MICA Expressed by Multiple Myeloma and Monoclonal Gammopathy of Undetermined Significance Plasma Cells Costimulates Pamidronate-Activated γδ Lymphocytes

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Abstract

Amino-biphosphonates (like pamidronate) activate human Vγ9/Vδ2 T lymphocytes and promote their cytotoxicity against multiple myeloma cells. T-cell receptor (TCR)–mediated effector functions of γδ cells are enhanced upon triggering of the activating receptor NKG2D by MICA, a stress-inducible antigen expressed by epithelial and some hematopoietic tumors, including multiple myeloma. Here we show that MICA was expressed not only by myeloma cell lines and by 6 of 10 primary multiple myeloma cells from patients but also by bone marrow plasma cells from all (six of six) patients with preneoplastic gammopathy (monoclonal gammopathy of undetermined significance, MGUS), a direct precursor of multiple myeloma. Moreover, compared with multiple myeloma plasma cells, MICA was expressed by MGUS plasma cells at significantly (*P* < 0.05) higher levels. MICA expressed by myeloma cell lines contributed to killing and IFN-γ production by Vγ9/Vδ2 cells only upon pamidronate treatment, suggesting a dual interaction between Vγ9/Vδ2 lymphocytes and multiple myeloma plasma cells involving both TCR triggering and NKG2D-mediated signals. Finally, MICA enhanced killing of freshly derived, pamidronate-treated multiple myeloma cells from patients by γδ cells, as indicated by the significantly (*P* < 0.05) higher γδ cytotoxicity against MICA-positive rather than MICA-negative multiple myeloma cells. Our results indicate that MICA expressed by monoclonal plasma cells is functional and correlates with disease stages, suggesting a role for the molecule in the immune surveillance against multiple myeloma. Moreover, pamidronate-activated Vγ9/Vδ2 lymphocytes can be exploited in the immune therapy of early stages multiple myeloma and possibly of premalignant disease. (Cancer Res 2005; 65(16): 7502-8)

Introduction

Lymphocytes bearing the Vγ9/Vδ2 T-cell receptor (TCR) have been implicated in the immune response against microbial infections and hematologic malignancies (1–7). Vγ9/Vδ2 T lymphocytes display a unique repertoire of antigen specificity; in particular, they recognize low molecular weight nonpeptide phosphate-containing molecules, including prenyl-pyrophosphate derivatives (8, 9) and naturally occurring alkilamines (10). Recognition of these antigens is TCR dependent and does not require processing and presentation by classic MHC molecules (11) thus being substantially different from MHC-restricted recognition of peptide antigens by αβ T lymphocytes.

Amino-biphoshonates (like pamidronate), drugs widely used to prevent bone resorption in several pathologic conditions including multiple myeloma, also promote proliferation, IFN-γ secretion, and killing of myeloma cell lines by Vγ9/Vδ2 T lymphocytes, in a TCR-dependent manner (12–14). Indeed, these compounds share structural homology with identified γδ T-cell ligands (12–14); moreover, they cause intracellular accumulation of phosphorylated mevalonate metabolites, which have been shown to mediate recognition of Daudi Burkitt's lymphoma, a prototypical target for Vγ9/Vδ2 T lymphocytes (15).

As for other lymphocyte subsets, effector functions of γδ lymphocytes can be modulated by triggering and inhibitory signals delivered by specific immune receptors (16, 17). Specifically, NKG2D, a type II C-lectin-like protein expressed by natural killer (NK) cells, most γδ cells and $CD8^+$ T lymphocytes (18, 19), has been shown to enhance cytolytic activity and cytokine production of Vγ9/Vδ2 T lymphocytes in response to mycobacterial antigens (20).

In humans, ligands of NKG2D are MHC class I–related chains (MIC) A and B and UL16-binding proteins (UBPL; refs. 21, 22). MICA and MICB are MHC class I homologues, with no role in antigen presentation, and a highly restricted expression in healthy individuals, including variable areas of the gastrointestinal epithelium (23, 24). However, MIC can be induced by certain viral and bacterial infections and by heat shock and is present on most epithelial as well as certain hematopoietic tumors (20, 25–28). MIC antigens may then be regarded as markers of cellular distress, whose expression is perceived by the immune system as a "danger" signal (18). Indeed, susceptibility of myeloma cells to NK cytotoxicity has been recently shown to depend on their MICA levels (29); the latter varied with disease stages, with MIC expressed in intramedullary myeloma but almost undetectable in plasma cells from late-stage extramedullary localizations (29).

Multiple myeloma is often preceded by monoclonal gammopathy of undetermined significance (MGUS), a condition that is frequently observed in elderly and is considered preneoplastic. Recent studies have shown that monoclonal plasma cells in MGUS

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carry most cytogenetic and genomic changes found in multiple myeloma plasma cells (30, 31), and a critical role of the host immune response in controlling malignant progression has been suggested (32, 33).

In the present study, we investigated MICA expression by MGUS versus multiple myeloma plasma cells and its relation with γδ effector functions.

Materials and Methods

Patients. Bone marrow samples were obtained from 10 patients with multiple myeloma and six patients with MGUS, who were admitted to the Department of Oncology and to the Clinical Immunology and Rheumatology Unit, S. Raffaele Hospital, granting their informed consent. Diagnosis and staging were based on reported criteria (34, 35). Samples were taken at the time of diagnosis before therapy. Patients' characteristics are summarized in Tables 1 and 2.

Cell lines, patient samples, and γδ T cells. The human myeloma cell lines U266 and LP1 were purchased from the American Type Culture Collection (Rockville, MD) and from DSMZ (Braunschweig, Germany), respectively, and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and antibiotics (tissue culture medium, TCM). Mononuclear cells were isolated from bone marrow samples by density centrifugation (Ficoll-Hypaque, Pharmacia, Piscataway, NJ). Enriched multiple myeloma plasma cell populations to be used in cytotoxicity experiments were obtained by depletion of adherent cells and of $CD2^+$ lymphocytes by the use of anti-CD2 monoclonal antibody (mAb)–conjugated beads (Dynal Biotech, Oslo, Norway); purity of plasma cell populations, as assessed by CD138/CD38 staining, was always >90%.

The human γδ cell lines and γδ T-cell clones were established, as described (36). Briefly, peripheral blood mononuclear cells (PBMC) from normal donors were stimulated with the γδ-specific phosphate antigen isopentenyl pyrophosphate (IPP, Sigma Chemical Co., St. Louis, MO, 35 μmol/L), and γδ cells were purified by negative depletion of $CD4^+$ and $CD8^+$ lymphocytes with anti-CD4 and anti-CD8 mAb-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA; ref. 37). γδ cell lines were propagated by cyclic restimulation with phytohemagglutinin A (PHA); clones were derived by limiting dilution of bulk γδ cell populations and cultured in TCM, in the presence of recombinant interleukin 2 (50 units/mL; EuroCetus, Amsterdam, The Netherlands; ref. 36). All γδ cell lines and clones expressed the Vγ9/Vδ2 TCR, as assessed by staining with the TiγA mAb, which specifically recognizes the Vγ9 epitope, and with the BB3 mAb, which recognizes specifically the Vδ2 epitope (kind gifts of Dr. Thierry Hercend, Villejuif, France and of Ermanno Ciccone, Genova, Italy, respectively).

Table 1. Clinical features of MM patients

Patient	Sex	Age (y)	Clinical stage	M protein	Plasma cells (%)
1	M	84	III B	IgGκ 58.47	60
2	F	52	III B	IgDλ NM	78
3	F	76	III A	IgAκ 14.8	68
4	F	78	I A	IgAκ 22.04	52
5	F	89	II A	IgGκ 20.3	64
6	F	80	II A	IgAκ 9.07	33
7	M	73	I A	IgGλ 11.9	36
8	M	77	II A	IgGλ 32	28
9	F	68	II A	IgGκ 11	56
10	M	65	III A	IgGκ 78.6	78

NOTE: The clinical stage was assessed according to ref. (35). Serum concentration of M component (expressed as g/L) and the percentage of MM plasma cells in bone marrow biopsies were determined at the time of diagnosis.
Abbreviation: NM, not measurable.

Table 2. Clinical features of patients with MGUS

Patient	Sex	Age (y)	M protein	Plasma cells (%)
1	M	67	IgGκ NM	5
2	F	83	IgAλ NM	8
3	F	73	IgGκ NM	4
4	F	57	IgGκ 14.71	8
5	M	76	IgGλ 7.85	9
6	F	65	IgAλ NM	3

NOTE: Serum concentration of M component (expressed as g/L) and the percentage of monoclonal plasma cells in the bone marrow were determined at the time of diagnosis.
Abbreviation: NM, not measurable.

Flow cytometric analysis. Expression of surface markers by multiple myeloma plasma cells was determined by immunofluorescence and flow cytometric (fluorescence-activated cell sorting, FACS) analysis, as described (36). Bone marrow plasma cells were identified by the use of a phycoerythrin-conjugated anti-CD38 mAb (Caltag Laboratories, Burlingame, CA) and a FITC-conjugated anti-CD138 mAb (IQ Products, Groningen, The Netherlands). MICA/B and NKG2D surface expression was assessed by staining with anti-MICA/B (clone 6G6, described in 38; 1:5,000 dilution of ascites), anti-MICA (clone AMO1, Immatics Biotechnologies, Tubingen, Germany, 10 μg/mL; ref. 28) or anti-NKG2D (R&D Systems, Minneapolis, MN, 1 μg/mL) mAbs, followed by incubation with a FITC-labeled goat anti-mouse IgG1 antiserum (Southern Biotechnology Associates, Birmingham, AL). Cells were then analyzed by flow cytometry (FACStar^{Plus}, Becton Dickinson, Sunnyvale, CA). ΔMean fluorescence intensity (MFI) of MICA staining was calculated by subtracting MFI obtained with the isotype control to MFI obtained with the specific anti-MICA mAb.

Cytotoxicity. Cytolytic activity of γδ lymphocytes was determined by a standard 4-hour ^{51}Chromium (Cr) release assay, as described in (36). Targets were ^{51}Cr-labeled multiple myeloma cell lines, either untreated or preincubated with pamidronate 1 mmol/L for 1.5 hours, or ^{51}Cr-labeled purified multiple myeloma plasma cells from patients, either untreated or preincubated ON with pamidronate 1 mmol/L. Specific lysis was calculated as: (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100. In selected experiments, the contribution of TCR- and NKG2D-mediated signaling to target cell lysis

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was assessed by preincubating pamidronate-treated myeloma cells with the AMO1 mAb (10 μg/mL) and γδ lymphocytes with the anti-TCR TiγA mAb (1:500 dilution of ascites), for 30 minutes at 4°C. Mouse IgG (Sigma Chemical) were used as negative control at 1 μg/mL. Percentage of inhibition was calculated as:

$$\% \text{ inhibition} = 1 - (\text{experimental lysis}/\text{control lysis}) \times 100$$

where control lysis was lysis in the absence of antibody.

In a separate set of experiments, the contribution of pamidronate-induced accumulation of mevalonate metabolites to U266 killing was determined by FACS analysis. Briefly, U266 cells were treated for 2 hours with mevastatin (Sigma, 25 μmol/L), a selective upstream inhibitor of the mavalonate pathway (15) before incubation with pamidronate (1 mmol/L for 1.5 hours); U266 cells were then washed, and γδ lymphocytes added at a 2:1 ratio. After a 6-hour coincubation, cells were labeled with a FITC-conjugated anti-TCRγδ mAb (Becton Dickinson, San Jose, CA), and the percentage of dead U266 cells, identified as TCRγδ-negative cells that failed to exclude the vital dye propidium iodide (36), was determined by FACS analysis. Stimulation with IPP, added at 15 μmol/L throughout the coincubation, served as positive control.

Determination of intracellular cytokine content. To induce IFN-γ production, γδ lymphocyte clones were coincubated for 6 to 8 hours at a 1:1 ratio with U266 cells, either untreated or pretreated with pamidronate 1 mmol/L for 1.5 hours. Brefeldin A (Sigma Chemical) was added at 10 μg/mL during the last 3 hours of culture to prevent cytokine secretion, then IFN-γ production was assessed as in ref. (37) using a FITC-conjugated anti-IFN-γ mAb (R&D Systems). γδ lymphocytes were identified by surface staining with a phycoerythrin-conjugated anti-TCRγδ mAb (Becton Dickinson, San Jose, CA). The percentage of IFN-γ^+ γδ T lymphocytes was determined by flow cytometry. In selected experiments, the contribution of TCR- and NKG2D-mediated signaling to IFN-γ production was assessed by preincubating pamidronate-treated myeloma cells with the AMO1 mAb (10 μg/mL) and γδ lymphocytes with the anti-TCR TiγA mAb (1:500 dilution of ascites). Percentage of inhibition was calculated as:

$$\% \text{ inhibition} = 1 - (\% \text{ IFN - } \gamma^{+}\gamma\delta \text{ cells with blocking mAb}/ \% \text{ IFN - } \gamma^{+}\gamma\delta \text{ cells without mAb}) \times 100.$$

Statistical analysis. To analyze differences in ΔMFI between patients with multiple myeloma and with MGUS, normality tests were used to assess Gaussian distribution. Nonparametric tests for independent samples were applied to evaluate the significance of the differences. To evaluate γδ cytotoxic activity against multiple myeloma cells expressing different levels of MICA, as well as the effect exerted by blocking mAbs, statistical analysis was done using the Student's *t* test for paired data. $P < 0.05$ was regarded as significant.

Results

Plasma cells from multiple myeloma and monoclonal gammopathy of undetermined significance express surface MIC. We first assessed the pattern of surface expression of MICA/B by myeloma cell lines by cytofluorimetric analysis using the 6G6 mAb, which recognizes an epitope on the α_3 domain of MICA and MICB (38), and the AMO1 mAb, which is specific for MICA (28). As shown in Fig. 1*A*, U266 and LP1 cells stained with both mAbs at comparable levels, indicating that MICA was expressed by these cells, in agreement with previous observations (28, 29). We next investigated MICA expression by multiple myeloma plasma cells freshly derived from bone marrow samples of 10 newly diagnosed, untreated patients (Table 1). Multiple myeloma cells, gated as CD138$^+$ cells, expressed surface MICA with a rather heterogeneous pattern, ranging from negligible staining (ΔMFI < 20) with the

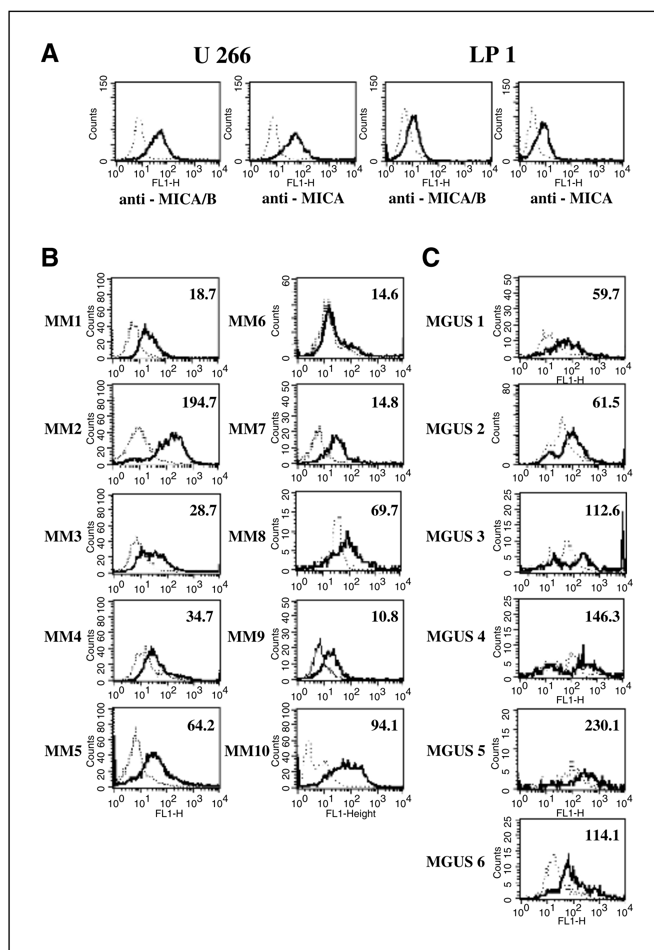

Figure 1. Multiple myeloma plasma cells express surface MICA antigen. *A,* myeloma cell lines U266 and LP1 were stained with the anti-MICA/B 6G6 mAb or the anti-MICA AMO1 mAb followed by a FITC-conjugated anti-mouse antiserum and analyzed by FACS. Results are expressed as green fluorescence intensity (arbitrary units, *x*-axis) versus cell number (*y*-axis). *Dotted lines,* negative control obtained with the FITC-labeled anti-mouse antiserum only. Cytofluorometric analyses of MICA expression by plasma cells freshly derived from bone marrow samples of (*B*) 10 multiple myeloma patients (MM1-MM10) and of (*C*) six patients with MGUS (MGUS1-MGUS6). Plasma cells were identified based on CD138/CD38 surface expression, as evaluated by FACS analysis. Results and negative control as in (*A*). Numbers indicate ΔMFI of MICA staining, which was calculated as described in Materials and Methods.

AMO1 mAb in four cases (multiple myeloma samples 1, 6, 7, and 9), to very high expression in sample MM2 (ΔMFI = 194.7) and MM10 (ΔMFI = 94.1; Fig. 1*B*). Similar results were obtained with the anti MICA/B mAb 6G6 (data not shown). Bone marrow infiltrating lymphocytes did not stain with the AMO1 mAb (data not shown), in agreement with previous reports that failed to detect MICA/B expression on PBMC from healthy donors (23) and on nonmalignant lymphocytes from leukemia patients (28).

Finally, when we analyzed MICA surface expression by CD38^{bright} or CD38$^+$/CD138$^+$ monoclonal plasma cells from bone marrow of patients with MGUS (Table 2), we found that in all (six of six) patients the molecule was present at high levels, with ΔMFI ranging from 59.7 to 230.1 (Fig. 1*C*). Statistical analyses indicate that ΔMFI of MICA expression by MGUS plasma cells were significantly (P < 0.05) higher than that of multiple myeloma plasma cells.

MICA expressed by multiple myeloma cell lines enhances effector functions of Vγ9/Vδ2 γδ lymphocytes. To investigate

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whether MICA expressed by multiple myeloma plasma cells may be involved in γδ cell/plasma cell interactions, we assessed the cytotoxic activity of Vγ9/Vδ2 T cell clones against U266 and LP1 myeloma cell lines. Whereas all γδ T-cell clones expressed high levels of surface NKG2D (Fig. 2*A, inset*) and U266 and LP1 cells expressed the ligand MICA (Fig. 1*A*), γδ cells displayed unremarkable cytolytic activity against both lines (Fig. 2*A*), indicating that MICA is not per se sufficient to promote target cell lysis. Preincubation of U266 and LP1 cells with the amino-biphosphonate pamidronate, which has been shown to activate Vγ9/Vδ2 T lymphocytes in a TCR-dependent manner (12–14), consistently increased killing by γδ T-cell clones, particularly in the case of U266 cells (Fig. 2*A*). The contribution of MICA engagement to lysis of pamidronate-treated MM cells was shown by inhibition experiments; as shown in Fig. 2*B*, preincubation of effector γδ cells with the specific anti-TCR mAb TiγA significantly (P < 0.005) inhibited killing of pamidronate-treated U266 cells. On the other hand, preincubation of target cells with the anti-MICA AMO1 mAb also significantly (P < 0.05) decreased γδ cytotoxic activity suggesting that MICA/NKG2D interaction enhances Vγ9/Vδ2 effector function, in agreement with previous observations (19). Comparable results were obtained with LP1 cells (data not shown).

U266 myeloma cells also promoted IFN-γ production by γδ lymphocytes only when preincubated with pamidronate (Fig. 2*C*). Again, inhibition experiments showed that cytokine production by pamidronate-activated γδ cells was mostly dependent on TCR triggering (61% inhibition of IFN-γ producing cells in the presence of TiγA mAb), but the addition of the anti-MICA mAb AMO1 could further decrease the percentage of IFN-γ^+ γδ cells (83% inhibition; Fig. 2*D*).

Killing of myeloma plasma cells by pamidronate-activated γδ lymphocytes is abrogated by upstream blockade of the mevalonate pathway. Amino-bisphosphonates share structural homology with identified γδ T-cell ligands (12–14); moreover, as a result of the blockade of the farnesyl pyrophosphate synthase along the mevalonate pathway (ref. 39; Fig. 3*A*), they may lead to intracellular accumulation of phosphorylated metabolites, which are in turn responsible for γδ cell activation (15). To determine the mechanism implicated in γδ cell recognition of pamidronate-treated U226 cells, we relied on mevastatin, a specific inhibitor of hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme of the mevalonate pathway (Fig. 3*A*). Pretreatment with mevastatin abrogated pamidronate-induced killing of U266 cells by γδ lymphocytes; conversely, cytotoxic activity elicited by IPP, a prototypical ligand of Vγ9/Vδ2 TCR which does not require to be processed or metabolized (11), was not affected by pretreatment with mevastatin, thus ruling out any toxic effect of the drug (Fig. 3*B*).

MICA enhances killing of plasma cells from multiple myeloma patients by Vγ9/Vδ2 T lymphocytes. We finally assessed the contribution of MICA to γδ cell killing of multiple myeloma plasma cells from patients. To this purpose, we evaluated cytotoxic activity of two bulk cultured Vγ9/Vδ2$^+$ populations, each against purified plasma cells freshly derived from bone marrow of four multiple myeloma patients and expressing different surface levels of MICA (see Fig. 1). In particular, we used as targets myeloma cells from MM10, which expressed high MICA levels (ΔMFI = 94.1), from MM7 and MM9, which expressed negligible MICA levels (ΔMFI = 14.8 and 10.8, respectively), and from MM3, expressing low levels (ΔMFI = 28.7) of MICA. γδ lymphocytes did not kill patients' multiple myeloma plasma cells, unless they were pretreated with pamidronate

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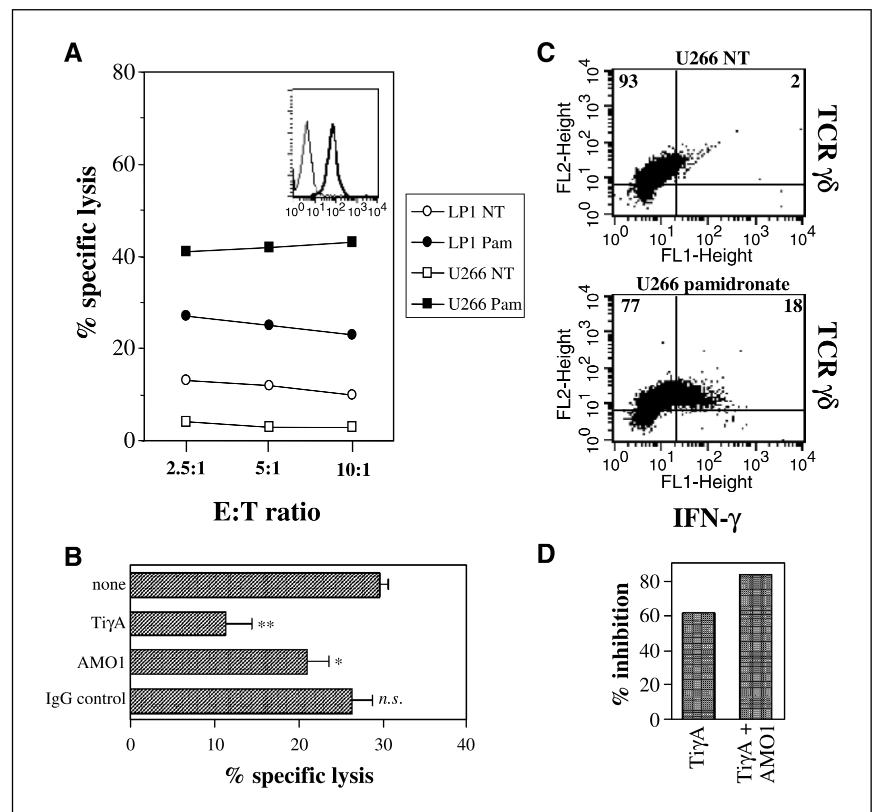

Figure 2. MICA engagement enhances effector functions of pamidronate-activated γδ lymphocytes. *A,* cytotoxic activity of γδ lymphocytes against untreated (*NT*) or pamidronate-treated (*Pam*) LP1 and U266 cell lines. Percentage of specific lysis at a given effector/target (E:T) ratio; representative of three independent experiments. *Inset,* surface expression of NKG2D receptor by γδ lymphocytes, as evaluated by FACS analysis. *B,* effect of blocking of γδ TCR with the TiγA mAb and of MICA with the AMO1 mAb on pamidronate-treated U266 cell lysis was evaluated by cytotoxicity assay. Mouse IgG were used as negative control. *Columns,* means of triplicate wells at an E:T ratio of 10:1; *bars,* ±SD. **, P < 0.005; *, P < 0.05; addition of the TiγA mAb and of the AMO1 mAb significantly reduced γδ cytotoxic activity; *n.s.,* not significant. Representative of three independent experiments. *C,* intracellular content of IFN-γ (green fluorescence, *x*-axis) in γδ lymphocytes was determined following 6-hour coincubation at a 1:1 ratio with either untreated (*top*) or pamidronate-treated (*bottom*) U266 cells by dual parameter FACS analysis. γδ cells were identified based on surface staining with a specific anti-TCRγδ mAb (red fluorescence, *y*-axis). Representative of four independent experiments. *D,* blocking of IFN-γ production by γδ lymphocytes stimulated with pamidronate-treated U266 cells using the anti-TCRγδ TiγA mAb and the anti-MICA AMO1 mAb. Determination of IFN-γ^+ γδ lymphocytes was done by dual-variable FACS analysis. Percentage of inhibition calculated as described in Materials and Methods. Percentage of IFN-γ^+ γδ cells in the absence of mAbs was 28.

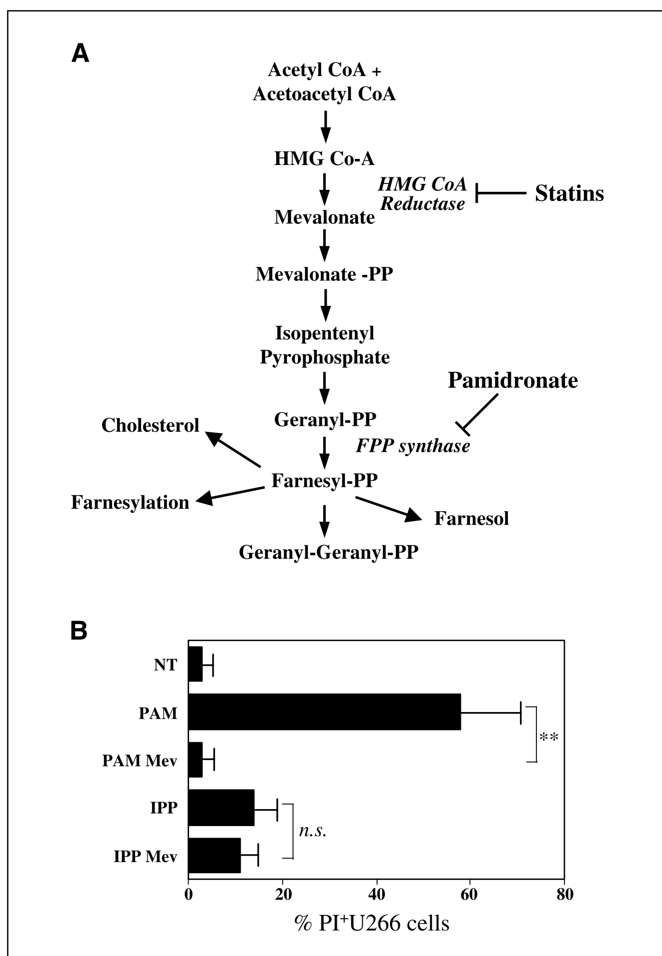

Figure 3. Killing of myeloma plasma cells by pamidronate-activated γδ lymphocytes is abrogated by blockade of the mevalonate pathway. *A,* schematic representation of the mevalonate pathway. Enzymatic blockade by mevastatin and by pamidronate is indicated. HMG-CoA, hydroxymethylglutaryl-CoA; PP, pyrophosphate; FPP, farnesyl pyrophosphate. *B,* effect of preincubation with mevastatin (*mev*) on U266 killing by γδ lymphocytes was evaluated by FACS analysis. U266 cells, either preincubated or not with 25 μmol/L mevastatin, were incubated with 1 mmol/L pamidronate or with 15 μmol/L IPP; untreated (*NT*) cells served as control. U266 cell death following coincubation with γδ lymphocytes was calculated as the percentage of TCRγδ-negative cells that failed to exclude propidium iodide. *Columns,* means of four independent experiments; *bars,* ±SD. **, $P < 0.005$, reduction in U266 killing by pretreatment with mevastatin was significant (Student's *t* test). *n.s.,* not significant.

(Fig. 4*A*). Notably, susceptibility of multiple myeloma cells to γδ cell killing varied according to their surface MICA levels, as indicated by the significantly ($P < 0.05$) higher specific lysis of MM10 compared with MM7 and MM9 (Fig. 4*A*). The contribution of MICA to killing by pamidronate-activated γδ lymphocytes was further strengthened by inhibition experiments (Fig. 4*B*), showing that addition of the anti-MICA AMO1 mAb significantly ($P < 0.005$) decreased γδ cytotoxic activity against multiple myeloma cells expressing high MICA levels (MM10) but not against those expressing low (MM3) or negligible (MM9) levels.

Discussion

In the present study, we show that MICA antigen is expressed not only by multiple myeloma but also by MGUS plasma cells; moreover, MICA enhances effector functions of pamidronate-activated γδ lymphocytes.

MICA expression has recently been reported in bone marrow–derived myeloma cell lines, as well as in primary multiple myeloma plasma cells from patients (29). We extend here the observation to bone marrow plasma cells freshly derived from most (6 of 10) additional patients with newly diagnosed multiple myeloma. The antigen was expressed with a rather heterogeneous pattern, without any obvious correlation with disease stage or other clinical features. Notably, we could also show MICA expression by monoclonal plasma cells from all (six of six) patients with MGUS; moreover, compared with multiple myeloma, MICA was expressed by MGUS plasma cells at significantly higher levels. Our data are in agreement with the reported loss of the antigen with disease progression (29), which was proposed to result from tumor shaping by the host immune response.

MGUS is considered a preneoplastic condition, and a role for the host immune response in controlling malignant progression has been hypothesized (32, 33). Indeed, in monoclonal gammopathies progression to clinical malignancy was found to be associated with an impairment in both $CD4^+$ and $CD8^+$ and NKT cell function (32, 33). Our present data of a decreased expression of MICA in

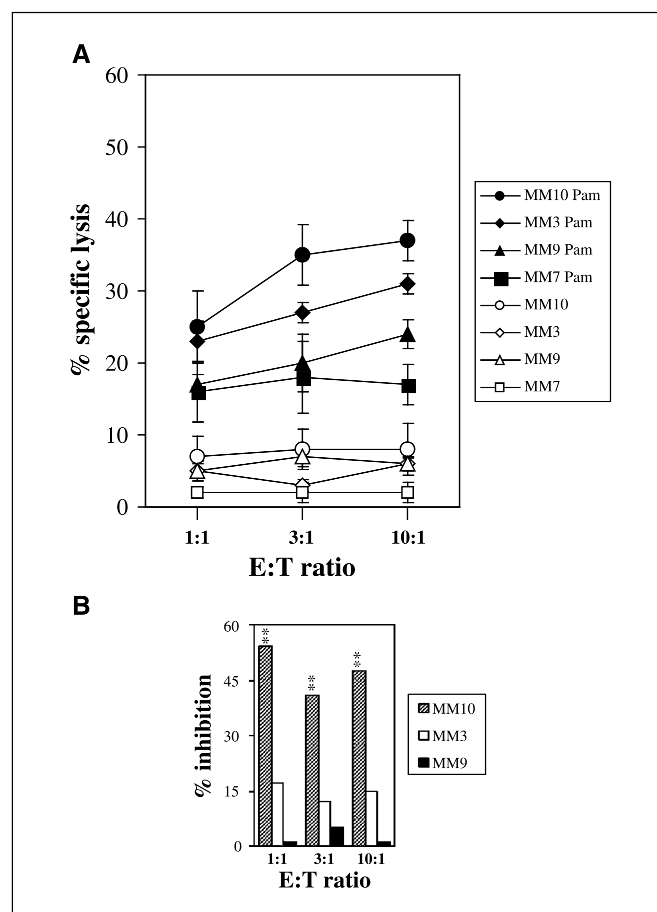

Figure 4. MICA enhances γδ lymphocytes killing of multiple myeloma plasma cells from patients. *A,* cytotoxic activity of γδ lymphocytes against untreated (○, □, △, ◇) or pamidronate-treated (●, ■, ▲, ◆) plasma cells derived from bone marrow of multiple myeloma patients. Percentage of specific lysis at a given effector/target (E:T) ratio. *Points,* means of experiments done with two bulk cultures; *bars,* ±SD. *B,* effect of blocking of MICA with the AMO1 mAb on pamidronate-treated multiple myeloma cell lysis was evaluated by cytotoxicity assay. Percentage of inhibition calculated as described in Materials and Methods. **, $P < 0.005$, Specific lysis of MM10 plasma cells in the presence or absence of AMO1 mAb was significantly different (Student's *t* test).

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multiple myeloma compared with MGUS plasma cells suggest that also this system may be implicated. MICA is a stress-inducible antigen (23), and its expression may be perceived by the immune system as a "danger" signal (18). The relevance of NKG2D/NKG2D ligands in tumor immune surveillance has been outlined in animal models (40, 41) and also in the immunity against human hematologic malignancies (27–29).

We show here that Vγ9/Vδ2 T lymphocytes also interact with multiple myeloma plasma cells via MICA. At variance with NK cells (18), MICA was not sufficient for γδ activation, as indicated by the lack of killing activity and cytokine production when γδ lymphocytes were coincubated with MICA-expressing myeloma cells in the absence of pamidronate. Pamidronate-activated γδ cells kill multiple myeloma plasma cells both *in vitro* and *ex vivo* (12), and immune therapy of multiple myeloma through pamidronate administration was shown to lead to objective tumor responses (42). Our present data further confirm the efficacy of amino-biphosphonates; in fact, pamidronate treatment of both myeloma cell lines and multiple myeloma plasma cells freshly derived from patients substantially increased γδ effector function. The mechanisms whereby amino-biphosphonates activate Vγ9/Vδ2 T lymphocytes are at present not completely elucidated. Amino-biphosphonate treatment leads to intracellular accumulation of phosphate metabolites as a result of the downstream blockade of the mevalonate pathway (Fig. 3*A*). Endogenous mevalonate metabolites are responsible for the activation of Vγ9/Vδ2 T lymphocytes by Daudi Burkitt's lymphoma (15). Our present data on the abrogation of γδ cytotoxic activity by pretreatment with mevastatin suggest that this mechanism is also responsible for pamidronate-induced recognition of myeloma cells.

In addition to TCR triggering, results from inhibition experiments underlie a role for MICA antigen in γδ/plasma cells interactions. This is further suggested by the significantly higher cytotoxicity of pamidronate-activated γδ lymphocytes against MICA-expressing versus MICA-negative multiple myeloma plasma cells from patients. Our data are then compatible with a costimulatory effect by MICA on TCR-induced functions of γδ lymphocytes, in agreement with the mechanisms described for Vγ9/Vδ2 T lymphocytes recognizing mycobacterium-infected dendritic cells (20). The dual interaction between Vγ9/Vδ2 T lymphocytes and multiple myeloma plasma cells may have implications for the design of immunotherapeutic interventions. It should be noted, however, that not only MIC is lost in late-stage multiple myeloma (29), but that it can be shed from advanced tumors as a soluble form, which may cause NKG2D down-regulation and hence impairment of antitumor immunity (43). This indicates that the antineoplastic potential of pamidronate-activated γδ lymphocytes could best be exploited in the immune therapy of early-stage multiple myeloma, and in perspective, in a prophylactic setting (44), of premalignant disease.

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