Clearing soluble MIC reverses the impaired function of natural killer cells from patients with multiple myeloma


ABSTRACT

Background Major histocompatibility complex (MHC) class I chain-related protein (MIC) is a stress-induced ligand released from multiple myeloma (MM) cells during progression, and soluble MIC impairs natural killer group 2D (NKG2D) activating receptor-mediated recognition and function of natural killer (NK) cells. However, whether clearing soluble MIC with a monoclonal antibody (mAb) can restore NK cell activity of MM patients remains undetermined.

Methods We analyzed The Cancer Genome Atlas (TCGA) Multiple Myeloma Research Foundation (MMRF) CoMMpass data set to examine the prognostic significance of MIC expression in MM. We examined the level of soluble MIC in paired peripheral blood (PB) and bone marrow (BM) plasma of patients with MM at diagnosis by ELISA. We evaluated the correlation between the level of soluble MIC and immunophenotype of NK cells from MM patients by multicolor flow cytometry. We also generated MIC-overexpressing MM cell line and characterized the cytotoxic function of patient NK cells in the presence of soluble MIC, and examined the impact of clearing soluble MIC with a humanized mAb (huB10G5).

Results We characterize the importance of MICA in MM by revealing the significantly better overall survival of patients with high MICA expression from TCGA MMRF CoMMpass data set. The level of soluble MICA is more highly elevated in MM than in precursor stages, and the concentration of soluble MICA is higher in BM plasma than in PB. The concentration of soluble MICA in BM was correlated with myeloma burden, while it was negatively correlated with the frequency of NKG2D+ NK cells in diagnostic BM aspirates of MM patients. Soluble MICA downregulated NKG2D expression and decreased cytotoxicity of MM patient NK cells ex vivo, which were reversed by a humanized soluble MIC-clearing mAb (huB10G5) with enhanced degranulation of NK cells.

Conclusions Our findings indicate targeting soluble MIC with huB10G5 might be a viable therapeutic approach to promote NKG2D-dependent cellular immunotherapy outcome in MM.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Major histocompatibility complex class I chain-related protein (MIC) is shed from multiple myeloma (MM) cells during the progression, and soluble MIC impairs natural killer group 2D (NKG2D)-mediated recognition and function of natural killer (NK) cells.

WHAT THIS STUDY ADDS

⇒ Clearing soluble MIC with an MIC-neutralizing monoclonal antibody (huB10G5) restored downregulated NKG2D and reversed impaired cytotoxicity of NK cells from MM patients with enhanced degranulation against MM cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our work provides insights into NK cell dysfunction during myeloma progression and supports further preclinical and clinical development of huB10G5 to improve NKG2D-mediated cellular immunotherapy outcomes in MM.

INTRODUCTION

Multiple myeloma (MM) is a malignancy of plasma cells associated with the dysregulated bone marrow (BM) immune microenvironment.1 Patients receive a combination treatment of proteasome inhibitors, immunomodulatory drugs (IMiDs), and monoclonal antibodies (mAbs), followed by high-dose chemotherapy with autologous stem cell transplantation if transplant-eligible.2 Despite continuous therapy and maintenance treatment, many patients ultimately experience relapse, and patients with relapsed or refractory MM (RRMM) may benefit from subsequent therapies with chimeric antigen receptor (CAR) T cells or bispecific T-cell engagers although with a sustained risk of disease progression.3–5 Therefore, treatments that yield durable responses still require further development, and there is a need for more studies on the implementation of new therapeutic approaches to overcome the immunosuppressive myeloma microenvironment.6–8 Myeloma cell survival depends on the interplay with the immune cells of BM where they
It is becoming evident that the BM cellular components, in addition to the cytokines and soluble molecules, undergo considerable alterations toward immune evasion during myeloma progression from monoclonal gammapathy of undetermined significance (MGUS), smoldering MM (SMM), and MM. In particular, natural killer (NK) cell differentiation and function are impaired in MM as well, as patients with MM have higher proportion of terminally differentiated CD57+ NK cells with dysregulated expression of genes related to their effector function. Therefore, NK cells are regarded as one of the important components of BM microenvironmental changes during myelomagenesis, and myeloma treatments such as IMiDs and mAbs at least partially rely on NK cells in potentiating their antitumor activity.

Our group and others have also shown that adaptive NK cells represent a distinct subset of NK cells in MM and that they robustly mediate antibody-dependent cellular cytotoxicity (ADCC). However, the mechanism underlying NK cell dysfunction during myeloma progression is largely unexplored.

NK cell effector functions are regulated by various activating and inhibitory signals. Above all, natural killer group 2D (NKGD2) is one of the major activating receptors on NK cells that recognizes stress-induced ligands: major histocompatibility complex class I chain-related molecules A/B (MICA/B) and the UL16-binding proteins 1 to 6 (ULBP1–6). NKGD2 ligands are ideal immunotherapeutic targets as their expression is induced by cellular stress, whereas it is restricted in healthy tissues. However, advanced-stage tumors release surface MICA to produce soluble MIC (sMIC) through proteolysis and tumor-derived extracellular vesicles, leading to escape from NKGD2-mediated immune surveillance. Moreover, evidence from observational human studies and mouse tumor models have shown that soluble NKGD2 ligands downregulate NKGD2 on antitumor effector cells and impair their function.

In MM, MICA expression is observed on myeloma cells and the release of sMICA was shown to dampen NKGD2-mediated cytotoxicity of NK cells. Furthermore, high level of sMICA in peripheral blood (PB) serum of MM patients was shown to correlate with poor prognosis.

Considering the growing appreciation of the immunosuppressive effect of sMIC, therapy with the sMIC-clearing mAb B10G5 was developed and shown to effectively relieve sMIC-mediated immune suppression in relevant preclinical solid tumor models. The murine B10G5 enhanced the effector functions of cytotoxic lymphocytes in humanized mouse models of prostate cancer and melanoma in large by restoring NK cell homeostasis and function in vivo. However, whether clearing sMIC with the humanized B10G5 (huB10G5) in MM patients can restore NK cell cytotoxic activity against MM cells is unknown.

Here, we investigated the clinical significance of NKGD2 ligand expression in MM by analysis of The Cancer Genome Atlas (TCGA) MM data set. We also examined the levels of soluble NKG2D ligands in paired PB and BM plasma of patients with MGUS, SMM, and newly diagnosed MM (NDMM). We further show that sMICA selectively downregulates NKGD2 on NK cells and impairs their cytotoxicity against MM cells. We demonstrate that clearing sMIC with huB10G5 restores NKGD2 expression and cytotoxicity of MM patient NK cells associated with increased degranulation. Our study suggests that clearing sMIC with huB10G5 can potentially enhance the outcome of NKGD2-mediated cellular immunotherapy for MM.

**Results**

**High MICA expression is associated with better prognosis in MM**

To gain insight into the levels of NKGD2 ligand in MM, we first analyzed the RNA sequencing (RNA-seq) data sets available from TCGA across three different types of lymphoid malignancies. These included gene expression data of the NDMM from the Multiple Myeloma Research Foundation (MMRF)-CoMMpass data set, diffuse large B-cell lymphoma (DLBCL) from the National Cancer Institute Center for Cancer Research (NCICCR)-DLBCL data set, and acute lymphoblastic leukemia (ALL) from the TARGET-ALL-P2 data set. The CoMMpass is a longitudinal prospective study of patients with MM containing transcriptomic data of sorted BM CD138⁺ plasma cells at the time of MM diagnosis (NCT01454297), NCICCR-DLBCL includes transcriptome sequencing data of DLBCL lymph node (LN) biopsies, and TARGET-ALL-P2 contains transcriptomic data of BM or PB of ALL. Notably, the expression level of MICA gene was significantly higher in BM of patients with NDMM compared with the LN of DLBCL, BM or PB of B-cell ALL (B-ALL), or BM of T-cell ALL (T-ALL) (figure 1A). Further analysis of BM CD138⁺ plasma cells from MMRF-CoMMpass data set revealed the enriched messenger RNA (mRNA) expressions of MICA and MICB in patients with MM (figure 1B). Indeed, significant correlation between the level of MICA and MICB genes were observed (online supplemental figure 1A), supporting the previous data indicating strong homology between them. However, the levels of ULBP1–6 expressions were negligible, and no correlation between MICA and ULBP1–3 was observed (figure 1B and online supplemental figure 1B–D).

To examine the clinical relevance of MICA gene expression in MM, we determined an optimal cut-off of 2.896 fragments per kilobase of exon per million (FPKM) to distinguish high and low expression of MICA from the receiver operating characteristic (ROC) curve in MMRF-CoMMpass data set (online supplemental figure 1E). Importantly, patients with NDMM with a high level of MICA expression demonstrated significantly better overall survival (OS) compared with patients with low level of MICA expression (figure 1C). The OS benefit in patients with high MICA expression remained significant across the International Staging System (ISS) stage I, II, and III.
Figure 1  High MICA expression is associated with better survival of MM patients. (A) Comparison of MICA gene expression in fragments per kilobase million (FPKM) from TCGA MMRF-CoMMpass data set (NCT01454297) in bone marrow (BM) of patients with newly diagnosed multiple myeloma (MM) (n=565), lymph node (LN) of diffuse large B-cell lymphoma (DLBCL) (n=481) from the National Cancer Institute Center for Cancer Research-DLBCL data set, BM (n=111) or peripheral blood (PB) (n=54) of B-cell acute lymphoblastic leukemia (B-ALL), and BM of T-cell acute lymphoblastic leukemia (T-ALL) from the TARGET-ALL-P2 data set (n=202). Each dot indicates a value obtained from one patient. P values were determined versus MM using the Mann-Whitney U test. (B) Expression of natural killer group 2D ligands in TCGA MMRF-CoMMpass cohort (n=565). Each dot indicates a value obtained from one patient. (C–F) Kaplan-Meier plot for overall survival (OS) according to the level of MICA expression in the entire MM cohort (n=565) (C), and across the International Staging System (ISS) stages (D–F). P values were determined using a log-rank test. MICA, major histocompatibility complex class I chain-related molecule A; MMRF, Multiple Myeloma Research Foundation; ULBP1–6, UL16-binding proteins 1 to 6; TCGA, The Cancer Genome Atlas; No., number.
disease, but the difference in survival was most prominent in ISS stage I disease (figure 1D–F), supporting the previous work demonstrating the importance of NKG2D-mediated immune surveillance in the early stage of MM progression.43 On the other hand, no significant differences in OS were observed according to the expression levels of MICB or ULBP1–3 (online supplemental figure 1F–I).

**sMICA is enriched in the BM plasma of MM patients**

To better define the role of sMICA during myeloma progression, we examined the levels of sMICA by ELISA in plasma from paired PB and BM of healthy donors (n=9), patients with MGUS (n=30), smoldering multiple myeloma (SMM) (n=16), and multiple myeloma (MM) (n=74) at diagnosis (figure 2A). The baseline characteristics of patients with NDMM according to the BM plasma level of sMICA are described in online supplemental table 1. Of note, plasma levels of sMICA in PB and BM were significantly higher in patients with MM compared with those of healthy donors, MGUS, or SMM (figure 2A). Indeed, the level of sMICA was higher in SMM than in MGUS (figure 2A). Particularly, sMICA was higher in plasma of BM than in paired PB across all patients (figure 2A). BM plasma level of sMICB was also significantly higher in patients with MM compared with healthy donors (figure 2B). However, there were no significant differences in BM plasma levels of soluble ULBP1 and ULBP2 between healthy donors and patients with MM, and soluble ULBP3 was not detected in BM plasma of both healthy donors and patients with MM (figure 2B). When we stratified patients with NDMM according to the low and high level of soluble NKG2D ligands, patients with high level of sMICA seemed to have inferior survival in terms of progression-free survival (PFS) and OS compared with patients with low level of sMICA, but this was not statistically significant (online supplemental figure 2A). Additionally, no differences in survival were observed according to the BM plasma levels of sMICB, soluble ULBP1, nor soluble ULBP2 (online supplemental figure 2B–D).

We also analyzed the cell surface expression of NKG2D ligands on BM plasma cells with multicolor flow cytometry (online supplemental figure 3A). The surface expression...
levels of NKG2D ligands on CD38<sup>+</CD138</sup> plasma cells were heterogeneous, but plasma cells of patients with MM had higher surface MICA expression compared with those of patients with MGUS or SMM (online supplemental figure 3B,C). However, surface expressions of other NKG2D ligands including MICB and ULBP1–3 on plasma cells were not significantly different across patients with MGUS, SMM and MM (online supplemental figure 3B,C).

The level of sMICA is negatively correlated with the frequency of NK2G<sup>+</</sup> NK cells in diagnostic BM aspirates of MM patients

As the progression of MM has been shown to be associated with MICA shedding,<sup>43</sup> we next analyzed the level of soluble NKG2D ligands in BM plasma in correlation with the surface expression of NKG2D ligands on malignant plasma cells from the paired BM plasma and BM mononuclear cell (BMMC) samples from the same patient. ULBP3 was excluded from the subsequent analysis as soluble ULBP3 was not detected in both PB and BM of patients with MM (figure 2B). We did not observe significant correlation between soluble NKG2D ligands of BM plasma and the surface NKG2D ligand expressions on the BM plasma cells of paired samples (figure 3A). However, plasma cell percentage among BMMCs assessed from the diagnostic BM aspirates, which is integral to MM diagnosis,<sup>48</sup> exhibited significant correlation with the level of sMICA, but not sMICB or soluble ULBP1–2 (figure 3B). Consistently, patients with MM with elevated BM plasma level of sMICA had significantly higher percentage of BM plasma cells at diagnosis compared with MM patients with low BM plasma level of sMICA, although other patient characteristics were evenly distributed between the groups (online supplemental table 1).

We then examined the frequency of NK cells in BM aspirates by multicolor flow cytometry, as MICA and NKG2D-dependent antitumor immunity was shown to be mediated by cytotoxic lymphocytes (online supplemental figure 4).<sup>46</sup> We analyzed CD56<sup>dim</sup>CD16<sup>+</sup> NK cells as this subset predominantly mediates cytotoxicity.<sup>50</sup> We did not observe any correlation between the frequency of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells among BMMCs and the level of soluble NKG2D ligands in the corresponding BM plasma (figure 3C). However, the frequency of NKG2D<sup>+</sup> NK cells among CD56<sup>dim</sup>CD16<sup>+</sup> NK cells significantly and negatively correlated with the level of sMICA in the BM plasma, although this correlation was not observed for sMICB nor soluble ULBP1–2 (figure 3D).

In addition to NKG2D, DNAX accessory molecule-1 (DNAM-1) is another activating receptor expressed by NK cells, but the frequency of DNAM-1<sup>+</sup> NK cells among CD56<sup>dim</sup>CD16<sup>+</sup> NK cells did not show significant correlation with the examined soluble NKG2D ligands (figure 3E). These results suggest that sMICA is preferentially and negatively correlated with the expression of NKG2D on NK cells.

Recombinant human MICA selectively downregulates NKG2D on NK cells

To better define the role of sMICA on NK cells of patients with MM, we treated NK-92MI cell line with soluble recombinant human MICA (rhMICA) and examined the changes in the level of activating and inhibitory receptors of NK cells by flow cytometry. Notably, rhMICA selectively and significantly reduced the expression of NKG2D on NK cells in a concentration-dependent manner, while no significant changes were observed for other activating receptors such as DNAM-1, and natural cytotoxicity triggering receptors Nkp46 and Nkp30 (online supplemental figure 5A). In addition, the level of inhibitory receptors including killer cell immunoglobulin-like receptor (KIR), NKG2A, T cell immunoreceptor with immunoglobulin and mucin domain-3 (TIM-3) on NK cells were not altered on treatment with rhMICA (online supplemental figure 5B). We did not examine lymphocyte-activation gene 3 (LAG-3) and programmed cell death protein 1 (PD-1), as we have previously shown minimal expression of these receptors on NK cells from MM patients.<sup>25</sup>

Clearing sMICA with huB10G5 restores NKG2D expression on NK cells of patients with MM

To assess whether clearing sMIC with huB10G5 could restore the downregulated NKG2D on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells, we treated BM cells from patients with NDMM ex vivo with rhMICA, corresponding patient plasma containing sMIC, or the patient plasma with both rhMICA and huB10G5 (figure 4A,B). Notably, rhMICA, but not plasma, selectively and significantly downregulated NKG2D expression on NK cells of patients with NDMM (figure 4A,B), presumably due to the limited concentration of sMICA in the plasma and the presence of additional soluble factors, chemokines, and cytokines that may regulate the NKG2D expression.<sup>51–54</sup>

Importantly, treatment with huB10G5 in the presence of both rhMICA and plasma reversed the downregulated NKG2D expression on CD56<sup>dim</sup>CD16<sup>+</sup> NK cells of patients with MM, while no significant changes were observed for other NK receptors (figure 4A,B). Similar trend was observed when we examined the population of CD56<sup>high</sup>CD16<sup>−</sup> NK cells (online supplemental figures 4,6). However, the expression of Nkp46 increased in the presence of plasma regardless of rhMICA in CD56<sup>dim</sup>CD16<sup>+</sup> NK cells and rhMICA slightly increased the proportion of Nkp46<sup>+</sup> cells among CD56<sup>high</sup>CD16<sup>−</sup> NK cells (figure 4A,B and online supplemental figure 6), suggesting their differential responsiveness to NKG2D downregulation associated with rhMICA.<sup>55–56</sup>

Beyond clearing sMIC, the huB10G5 was formatted as a human IgG1 which can mediate ADCC by NK cells. As we and others have recently shown that FcRγ<sup>+</sup> or NKG2C<sup>+</sup> adaptive NK cells with higher CD57 expression mediate superior ADCC compared with FcRγ<sup>−</sup> or NKG2C<sup>−</sup> conventional NK cells with lower CD57 expression in MM,<sup>23–26</sup> we divided NK cells into these two subsets and analyzed


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Figure 3  sMICA concentration is positively correlated with plasma cell burden but negatively correlated with NKG2D+ NK cells in MM patients. (A) Correlation of the NKG2D ligand positive cells among the gated plasma cells and the level of soluble NKG2D ligands in BMMCs and paired plasma of pooled patients with MGUS, SMM, and MM at diagnosis (n=22). (B–E) Correlation of BM plasma levels of soluble NKG2D ligands in diagnostic BM aspirates of patients with MM (n=74) examined with an ELISA and percentage of BM plasma cells in diagnostic BM aspirates (B) CD56dimCD16+ NK cells among BMMCs (C) NKG2D+ NK cells among CD56dimCD16+ NK cells (D) and DNAM-1+ NK cells among CD56dimCD16+ NK cells (E) in paired samples assessed by flow cytometry analysis. Each dot indicates a value obtained from one patient. Correlation coefficients (R) and P values were determined by Spearman’s rank-order correlation test. BM, bone marrow; BMMC, BM mononuclear cell; DNAM-1, DNAX accessory molecule-1; MM, multiple myeloma; NK, natural killer; NKG2D, natural killer group 2D; sMICA/B, soluble major histocompatibility complex class I chain-related molecule A/B; sULBP, soluble UL16-binding protein.
Figure 4  huB10G5 restored downregulated NKG2D by rhMICA in CD56<sup>dim</sup>CD16<sup>+</sup> NK cells from BMMCs of patients with MM. (A) Representative flow cytometry plots and (B) summary data showing percentage of positive cells among CD56<sup>dim</sup> CD16<sup>+</sup> NK cells after <em>ex vivo</em> treatment with IgG (5 µg/mL), rhMICA (10 µg/mL), BM plasma (20%), or combination of rhMICA (10 µg/mL), paired BM plasma (20%), and huB10G5 (5 µg/mL) for 6 hours in cultured BMMCs of patients with MM (n=10, except for n=9 for rhMICA) at diagnosis. Each dot indicates a value obtained from one patient. P values versus IgG, rhMICA, or plasma were determined using the Mann-Whitney U test. *P<0.05; n.s., not significant. BM, bone marrow; BMMC, BM mononuclear cell; DNAM-1, DNAX accessory molecule-1; FSC-H, Forward scatter height; KIR, Killer cell immunoglobulin-like receptor; MM, multiple myeloma; NK, natural killer; NKG2D, natural killer group 2D; NKp46/30, natural cytotoxicity triggering receptor 1/3; rhMICA, recombinant human major histocompatibility complex class I chain-related molecule A; TIGIT, T cell immunoreceptor with immunoglobulin and ITIM domain.
whether there are differences in the degree of NKG2D restoration after treatment with huB10G5 in the presence of rhMICA and patient plasma. There were no differences in the percentage of conventional and adaptive NK cells among CD56dimCD16+ NK cells after the treatment with rhMICA, plasma, or huB10G5 in the presence of both rhMICA and plasma (online supplemental figure 7A). Of note, huB10G5 restored downregulated NKG2D expression by rhMICA and plasma in both conventional and adaptive NK cells (online supplemental figure 7B).

As NKG2D has been shown to provide co-stimulatory signal for CD8+ T-cell activation, we also examined CD8+ and CD4+ T-cell subsets based on differentiation status including CCR7+CD45RA− naïve T (TNaive), CCR7+CD45RA+ central memory T (TCM), CCR7−CD45RA− effector memory T (TEM), and CCR7−CD45RA+ terminally differentiated effector memory T (TEMRA) cells (online supplemental figure 9A, B). We then examined the levels of the presence of both rhMICA and plasma (online supplemental figure 9C). We noted minor expression of NKG2D in combination with rhMICA and plasma (online supplemental figure 7A). Of note, huB10G5 restored downregulated NKG2D expression by rhMICA and plasma in both conventional and adaptive NK cells (online supplemental figure 7B).

Bortezomib enhances MICA expression and increases the level of sMICA

We then examined the changes in the level of surface and soluble NKG2D ligands in MM cell lines including SKO-007-MICA after treatment with anti-myeloma agents in doses that minimally affect cell viability (online supplemental figure 13A). Bortezomib or lenalidomide increased surface expression of MICA in U266B1 and SKO-007 cells, while the level of MICA expression remained high in SKO-007-MICA cells after the drug treatment (online supplemental figure 13B, C and figure 5A and B). However, the level of surface MICA remained unchanged in RPMI 8226 after treatment with bortezomib or lenalidomide, consistent with the previous study demonstrating the diversity of bortezomib-mediated responses in different MM cell lines. Other NKG2D ligands including MICB and ULBP1–3 were not expressed even after the drug treatment (online supplemental figure 13D–G).

huB10G5 clears bortezomib-induced increase in sMICA

Of note, bortezomib significantly increased the concentration of sMICA, while lenalidomide, dexamethasone, or daratumumab showed minimal impact on the level of sMICA in culture supernatant of SKO-007-MICA cells (figure 5C). When SKO-007-MICA cells were treated in combination with bortezomib, lenalidomide dexamethasone, and daratumumab, minimal...
A representative flow cytometry plots of surface MICA expression in SKO-007-MICA cells after treatment with DMSO (0.2%), bortezomib (V, 2.5 nM), lenalidomide (R, 20 µM), dexamethasone (d, 10 µM), daratumumab (D, 10 µg/mL), VRd, or D-VRd for the indicated time period. (B) Percent MICA positive cells among gated live cells measured by flow cytometry after treatment with indicated drugs. (C) Concentration of sMICA examined with ELISA in culture supernatant of SKO-007-MICA cells after treatment with IgG (5 µg/mL) or huB10G5 (5 µg/mL) for the indicated time period. P values for sMICA were determined versus DMSO using the Mann–Whitney U test. *P<0.05. DMSO, Dimethyl Sulfoxide; MICA, major histocompatibility complex class I chain-related molecule A; sMICA, soluble MICA.

Figure 5 huB10G5 cleared bortezomib-induced increase in sMICA in culture supernatant of SKO-007-MICA cells. (A) Expectedly, treatment with huB10G5 led to rapid and profound clearance of sMICA in culture supernatant of SKO-007-MICA cells even after treatment with bortezomib, lenalidomide, dexamethasone, and daratumumab (figure 5C).

huB10G5 reverses impaired cytotoxicity of patient with MM NK cells by clearing sMICA

To better delineate the role of huB10G5 on NK cell function, we assessed the cytotoxicity of NK cells from patients with MM against SKO-007-MICA cells in the absence or presence of huB10G5. For this purpose, we sorted NK cells from PB mononuclear cells (PBMCs) of patients with NDMM and co-cultured them with SKO-007-MICA cells in either fresh media, culture supernatant of 72 hours cultured SKO-007-MICA cells containing MICA, fresh media with low rhMICA (0.1 µg/mL), or fresh media with high rhMICA (10 µg/mL) at low (1:1) or high (3:1) effector-to-target (E:T) ratios (figure 6A).

Notably, the ex vivo cytotoxicity of NK cells from patient with MM was diminished on co-culture with culture supernatant of 72 hours cultured SKO-007-MICA cells containing MICA, fresh media with low rhMICA (0.1 µg/mL), or fresh media with high rhMICA (10 µg/mL) at low (1:1) or high (3:1) effector-to-target (E:T) ratios (figure 6A), in agreement with the dose-dependent downregulation of NKG2D on NK cells on rhMICA treatment (online supplemental figure 5A). However, treatment with huB10G5 abrogated the effects of co-culture in culture supernatant or fresh media with high rhMICA on the cytotoxicity.
of NK cells, which were evident in both low and high E:T ratios (figure 6A), suggesting the effective neutralization of the NK cell-suppressive effects of sMICA or rhMICA by huB10G5. Consistently, huB10G5 enhanced the NKG2D receptor expression on NK cells co-cultured in culture supernatant of SKO-007-MICA cells at both low and high E:T ratios (figure 6B,C). These results suggest that huB10G5 effectively restores sMICA-mediated or rhMICA-mediated inhibition of NKG2D receptor, which may contribute to the augmentation of NKG2D-dependent NK cytotoxicity on treatment with huB10G5.
huB10G5 promotes degranulation of patient with MM NK cells
To obtain further insights into the enhanced cytotoxic activity of NK cells by huB10G5 in the presence of sMICA, we characterized the effects of huB10G5 on NK cell effector functions. In this regard, we examined cytotoxic degranulation (CD107a) and expressions of cytotoxic molecules (perforin and granzyme B) and cytokines (interferon (IFN)-α and tumor necrosis factor (TNF)-α) in NK cells from BMMCs of patients with MM in either fresh media, culture supernatant, or fresh media containing high rhMICA, which were treated with either IgG or huB10G5. Similar to the cytotoxicity assay, degranulation of NK cells was diminished in culture supernatant or fresh media containing high rhMICA (figure 7A,B). However, huB10G5 reversed the decrease in degranulation of NK cells in the presence of cultured supernatant containing sMICA or high rhMICA (figure 7A,B), implying that triggering NK cell degranulation may be one of the rejuvenation mechanisms mediated by huB10G5. In spite of this, there were no differences in the expression levels of cytotoxic molecules or cytokines on treatment with huB10G5 (figure 7A,B). Taken together, we propose that sMICA inhibits degranulation and cytotoxicity of NK cells from MM patients in association with NK2D downregulation and clearing sMICA with huB10G5 can reverse these impairments (online supplemental figure 14).

DISCUSSION
In this study, we show the abundant expressions of MICA in MM and we demonstrate the prognostic significance of MICA by showing that patients with high level of MICA have significantly better OS compared with patients with low MICA expression according to the TCGA MMRF-CoMMpass data set, suggesting the importance of MICA-NKG2D axis in MM. TCGA analysis assesses MICA mRNA, not protein. The stressed MM cells may express surface MICA not as much as their mRNA level under higher endoplasmic reticulum stress.59 These tumor cells with more stress and high MICA mRNA may present a survival disadvantage, which may contribute to better clinical outcome of patients with MM. It is also possible that MM cells at the nascent stage express more surface-bound MICA and thus stimulate NK cells and T cells to control tumors. To distinguish these two possibilities, quantification of surface MICA in a large number of patients is needed for future investigations. We also reveal that sMICA is higher in plasma of BM than in PB and that sMICA is increased as MM progresses from MGUS and SMM. Patients with higher BM plasma level of sMICA at MM diagnosis seemed to have inferior prognosis in our cohort, although statistical significance was not reached. Nonetheless, this is supported by our data showing that the level of sMICA is positively correlated with the myeloma burden but negatively correlated with NK2D+ NK cells in diagnostic BM aspirates of patients with MM. sMICA downregulated the NKG2D receptor and impaired cytotoxicity and degranulation of NK cells from patients with MM. We show that clearing sMICA with huB10G5 restores NKG2D and repairs the defective NK cell cytotoxicity with enhanced degranulation (online supplemental figure 14).

The level of sMICA varied among patients, indicating that examining the level of sMICA at diagnosis especially for those with high BM tumor load will enable patient stratification to identify those that might benefit from sMICA clearance. Although we found no significant differences in survival according to the level of soluble NKG2D ligands in our cohort, a trend toward inferior survival was observed in patients with high sMICA. High level of sMICA was shown to be an independent adverse prognostic factor in NDMM.45 The minor difference in survival according to the level of sMICA in our study may be attributed to the different sample source; we examined both PB and BM plasma whereas the previous study only analyzed PB, but we found that the level of sMICA is higher in BM than in PB plasma. As most of the malignant plasma cells reside in the BM of patients with MM, the level of sMICA in BM plasma would better reflect both the actual MICA expression and sMICA level of patients with MM. Indeed, the previous work included both patients with NDMM and patients with RRMM while we examined only patients with NDMM.45 Moreover, significant advances in therapeutic strategies since then may have led to the different outcomes, as the patients in the earlier days of previous work were treated with regimens that are not currently used as frontline.45

Our data highlight sMICA as one of the BM microenvironmental modulator during MM progression, particularly in NK cells. MM evolves from the precursor states of MGUS and SMM, associated with the acquisition of additional genetic events in parallel with the initiating chromosomal alterations.1 However, major genetic changes occur early in the course of the disease, as noticeable by the fewer changes being identified between SMM and MM than between MGUS and SMM176083 indicating the importance of microenvironmental control. Our data suggest that sMICA may contribute to the NK cell dysfunction in MM by downregulating NK2D, and that sMICA may be targeted with huB10G5. We showed that huB10G5 reversed rhMICA-mediated NK2D downregulation in both conventional and adaptive NK cells. Our previous work demonstrated that the level of NK2D expression was not significantly different between conventional and adaptive NK cells in both PB and BM of patients with MM.45 In this regard, whether the NK2D restoration has differential impact on enhancing the ADCC of adaptive NK cells and promoting the natural cytotoxicity of conventional NK cells remains open question, as huB10G5 may also mediate ADCC.38

We demonstrated rapid clearance of sMICA by huB10G5 in MM cells producing large amounts of sMICA. Proteolytic shedding of MICA/B on myeloma cell is associated with the increased expression of ERp5 during myeloma progression,45 as ERp5 unfolds and exposes the proteolytic cleavage site of the α3 domain

Figure 7  Inhibition of NK cell degranulation by soluble major histocompatibility complex class I chain-related molecule A or rhMICA is relieved by huB10G5. (A) Representative flow cytometry plots of degranulation (CD107a) and expression of effector molecules (perforin, granzyme B, TNF-α, and IFN-γ) in NK cells from bone marrow mononuclear cells of patients with newly diagnosed multiple myeloma in the presence of IgG (5 µg/mL) or huB10G5 (5 µg/mL) for 6 hours in fresh media, 72 hours culture supernatant, or fresh media containing rhMICA (10 µg/mL). (B) Summary data of percentage of CD107a+, perforin+, granzyme B+, IFN-γ+, or TNF-α+ cells among gated NK cells from ex vivo treatment experiments described in (A). Each dot indicates a value obtained from one patient. P values versus IgG were determined using the Mann-Whitney U test. *P<0.05. n.s., not significant. IFN, interferon; NK, natural killer; rhMICA, recombinant human major histocompatibility complex class I chain-related molecule A; TNF, tumor necrosis factor.
of MICA/B. Patients with MGUS or SMM have higher level of surface MICA on myeloma cells compared with patients with MM, which is also associated with higher level of sMICA in MM.\(^\text{43}\) In addition to huB10G5, MICA/B α3 domain-targeting mAb (7C6), CAR NK cells, or vaccine preventing their cleavage showed efficacy in preclinical tumor models.\(^\text{49, 62-64}\) Whereas 7C6 inhibits the shedding of cell surface MICA/B and upregulates their basal expression with reduced serum concentration of sMICA/B,\(^\text{49}\) huB10G5 clears the shed sMICA/B from MM cells. Considering our data that patients with MM have high level of sMICA compared with precursor stage, huB10G5 may be suitable for the late-stage symptomatic disease, while α3 domain-targeting mAb, CAR NK cells, or vaccine may be beneficial in the early stages of myeloma such as high-risk SMM before increasing the concentration of shed sMICA/B. In addition, it would be valuable to assess the concentration of sMICA in BM of patients with RRMM to find out whether the level of sMICA may be useful to predict the NK2G2D-based cellular immunotherapy outcome. Because MICA/B are highly polymorphic and MICA genotype was shown to be associated with the level of sMICA and clinical response,\(^\text{46}\) whether huB10G5 differentially restores NK2G2D according to the MICA genotype is worth further investigation. In parallel, serial measurement of anti-sMIC antibodies may enable predicting response to evolving immunotherapies in MM, as anti-sMIC antibodies were shown to be higher in MGUS than in MM and effective immune control.\(^\text{43, 65}\) Furthermore, although we have mainly focused on the effect of NK cells in the restoration of antitumor immunity, more studies are needed to verify the effect of huB10G5 on CD8\(^+\) T cells and Vγ9/Vδ2 T cells.\(^\text{66}\)

We used rhMICA and paired BM plasma containing sMICA independently to examine the effect of sMICA on the level of NK2G2D expression in NK cells or T cells from BMMCs of patients with MM. rhMICA was employed because BM plasma contains confounding cytokines that might affect the expression of NK2G2D. Moreover, there are technical limitations in obtaining enough volume of first-pull BM aspirate from the patients, as dilution by PB must be minimized. We demonstrated that the patient NK cell cytotoxicity against MM cells is impaired in the presence of high sMICA or rhMICA, which is reversed by huB10G5. Our work is the first study incorporating patient with MM samples and huB10G5, showing the restoration of cytotoxic function in NK cells against MM cells. We performed ex vivo experiments with a predetermined concentration of sMIC, which was derived from the previous work demonstrating the impaired cytotoxicity of NK cells by sMIC.\(^\text{67}\) This concentration is higher compared with the level observed from patient plasma samples, but we employed this dose to minimize the effect of confounding factors in interpreting the functional consequence of clearing sMIC by huB10G5 in NK2G2D-mediated cytotoxicity of NK cells within a short period of incubation ex vivo. Our next approach with in vivo studies will examine this issue by exploring the effects of huB10G5 in physiologically relevant concentrations of sMIC in BM microenvironment of MM models or patients.

Recovery of NK cell cytotoxicity was associated with the enhanced NK cell degranulation, but not with the expression of cytotoxic molecules or cytokines, presumably due to the fast release of preformed granules containing perforin and granzymes from NK cells during the incubation. We revealed that bortezomib, one of the backbone therapies for MM, increases the concentration of sMICA in culture supernatant of SKO-007-MICA cells. This implies that sMICA may be one of the mechanisms of bortezomib-resistance in MM. We also showed that bortezomib enhances the surface expression of MICA in some MM cells as previously demonstrated,\(^\text{35}\) which may facilitate NK2G2D-mediated surveillance. Therefore, a better understanding of the alterations in sMICA dynamics relative to surface MICA and its functional consequence during or after bortezomib treatment is required to further devise combinatorial approaches employing huB10G5 to overcome bortezomib resistance.

Although there are two Food and Drug Administration-approved B cell maturation antigen (BCMA)-targeting CAR T cells for patients with RRMM, relapse after CAR T cell therapy is inevitable in MM.\(^\text{68}\) Moreover, many patients cannot get their CAR T cells in a timely manner as patients wait an average of 6 months due to the limited manufacturing slots.\(^\text{69}\) Considering the dismal prognosis of patients with RRMM with a median PFS of 4.6 months and even worse in patients with high-risk cytogenetics or extramedullary involvement,\(^\text{70}\) treatment delay can lead to poor outcomes in the real-world practice. Given these limitations, there is growing interest in bispecific antibodies and NK cells as an off-the-shelf immunotherapy platform, and NK2G2D CAR NK cells have lately shown preclinical efficacy against MM cells.\(^\text{27, 44, 71-73}\) Our data demonstrating that abundant MICA expression is correlated with better OS in patients with MM, whereas a high level of sMICA is related with poor prognosis, support further development of NK2G2D CAR NK cells in MM. However, our work suggests that the ability of NK2G2D CAR NK cells may be impaired in the context of large tumor volume and high level of sMICA, thus testing whether this could be overcome by huB10G5 might be a beneficial synergistic strategy to improve cellular immunotherapy outcome.\(^\text{74}\) This combination presents opportunities to improve NK2G2D-dependent NK cell-mediated immunotherapy strategies providing the advantage of not only clearing sMICA but also promoting ADCC, as huB10G5 and the NK2G2D receptor binds to MIC differently to allow engagement of NK2G2D on NK cells against MIC-expressing cells.\(^\text{38}\)

In conclusion, we show that the MICA/B gene expressions are enriched in MM and high level of MICA expression is associated with better OS. Clearing sMIC with huB10G5 restores downregulated NK2G2D and reverses impaired cytotoxicity of MM patient NK cells with enhanced degranulation against MM cells. Our results
provide clues regarding NK cell dysfunction in MM and support further preclinical and clinical development of huB10G5 to improve NKG2D-mediated cellular immunotherapy outcome in pathologies with high sMIC.

MATERIALS AND METHODS

Patient samples

We conducted the study in accordance with the Declaration of Helsinki and written informed consent was obtained from all study participants. We obtained paired PB plasma, PBMCs, BM plasma, and BMMCs at the time of MGUS, SMM, and MM diagnosis from treatment-naive patients between January 2018 and December 2022. BMMCs from healthy donors were acquired from diagnostic BM aspirates of healthy individuals. PB and BM samples were collected in EDTA-containing tubes and immediately centrifuged at 4000×g for 10 min for plasma collection. Plasma aliquots were stored at −80°C within 4 hours after sample collection. PBMCs and BMMCs were isolated by Ficoll (Cytiva, Massachusetts, USA) density-gradient centrifugation and cryopreserved for future use. The baseline characteristics of patients in this study are described in online supplemental table 1.

TCGA data analysis

Clinical data and transcriptomic profiling data were downloaded from MMRF-CoMMpass, NCICCR-DLBCL, and TARGET-ALL-P2 data sets (http://portal.gdc.cancer.gov/) using the R/Bioconductor package, TCGA Bio-views. The gene expression data on the TCGA database was obtained by RNAseq, and we used FPKM data transformed from count. To select patients with NDMM from the MMRF-CoMMpass data set (n=859), we selected 565 samples obtained from the BM of patients with primary MM within 1 month of initiation of treatment. According to the clinical data, among the 565 patients, 198 patients were ISS-I, 202 patients were ISS-II, 152 patients were ISS-III, and the others, 13 patients had no information regarding ISS. To determine the optimal cut-off value for dividing high and low groups of NKG2D ligand expression in patients with NDMM from MMRF-CoMMpass data set, ROC curves were analyzed by pROC R package (V.1.18). The best cut-point was determined at the point where the Youden’s Index was maximized. If the condition that area under the curve (AUC)>0.5 and P value<0.05 was satisfied, the cut-off value was set to the median value of gene expression. Kaplan-Meier survival curves were generated, and log-rank test was performed using survival R package (V.3.5). In the NCICCR-DLBCL data set (n=481), all samples were from the LNs of patients. Among the TARGET-ALL-P2 data set (n=532), 202 samples obtained from the BM of patients with T lymphoblastic leukemia/lymphoma were selected as T-ALL, and 165 samples obtained for BM (n=111), or PB (n=54) of precursor B-cell lymphoblastic leukemia were selected as B-ALL.

ELISA

Plasma samples from patients or supernatant of cultured cell lines were used for ELISA. The concentration of soluble NKG2D ligands in plasma or supernatant was measured by DuoSet ELISA (R&D Systems, Minnesota, USA) for human MICA (DY1306), MICB (DY1599), ULBP1 (DY1380), ULBP2 (DY1298) and ULBP3 (DY1517-05), using the procedures recommended by the manufacturers. Briefly, 96-well microplate was coated with the diluted capture antibody in phosphate-buffered saline without carrier protein. Then the plate was sealed and incubated overnight at room temperature (RT). Microplates were washed and the remaining buffer was removed by inverting the plate and blotting against the paper towel. Microplates were blocked by diluent and incubated at RT for an hour and washed again. Target samples were added to the plate and incubated for 2 hours at RT and then detection antibody was added to the washed microplates and incubated for 2 hours at RT. After washing, working dilution of streptavidin-horseradish peroxidase (HRP) was added and incubated for 20 min at RT and substrate solution was added for 20 min after washing. After addition of stop solution, absorbances were read at 450 nm using the VersaMax Microplate Reader (Molecular Devices). The concentrations of the standard controls were used to generate a standard curve using SoftMax Pro Software (V.6.2.2).

Flow cytometry

BMMCs or cell lines were stained using the Live/Dead Fixable Cell Stain Kit (Thermo Fisher Scientific, Massachusetts, USA) to exclude dead cells from the analysis. When analyzing plasma cells, BMMCs were immediately employed for flow cytometry staining without overnight resting in culture. After washing with flow cytometry (FACS) staining buffer, these cells were stained with the indicated fluorochrome-conjugated antibodies for 20 min at RT. For intracellular staining, surface-stained cells were fixed and permeabilized using an FoxP3 Staining Buffer Kit (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer’s instructions. Multicolor flow cytometry was performed using an FACSDlyric (BD Biosciences, New Jersey, USA) and the data was analyzed by FlowJo V.10 software (Treestar). The antibodies used for flow cytometry are detailed in online supplemental table 2, and the gating strategies are summarized in online supplemental figures 3A, 4 and 8.

Cell lines

RPMI 8226, U266B1, SKO-007, IM-9, Hs 602, RPMI 6666, Jiyoye, Daudi, HuT 78, H9, 293T, NK-92MI cell lines were obtained from Korean Cell Line Bank or American Type Culture Collection. According to the supplier’s recommendation, RPMI 8226, U266B1, and IM-9 cell lines were cultured in RPMI 1640 (Cytiva, Massachusetts, USA) supplemented 15% fetal bovine serum (FBS, Cytiva, Massachusetts, USA) with 1× Penicillin and Streptomycin (P/S, Thermo Fisher Scientific, Massachusetts, USA).
SKO-007 cell line was cultured in the same medium with 0.02 mg/mL 6-thioguanine (Merck, Massachusetts, USA), 1 mM sodium pyruvate and 2 mM L-glutamine (Corning, New York, USA) added. Hs 602, Daudi, and 293 T-cell lines were cultured in RPMI 1640 supplemented 10% FBS with P/S. RPMI 6666, Jiyoye, and H9 cell lines were cultured in RPMI 1640 supplemented 20% FBS with P/S. HuT 78 cell line was cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Lonza, Switzerland) supplemented 10% FBS with P/S. NK-92MI cell lines were cultured in RPMI 1640 supplemented 25% FBS with P/S. Cells were cultured at 37°C, 5% CO2 and confirmed to be Mycoplasma-negative with MycoAlert Detection Kit (Lonza, Switzerland).

Drug treatment
Cryopreserved BMMCs from patients with NDMM were thawed and incubated overnight in RPMI 1640 medium supplemented with 15% FBS and P/S. Cells were resuspended in RPMI 1640 medium supplemented with 15% FBS and 2 x 10^5 cells added to each well of a U-bottom 96-well plate. Plated BMMCs, MM cell lines, or NK-92MI cell line were cultured with rhMICA (Sino Biologicals, China), BM plasma, huB10G5 (provided by CanCure Bio, Japan) according to the supplier’s instruction. Briefly, lentiviral plasmid DNA diluted sterile water was added to a tube of Lenti-X Packaging Single Shots to form nanoparticle complex. The complex solution was added dropwise to 293 T cells preincubated overnight in a plate. The cells were incubated for 4 hours at 37°C, 5% CO2, and supplied additional fresh growth media. The lentiviral supernatant was collected 48–72 hours after transfection and filtered through a 0.45 μm filter, and then infected into SKO-007 cells with 8 μg/mL polybrene. Live MICA+ and ZsGreen+ transduced SKO-007 cells were sorted using an FACSAria III (BD Biosciences, New Jersey, USA).

Lentivirus production and transduction
SKO-007 cell line was transduced with lentiviral vector pHAGE-EF1a MICA*009-IRES-ZsGreen (Addgene, Massachusetts, USA), which consists of pHAGE-CMV-fullEF1a as backbone, MICA*009 as insert, and ZsGreen as reporter. Lentiviruses were produced in 293 T cells using Lenti-X Packaging Single Shots (VSV-G) (Takara Bio, Japan) according to the supplier’s instruction. Briefly, lentiviral plasmid DNA diluted sterile water was added to a tube of Lenti-X Packaging Single Shots to form nanoparticle complex. The complex solution was added dropwise to 293 T cells preincubated overnight in a plate. The cells were incubated for 4 hours at 37°C, 5% CO2, and supplied additional fresh growth media. The lentiviral supernatant was collected 48–72 hours after transfection and filtered through a 0.45 μm filter, and then infected into SKO-007 cells with 8 μg/mL polybrene. Live MICA+ and ZsGreen+ transduced SKO-007 cells were sorted using an FACSAria III (BD Biosciences, New Jersey, USA).

NK cell sorting
NK cells were isolated from PBMCs of patients with NDMM using a human NK Cell Isolation Kit (Miltenyi Biotec, Germany). After magnetic bead separation according to the manufacturer’s protocol, enriched cells were labeled with a mixture of BV510–CD3, APC-H7–CD14/CD19 and FITC–CD56 antibodies. Purity of CD3+CD14+CD19+CD56+ NK cell was >98% as checked by an FACSLyric (BD Biosciences, New Jersey, USA). The gating strategy for NK-cell is provided in online supplemental figure 4.

Cytotoxicity assay
Cytotoxicity assays were performed using SKO-007-MICA cells cultured in RPMI 1640 supplemented with 15% FBS, P/S, 0.02 mg/mL 6-thioguanine, 1 mM sodium pyruvate and 2 mM L-glutamine. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C and confirmed to be Mycoplasma-negative with MycoAlert Detection Kit (Lonza, Switzerland). The viability of SKO-007-MICA cells just before the initiation of the experiment was >95%. Target SKO-007-MICA cells were labeled with PKH26 dye (Merck, Massachusetts, USA) according to the manufacturer’s protocol, and then co-cultured with sorted NK cells with low and high E:T ratios (1:1 or 3:1) in the absence or presence of IgG (5 μg/mL) or huB10G5 (5 μg/mL) for 4 hours. Cells were then harvested and stained with TO-PRO-3 (Thermo Fisher Scientific, Massachusetts, USA) and monensin (BD Biosciences, New Jersey, USA) were added 1 hour after the initiation of co-culture, and the co-culture was maintained for 5 hours in the presence of antibody against CD107a. Cytotoxic molecules and cytokine production were assessed by intracellular staining using antibodies against perforin, granzyme B, IFN-γ, and TNF-α.

Statistical analysis
No statistical methods were used to predetermine sample size. PFS was measured from the time of diagnosis to disease progression or death. OS was defined from the time of diagnosis to the last follow-up or death. Survival was analyzed with the Kaplan-Meier method and log-rank test for comparison. The experiments were randomized, and investigators were blinded to allocation during experiments and outcome analyses. All values are presented as mean±SD. Categorical variables and continuous variables were compared as indicated in the figure legends. Significance was determined by the two-sided Mann-Whitney U test to compare two groups or the two-way analysis of variance with Holm-Sidak’s multiple comparisons test for multiple-group comparisons. Statistical analyses were performed using GraphPad Prism V.9.2 (GraphPad Software). Significance was set at P<0.05.

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Acknowledgements We appreciate Sujin Kim and Sujin Song (ABMRC) for technical support; Yugeon Hwang, Sowon Moon, Jisun Kim, Hee Won Ju, Soonhee Yu, Sunyoung Park, Sunhee Lee, Joo Hyoong Hwang and Minji Kim (Severance Hospital) for patient care and collection of samples; MID (Medical Illustration...


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increases NK-cell cytokine release capabilities.


