The Regulatory Effect of UL-16 Binding Protein-3 Expression on the Cytotoxicity of NK Cells in Cancer Patients

Xiao Mou1,2*, Yuepeng Zhou1,2*, Peng Jiang3#, Gianjiang1, Chengcheng Xu1,2, Hongli Liu1,2, Tingting Zheng1,2, Guoyue Yuan1, Yanyun Zhang5, Deyu Chen1,2 & Chaoming Mao1,2

1Department of Nuclear Medicine, The Hospital Affiliated to Jiangsu University, Zhenjiang, China, 2Institute of Oncology, The Hospital Affiliated to Jiangsu University, Zhenjiang, China, 3Department of anesthesiology, The Hospital Affiliated to Jiangsu University, Zhenjiang, China, 4Department of Pediatrics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, 5Key Laboratory of Stem Cell Biology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.

The activating immunoreceptor NKG2D (natural killer group 2, member D) and its ligands play important roles in the innate and adaptive immune responses. UL16-binding protein 3 (ULBP3), an NKG2D ligand, is overexpressed on certain epithelial tumor cells. In this study, we investigated the effect of ULBP3 expression on the cytotoxic activity of natural killer (NK) cells. ULBP3 were measured by flow cytometry analysis, immunohistochemistry, and time-resolved fluoroimmunoassay. The cytotoxicity of NK cells was determined with the lactate dehydrogenase release assay. We found that ULBP3 was overexpressed on tumor cell lines and tumor tissues. Serum from cancer patients, but not from healthy donors, contained elevated levels of soluble ULBP3 (sULBP3). Importantly, high expression of ULBP3 on the cell surface of tumor cells augmented NKG2D-mediated NK cell cytotoxicity. However, low levels of sULBP3 (<15 ng/ml) weakened the cytotoxicity of NK cells by decreasing NKG2D expression on NK cells. Further analysis showed that serum samples from most cancer patients (>70%) contained the low level of sULBP3. Our results demonstrate that tumor cells express surface and soluble ULBP3, which regulate NK cell activity. Thus, ULBP3 is a potential therapeutic target for improving the immune response against cancer.

Natural killer (NK) cells, components of the innate immune system, contribute to the elimination of virus-infected cells as well as to antitumor immune responses1. NK cell reactivity is guided by the principles of “missing-self” and “induced-self,” in which NK cells are activated by the downregulation or absence of major histocompatibility complex (MHC) I expression (“missing-self”) and/or by the stress-induced expression of ligands that bind activating NK receptors (“induced-self”). The balance of various activating and inhibitory signals determines whether NK cell responses are initiated2–5.

Among the activating NK receptors, NKG2D (natural killer group 2, member D) is particularly relevant for tumor cell recognition and killing. NKG2D is a C-type lectin-like activating receptor expressed on the cell surface of almost all NK cells, some cytotoxic CD8+ T cells, NK T cells, and γδ T cells, and a small subset of CD4+ γδ T cells6–8. NKG2D mediates NK cell activation by overcoming inhibitory signals from self recognition9,10. Malignant transformation induces the expression of NKG2D ligands (NKG2DL), as documented in a variety of human and mouse tumors. The activating immunoreceptor NKG2D endows cytotoxic lymphocytes with the capacity to recognize and eliminate malignant cells, and it plays a critical role in immune surveillance11. For example, NKG2DL-expressing tumor cell grafts were efficiently rejected, whereas parental NKG2D-ligand negative tumor cells formed tumors2–5. A distinctive feature of the NKG2D recognition system is that NKG2D can interact with a number of distinct ligands with affinities ranging from 4 to 400 nM11–16. The ligands recognized by NKG2D, which belong to distinct and relatively distantly related families, include major histocompatibility complex class-I related chain (MIC) A, MICB, and UL16-binding proteins (ULBPs) in humans16,17. NKG2DLs are generally not expressed on benign cells, but are induced by cellular stress, genotoxic stress, and infection18,19. The human ULBP proteins are widely expressed by various tumor types, including leukemia, and primary solid tumors20–22.

In addition to expressing NKG2DLs on their surface, tumors spontaneously release soluble ligands23. Soluble MICA secreted by tumor cells downregulated surface NKG2D expression on T cells to induce the functional impairment of anti-tumor immune effector cells, suggesting that shedding may reduce the expression of
NKG2DLs on the tumor cell surface and contribute to tumor escape from immunosurveillance. Soluble MICA induced the internalization and lysosomal degradation of the NKG2D receptor in CD8+ T and NK cells, further reducing the efficiency of NKG2D recognition. Elevated serum levels of soluble MICA have been detected in patients with various types of cancer and may represent a diagnostic marker in patients with suspected malignancies.

Unlike other NKG2DLs, ULBP3 has a moderate affinity for NKG2D. However, the regulatory function of ULBP3 in NK cells and its significance in cancer patients are largely unknown. In the present study, ULBP3 expression in several tumor cell lines and tumor tissue cells from common cancer types was analyzed. The effects of surface and soluble forms of ULBP3 on the interaction between tumor cells and NK cells were examined. Our results showed that ULBP3 regulated the activity of NK cells against tumors. Thus, ULBP3 provides a target for tumor immunotherapy.

Results
Elevated expression of ULBP3 in tumor cell lines and tumor tissues. To evaluate the distribution of the NKG2DL ULBP3 in tumor cells from common cancers, the surface expression of ULBP3 in SW620, K562, 7721, A549, and ECA109 cell lines was analyzed by flow cytometry (FCM) analysis. The colorectal cancer cell line CD133+ SW620 expressed high levels (>50%) of ULBP3 (59.0 ± 2.6%, n = 3), and CD133+ SW620 cells expressed moderate levels (20%–50%) of ULBP3 (22.0 ± 1.4%, n = 3). The liver cancer cell line 7721 also expressed a moderate level of ULBP3 protein (30.0 ± 3.7%, n = 3). However, surface ULBP3 protein was undetectable on the lung cancer cell line A549 and esophageal carcinoma cell line ECA109. The leukemic cell line K562, which does not express surface ULBP3, was used as a negative control (Figure 1A). We then examined the expression of ULBP3 in different tumor tissues. In cancer patients with colorectal cancer (n = 5), liver cancer (n = 3), lung cancer (n = 3), and gastric cancer (n = 7), FCS indicated that ULBP3 expression was much higher in the tumor tissue than in the adjacent non-tumor tissue (ANTT). Representative dot graphs are shown in Figure 1B. Immunohistochemistry results also showed that ULBP3 expression was upregulated in colorectal (n = 5), liver (n = 3), lung (n = 5), and gastric (n = 5) cancer tissue (Figure 1C). The results of RT-PCR analysis demonstrated that the ULBP3 gene was expressed in ULBP3-expressing tumor cell lines and in fresh tumor tissues from cancer patients, but not in ANTT (Figure 1D).

Spontaneous release of soluble ULBP3 (sULBP3) from tumor cell lines and elevated serum sULBP3 in cancer patients. To determine whether tumor cells release sULBP3, we used a time-resolved fluoroimmunoassay (TRFIA) with a Eu3+-labeled anti-ULBP3 monoclonal antibody. The detection limit of the TRFIA method for ULBP3-Fc was approximately 0.05 ng/ml (Figure 2A). After 48 h of culture, sULBP3 levels in the supernatants of SW620, K562, 7721, A549, and ECA109 cells were measured. No sULBP3 was detectable in the culture supernatants of K562, A549, and ECA109 cells or in the culture supernatants of resting or activated NK cells. However, the levels of sULBP3 were detected in the supernatants of the tumor cell lines CD133+ SW620 (3.6 ± 0.08 ng/ml), CD133+ SW620 (6.0 ± 0.1 ng/ml), and 7721 (4.3 ± 0.1 ng/ml) (Figure 2B). To determine whether ULBP3 was released by human tumors in vivo, we analyzed serum sULBP3 levels in 116 patients with various malignancies and 48 healthy volunteers. Compared with healthy volunteers (1.5 ± 0.1 ng/ml), the serum concentration of sULBP3 was significantly higher in cancer patients (colorectal cancer: n = 45, 14.4 ± 2.5 ng/ml, P < 0.001; gastric cancer: n = 38, 8.4 ± 1.1 ng/ml, P < 0.001; lung cancer: n = 33, 9.6 ± 1.4 ng/ml, P < 0.001) (Figure 2C). Further analysis showed that 73.3% of colorectal cancer patients, 83.3% of gastric cancer patients, and 82.3% of lung cancer patients were distributed in the low level of serum ULBP3 (<15 ng/ml), as shown in Table 1.

Differences in the functional effects of surface ULBP3 and sULBP3 on NK cell activity. Next, we assessed the effect of ULBP3 cell surface expression on the lytic capacity of effector NK cells. CD133+ SW620 (moderate expression of surface ULBP3), CD133+ SW620 (high expression of surface ULBP3), and CD133+ SW620 (no/low expression of surface ULBP3; Figure 3A) tumor cells were used as target cells, and NK cells were freshly isolated from healthy volunteers for use as effector cells in cytotoxicity assays. With CD133+ SW620, ULBP3 levels, the efficacy of NK-cell mediated killing was low when the effector to target cell (E:T) ratio was low (5:1 or 1:1) or high (10:1). However, a high level of cytotoxicity was observed with CD133+ SW620 cells, and a moderate level of cytotoxicity was found with CD133+ SW620 cells. These results suggested that the amount of ULBP3 expressed on the cell surface of tumor cells affected the cytotoxicity of NK cells (Figure 3B). In these experiments, blocking NKG2D on the surface of NK cells with a specific neutralizing monoclonal antibody strongly reduced the cytolytic activity of NK cells against the target cells at an E:T ratio of 10:1, indicating that the NKG2D/NKG2DL pathway was involved in modulating the cytotoxic activity of NK cells (Figure 3C).

To determine the effect of sULBP3 on the lytic capacity of NK cells, different concentrations of soluble recombinant ULBP3-Fc were added to the culture medium of tumor cells and NK cells, and cytotoxicity was measured. In the absence of surface ULBP3 expression on K562 and A549 cells, the addition of a low dose of sULBP3 (<15 ng/ml) reduced NK cell-mediated cytotoxicity against K562 cells at an E:T ratio of 10:1. However, no significant increase in NK cell cytotoxicity was observed when high concentrations of sULBP3 (>15 ng/ml) were added to co-culture of NK and target cells (Figure 4A and 4B). With 7721 and CD133+ SW620 cells expressing moderate to high levels of surface ULBP3, similar results were observed (Figure 4C and 4D). Then, we studied the effect of soluble ULBP3 on the lytic capacity of NK cells on different E:T ratios, and we found that, firstly, in the non-expression of surface ULBP3 on K562 and A549 target cells, the presence of low dose of sULBP3 (1 ng/ml) caused a statistically reduction of NK cell cytotoxicity against target cells at E:T ratio of 5:1 and 10:1. However, no significant increase of NK cell cytotoxicity was observed when high concentration of sULBP3 (30 ng/ml) was added in co-culture of NK and target cells (Figure 4E and 4F). Secondly, in the moderate/high expression of surface ULBP3 on 7721 and CD133+ SW620 target cells, the similar results were observed (Figure 4G and 4H).

To confirm that sULBP3 regulated NK cell-mediated cytotoxicity, we incubated sULBP3-containing supernatants for 30 min in the absence or presence of an NKG2D-Fc fusion protein (5 ng/ml) before adding the supernatants to co-culture of NK cells and tumor cells. Neutralization of sULBP3 by NKG2D-Fc diminished the inhibitory effect of the sULBP3-containing supernatants on NK cell cytotoxicity against K562 (Figure 5A), 7721 (Figure 5B), and CD133+ SW620 (Figure 5C) cells, and no significant effects were detected in the NKP44-Fc control group.

To determine the mechanism by which sULBP3 affects the activity of NK cells, NK cells were pre-incubated with different concentrations of ULBP3-Fc for 6 h, and NKG2D expression was measured. When ULBP3-Fc was added to the culture medium, NKG2D expression on NK cells decreased, particularly with 1 ng/ml ULBP3-Fc. A representative graph depicting NKG2D expression is shown in Figure 5D, and combined data are shown in Figure 5E.
and the percentage of NK cells in tumor tissues from 10 cancer patients (4 colorectal cancer, 3 lung cancer, and 3 gastric cancer). The percentage of infiltrating NK cells negatively correlated with ULBP3 expression ($R^2 = 0.759$, $P = 0.011$). Representative data from 4 patients are shown in Figure 6A, and combined data are shown in Figure 6B. We then compared NK cytotoxicity in healthy individuals and in cancer patients with low (<15 ng/ml) or high (>15 ng/ml) levels of sULBP3. Freshly isolated NK cells from the peripheral blood mononuclear cells (PBMCs) of healthy individuals ($n = 3$) and pre-therapy cancer patients (3 colorectal cancer, 2 lung cancer, and 3 gastric cancer) were used as effector cells. K562 cells served as target cells. The cytotoxicity of NK cells from cancer
patients with a low level of sULBP3 were decreased (n = 5, lysis% = 12.3 ± 3.6%, P < 0.05), compared with healthy individuals (lysis% = 27.9 ± 2.4%). However, the cytotoxicity of NK cells from cancer patients with a high level of sULBP3 were normal (n = 3, lysis% = 25.4 ± 3.7%).

Given that tumor cells generally express surface ULBP3 and sULBP3, we investigated whether an anti-ULBP3 antibody altered the cytotoxic activity of NK cells against ULBP3-expressing tumor cells. The lysis efficiency of the tumor cell lines CD133+SW620 (Figure 6B) and 7721 (Figure 6C) at an E:T ratio of 10:1 was enhanced in the presence of anti-ULBP3 monoclonal antibody (B2-F1-F1). Furthermore, when an antibody against CD16 receptor was added, the effect was blocked, suggesting that the elevated activity of NK cells resulted from ADCC. Consistently, the downregulation of NK cytotoxicity by sULBP3 in K562 (Figure 6D) or A549 (Figure 6E) cells lacking sULBP3 was also reversed by B2-F1-F1. Together, these results suggested that B2-F1-F1 abrogated the sULBP3-induced inhibition of NK cell cytotoxicity and induced surface ULBP3-mediated ADCC to enhance the cytotoxicity of NK cells.

Discussion

By regulating the innate and adaptive immune response through ligation of NKG2D, NKG2DLs are thought to be important for tumor initiation and development. For example, NKG2DL expression has been associated with prognosis, immunoeediting, and the control of an immunological checkpoint that relies on NKG2D-mediated immune responses during the epithelial-to-mesenchymal transition. NKG2DL expression is of particular interest because previous experiments demonstrated that the shedding of sULBP3 from tumor cells is directly related to the activity of NK cells against tumor cells. 

In our study, only a low dose of sULBP3 (15 ng/ml) decreased NK cytotoxicity by sULBP3. Furthermore, the percentage of infiltrating NK cells negatively correlated with ULBP3 expression. These results suggest that a low concentration of sULBP3 downregulated NK cell activity in vitro were consistent with the discovery that more than 70% of cancer patients had a low concentration (<15 ng/ml) of serum sULBP3. Furthermore, the percentage of infiltrating NK cells negatively correlated with ULBP3 expression. These results suggest that a low concentration of sULBP3 downregulated NK cell activity. These findings that a low concentration of sULBP3 in vivo is consistent with the discovery that more than 70% of cancer patients had a low concentration (<15 ng/ml) of serum sULBP3. Furthermore, the percentage of infiltrating NK cells negatively correlated with ULBP3 expression. These results suggest that a low concentration of sULBP3 downregulated NK cell activity. These findings that a low concentration of sULBP3 in vivo is consistent with the discovery that more than 70% of cancer patients had a low concentration (<15 ng/ml) of serum sULBP3.

Table 1 | sULBP3 distribution in serum of cancer patients

| Cancer Type       | Number | 15 ng/ml (%) | >15 ng/ml (%) |
|-------------------|--------|--------------|---------------|
| Colorectal cancer | 45     | 33(73.3)     | 12(26.7)      |
| Gastric cancer    | 38     | 31(83.3)     | 7(16.7)       |
| Lung cancer       | 33     | 28(82.3)     | 5(17.7)       |
soluble ULBP3 may have additive/synergistic effects and cooperate to reduce NK cell reactivity, thus enabling human tumor cells to evade NK-mediated surveillance. Given that tumor cells generally expressed surface ULBP3 and sULBP3, an anti-ULBP3 antibody could contribute to the activity of NK cells against ULBP3-expressing tumor cells. The neutralization of sULBP3 in patients might be a therapeutic strategy to enhance the immune response by promoting NK cell reactivity. Intriguingly, the addition of an anti-ULBP3 antibody prepared in our laboratory triggered ULBP3-mediated ADCC and increased NK cytotoxicity against ULBP3-expressing tumor cells, suggesting the antibody has immunotherapeutic value for cancer treatment.

Methods

Patient characteristics and clinical samples. The patients in this study were recruited from The Hospital Affiliated to Jiangsu University and were diagnosed through standard clinical and laboratory examinations. Healthy volunteers were selected based on normal physical and tumor marker examination. Peripheral blood
samples were obtained from patients before therapy. Serum was frozen at -80°C for subsequent analysis. Fresh tumor tissue and ANTT were obtained during surgery from cancer patients before they received treatment. The tissue was cleaved and digested with collagenase and pancreatin. Single tumor cell suspensions were washed with PBS and resuspended. All patients and healthy volunteers provided written informed consent in accordance with the guidelines of the Ethics Committee of The Hospital Affiliated to Jiangsu University. This study has been approved by the Ethics Committee of The Hospital Affiliated to Jiangsu University, and was performed in accordance with the ethical standards laid down in the 1964 declaration of Helsinki and all subsequent revisions.

Cell culture and purification. The human tumor cell lines CD133+ SW620, CD133+ SW620 (colorectal cancer line), K562 (erythroleukemia cell line), 7721 (liver cancer cell line), A549 (lung cancer cell line), and ECA109 (esophageal carcinoma cell line) were used. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics.

Figure 5 | NKG2D reverses the sULBP3-induced downregulation of NK cell cytotoxicity. K562 (A), 7721 (B), or CD133+ SW620 (C) cells were co-cultured with normal NK cells, with or without ULBP3-Fc (1 ng/ml), NKG2D-Fc (5 ng/ml), and Nkp44-Fc (5 ng/ml). The cytotoxicity of NK cells was measured by LDH release after co-culture for 6 h at the indicated E:T ratios. (D) Normal NK cells were incubated with the indicated concentrations of ULBP3-Fc for 6 h, and NKG2D expression in NK cells was measured by FCM. A representative graph of NKG2D expression is shown. (E) Combined data of NKG2D expression are shown. The data are representative of results obtained from at least 3 independent experiments. The results are expressed as the mean ± SEM. *P < 0.05, **P < 0.01.

Figure 6 | Anti-ULBP3 monoclonal antibody enhances the activity of NK cells from cancer patients via ADCC. ULBP3 expression and the percentage of infiltrating NK cells in tumor tissues from 10 patients (4 colorectal cancer, 3 lung cancer, and 3 gastric cancer) were determined by FCM. A representative graph is shown in (A). The relationship between ULBP3 expression and infiltrating NK cells in tumor tissues is shown in (B). Normal NK cells were co-cultured with CD133+ SW620 (C) or 7721 (D) cells at the indicated E:T ratios, and cytotoxicity was measured by LDH release after 6 h of co-culture, with or without the addition of isotype IgG, anti-ULBP3 (B2-F1-F1), and/or anti-CD16 (5 μg/ml). K562 (E) or A549 (F) cells were co-cultured with normal NK cells, and cytotoxicity was measured by LDH release after 6 h of co-culture at the indicated E:T ratios, with or without the addition of ULBP3-Fc (1 ng/ml) and/or B2-F1-F1 (5 μg/ml). The data are representative of results obtained from at least 3 independent experiments. The results are expressed as the mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.
AutoDEFLIA 1235 immunoassay system (EGG&G Wallace), and data were analyzed using the MultiCalc software.

**Statistics analysis.** The results are expressed as the mean ± SEM. Comparisons between 2 groups were performed with Student’s t-test. Differences among groups were assessed using one-way analysis of variance followed by the Tukey post hoc multiple comparisons test. P < 0.05 was considered statistically significant.

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Author contributions
X.M., Y.Z. and P.J. performed the experiments and analyzed the data. C.M. designed the project and wrote the manuscript. Y.Z. and T.Z. edited various parts of the manuscript. Q.J., X.C., H.L. and T.Z. helped with the experimental design. G.Y. and D.C. supervised the data analysis and edited the manuscript. All authors reviewed the manuscript.

Additional information
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