C. Fauriat, S. Sivori, E. Marcenaro, and D. Olive. 2004. NK cells: innate immunity against hematological malignancies*? Trends Immunol.* 25: 328–333.
8. Blanca, I. R., E. W. Bere, H. A. Young, and J. R. Ortaldo. 2001. Human B cell activation by autologous NK cells is regulated by CD40-CD40 ligand interaction: role of memory B cells and $CD5^+$ B cells. *J. Immunol.* 167: 6132–6139.

9. Carbone, E., G. Terrazzano, G. Ruggiero, D. Zanzi, A. Ottaiano, C. Manzo, K. Kärre, and S. Zappacosta. 1999. Recognition of autologous dendritic cells by human NK cells. *Eur. J. Immunol.* 29: 4022–4029.
10. Carbone, E., G. Terrazzano, A. Melián, D. Zanzi, L. Moretta, S. Porcelli, K. Kärre, and S. Zappacosta. 2000. Inhibition of human NK cell-mediated killing by CD1 molecules. *J. Immunol.* 164: 6130–6137.
11. Martín-Fontecha, A., E. Assarsson, E. Carbone, K. Kärre, and H. G. Ljunggren. 1999. Triggering of murine NK cells by CD40 and CD86 (B7-2). *J. Immunol.* 162: 5910–5916.
12. Carbone, E., G. Ruggiero, G. Terrazzano, C. Palomba, C. Manzo, S. Fontana, H. Spits, K. Kärre, and S. Zappacosta. 1997. A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. *J. Exp. Med.* 185: 2053–2060.
13. Farag, S. S., T. A. Fehniger, L. Ruggeri, A. Velardi, and M. A. Caligiuri. 2002. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood* 100: 1935–1947.
14. Martín-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for T_H1 priming. *Nat. Immunol.* 5: 1260–1265.
15. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47: 187–376.
16. Bottino, C., L. Moretta, D. Pende, M. Vitale, and A. Moretta. 2004. Learning how to discriminate between friends and enemies, a lesson from natural killer cells. *Mol. Immunol.* 41: 569–575.
17. Bottino, C., L. Moretta, D. Pende, M. Vitale, and A. Moretta. 2001. Human natural killer cell receptors and co-receptors. *Immunol. Rev.* 181: 203–214.
18. Long, E. O. 1999. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol.* 17: 875–904.
19. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 19: 197–223.
20. Pende, D., S. Parolini, A. Pessino, S. Sivori, R. Augugliaro, L. Morelli, E. Marcenaro, L. Accame, A. Malaspina, R. Biassoni, et al. 1999. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J. Exp. Med.* 190: 1505–1516.
21. Gonzalez, S., V. Groh, and T. Spies. 2006. Immunobiology of human NKG2D and its ligands. *Curr. Top. Microbiol. Immunol.* 298: 121–138.
22. Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier, and T. Spies. 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 285: 727–729.
23. Sutherland, C. L., N. J. Chalupny, and D. Cosman. 2001. The UL16-binding proteins, a novel family of MHC class I-related ligands for NKG2D, activate natural killer cell functions. *Immunol. Rev.* 181: 185–192.
24. Pende, D., C. Bottino, R. Castriconi, C. Cantoni, S. Marcenaro, P. Rivera, G. M. Spaggiari, A. Dondero, B. Carnemolla, N. Reymond, et al. 2005. PVR (CD155) and Nectin-2 (CD112) as ligands of the human DNAM-1 (CD226) activating receptor: involvement in tumor cell lysis. *Mol. Immunol.* 42: 463–469.
25. Qin, X. F., D. S. An, I. S. Chen, and D. Baltimore. 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. USA* 100: 183–188.
26. Pende, D., P. Rivera, S. Marcenaro, C. C. Chang, R. Biassoni, R. Conte, M. Kubin, D. Cosman, S. Ferrone, L. Moretta, et al. 2002. Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. *Cancer Res.* 62: 6178–6186.
27. Bottino, C., R. Castriconi, D. Pende, P. Rivera, M. Nanni, B. Carnemolla, C. Cantoni, J. Grassi, S. Marcenaro, N. Reymond, et al. 2003. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J. Exp. Med.* 198: 557–567.
28. Seliger, B., T. Cabrera, F. Garrido, and S. Ferrone. 2002. HLA class I antigen abnormalities and immune escape by malignant cells. *Semin. Cancer Biol.* 12: 3–13.
29. Pellegrino, M. A., A. K. Ng, C. Russo, and S. Ferrone. 1982. Heterogeneous distribution of the determinants defined by monoclonal antibodies on HLA-A and B antigens bearing molecules. *Transplantation* 34: 18–23.
30. Stam, N. J., T. M. Vroom, P. J. Peters, E. B. Pastoors, and H. L. Ploegh. 1990. HLA-A- and HLA-B-specific monoclonal antibodies reactive with free heavy chains in Western blots, in formalin-fixed, paraffin-embedded tissue sections and in cryo-immuno-electron microscopy. *Int. Immunol.* 2: 113–125.
31. Perosa, F., G. Luccarelli, M. Prete, E. Favoino, S. Ferrone, and F. Dammacco. 2003. β_2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J. Immunol.* 171: 1918–1926.
32. Wang, X., M. Campoli, H. S. Cho, T. Ogino, N. Bandoh, J. Shen, S. Y. Hur, T. Kageshita, and S. Ferrone. 2005. A method to generate antigen-specific mAb capable of staining formalin-fixed, paraffin-embedded tissue sections. *J. Immunol. Methods* 299: 139–151.
33. Ogino, T., X. Wang, S. Kato, N. Miyokawa, Y. Harabuchi, and S. Ferrone. 2003. Endoplasmic reticulum chaperone-specific monoclonal antibodies for flow cytometry and immunohistochemical staining. *Tissue Antigens* 62: 385–393.
34. Bandoh, N., T. Ogino, H. S. Cho, S. Y. Hur, J. Shen, X. Wang, S. Kato, N. Miyokawa, Y. Harabuchi, and S. Ferrone. 2005. Development and characterization of human constitutive proteasome and immunoproteasome subunit-specific monoclonal antibodies. *Tissue Antigens* 66: 185–194.
35. McGinnes, K., C. G. Marks, and R. Penny. 1986. A fluorescence NK assay using flow cytometry. *J. Immunol. Methods* 86: 7–15.
36. Nisticò, P., R. Mortarini, B. L. De Monte, A. Mazzocchi, M. Mariani, F. Malavasi, G. Parmiani, P. G. Natali, and A. Anichini. 1992. Cell retargeting by bispecific monoclonal antibodies: evidence of bypass of intratumor susceptibility to cell lysis in human melanoma. *J. Clin. Invest.* 90: 1093–1099.
37. Castelli, C., A. Mazzocchi, S. Salvi, A. Anichini, and M. Sensi. 1992. Use of the Vδ1 variable region in the functional T-cell receptor α chain of a $WT31^+$ cytotoxic T lymphocyte clone which specifically recognizes HLA-A2 molecule. *Scand. J. Immunol.* 35: 487–494.
38. Mazzocchi, A., A. Anichini, C. Castelli, M. Sensi, F. Poli, C. Russo, and G. Parmiani. 1990. T lymphocytes can mediate lysis of autologous melanoma cells by multiple mechanisms: evidence with a single T cell clone. *Cancer Immunol. Immunother.* 32: 13–21.
39. Ikeda, H., B. Lethé, F. Lehmann, N. van Baren, J. F. Baurain, C. de Smet, H. Chambost, M. Vitale, A. Moretta, T. Boon, and P. G. Coulie. 1997. Characterization of an antigen that is recognized on a melanoma showing partial HLA loss by CTL expressing an NK inhibitory receptor. *Immunity* 6: 199–208.
40. Bakker, A. B., J. H. Phillips, C. G. Figdor, and L. L. 1998. Killer cell inhibitory receptors for MHC class I molecules regulate lysis of melanoma cells mediated by NK cells, $\gamma\delta$ T cells, and antigen-specific CTL. *J. Immunol.* 160: 5239–5245.
41. Seliger, B., M. J. Maeurer, and S. Ferrone. 2000. Antigen-processing machinery breakdown and tumor growth. *Immunol. Today* 21: 455–464.
42. Yewdell, J. 2002. To DRiP or not to DRiP: generating peptide ligands for MHC class I molecules from biosynthesized proteins. *Mol. Immunol.* 39: 139–146.
43. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.* 74: 181–273.
44. Ferrone, S., and F. M. Marincola. 1995. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol. Today* 16: 487–494.
45. Nasca, R., and E. Carbone. 1999. Natural killer cells as potential tools in melanoma metastatic spread control. *Oncol. Res.* 11: 339–343.