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NK Cells Infiltrating a MHC Class I-Deficient Lung Adenocarcinoma Display Impaired Cytotoxic Activity toward Autologous Tumor Cells Associated with Altered NK Cell-Triggering Receptors[1]

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NK cells are able to discriminate between normal cells and cells that have lost MHC class I (MHC-I) molecule expression as a result of tumor transformation. This function is the outcome of the capacity of inhibitory NK receptors to block cytotoxicity upon interaction with their MHC-I ligands expressed on target cells. To investigate the role of human NK cells and their various receptors in the control of MHC-I-deficient tumors, we have isolated several NK cell clones from lymphocytes infiltrating an adenocarcinoma lacking β_2-microglobulin expression. Unexpectedly, although these clones expressed NKG2D and mediated a strong cytolytic activity toward K562, Daudi and allogeneic MHC-class I^+ carcinoma cells, they were unable to lyse the autologous MHC-I^- tumor cell line. This defect was associated with alterations in the expression of natural cytotoxicity receptor (NCR) by NK cells and the NKG2D ligands, MHC-I-related chain A, MHC-I-related chain B, and UL16 binding protein 1, and the ICAM-1 by tumor cells. In contrast, the carcinoma cell line was partially sensitive to allogeneic healthy donor NK cells expressing high levels of NCR. Indeed, this lysis was inhibited by anti-NCR and anti-NKG2D mAbs, suggesting that both receptors are required for the induced killing. The present study indicates that the MHC-I-deficient lung adenocarcinoma had developed mechanisms of escape from the innate immune response based on down-regulation of NCR and ligands required for target cell recognition. ***The Journal of Immunology,*** **2005, 175: 5790–5798.**

The cytolytic and cytokine functions of NK cells are regulated by a complex balance between inhibitory and activating signals that are triggered via the engagement of different NK cell surface receptors with their specific ligands (1–3). Target cells expressing MHC class I (MHC-I)[3] molecules are protected from NK cell lysis by interactions between these molecules and inhibitory NK receptors. Human inhibitory NK cell receptors (iNKRs) include Ig-like killer inhibitory receptors (KIRs), which interact with HLA-A, -B, or -C, the CD94/NKG2A heterodimer, specific for HLA-E and ILT2/LIR1, characterized by broad specificities for different MHC-I molecules (4–8). In contrast, in the absence of these interactions, target cells become sensitive to NK lysis by triggering of NK cell-activating receptors.

Human triggering receptors responsible for NK cell activation have been recently identified (9, 10). These receptors include a heterogeneous family of molecules composed of natural cytotoxicity receptors (NCRs), which are exclusively expressed by NK cells and NKG2D, which is expressed by both NK cells and CTLs. NCRs belong to the Ig superfamily and include NKp46 and NKp30, which are expressed by both resting and activated NK cells, and NKp44, which is expressed only by activated NK cells (11–14). They associate with different ITAM-transducing polypeptides, including CD3ζ and FcRγ (for NKp30 and NKp46) and KARAP/DAP12 (for NKp44), which permit delivery of intracellular signals resulting in induction of cytotoxic activity (9, 15, 16). Thus far, the tumor ligands of NCR remain to be identified. NKG2D, a c-type lectin molecule, also plays an important role in induction of NK-mediated cytolytic activity. Human NKG2D associates with the DAP10 transmembrane adaptor, which bears a YxxM motif and activates the PI3K pathway (16–20). Unlike NCRs, the target cell ligands for NKG2D were identified and include MHC-I-related chains (MIC)A and MICB (21), up-regulated upon cell stress such as tumor transformation, and UL16-binding

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[3] Abbreviations used in this paper: MHC-I, MHC class I; iNKR, inhibitory NK receptor; KIR, Ig-like killer inhibitory receptor; NCR, natural cytotoxicity receptor; MIC, MHC-I-related chain; ULBP, UL16-binding protein; ADC, adenocarcinoma; TIL, tumor-infiltrating lymphocyte; NSCLC, non-small cell lung cancer; LCC, large cell carcinoma; β_2m, β_2-microglobulin; MFI, mean fluorescence intensity.

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proteins (ULBP)1, 2, and 3 (22). Functional studies indicated that these ligands are expressed by most tumor cells (9), in particular those of epithelial origin (23).

Altered MHC-I expression has been frequently observed in many human solid tumors, including lung carcinomas (24). Deficient HLA-class I expression may correspond to a tumor escape mechanism from the CTL response, and therefore, NK cells may play an important role in the control and eradication of cancer cells. However, little is known about the magnitude of the NK cell response to autologous HLA class I-deficient tumor cells and the role of the various triggering receptors in NK cell-mediated lysis. In this report, we have established a MHC-class I-deficient tumor cell line from a lung adenocarcinoma (ADC), and we have isolated several NK cell clones from autologous tumor-infiltrating lymphocytes (TILs). The susceptibility of this tumor cell line to autologous and allogeneic NK cell-mediated cytotoxicity was analyzed, and the expression and role of the various NK-activating receptors were investigated.

Materials and Methods

Patient characteristics and clinical course

Patient Coco, a 50-year-old Caucasian man, presented in February 1999 with an ADC of the right lung. No other tumoral localizations were found, except at proximal lymph nodes and the pleural membrane, which were invaded. The primary tumor (pT3, N1) was resected before chemotherapy and the patient received postsurgical radiotherapy. He was without evidence of cancer until March 2002, when he developed cerebral metastasis of the right frontal lobe. This lesion was rapidly resected and an encephaloradiotherapy was performed. At the time of surgery and 4 years later, blood samples were collected, and mononuclear cells were isolated and frozen.

Derivation of tumor cell line, polyclonal NK cell cultures, and NK cell clones

The non-small cell lung carcinoma (NSCLC) cell line ADC-Coco was derived from the primary tumor of patient Coco (A25/A29, B5101/B4501, and Cw4/Cw6). Briefly, a fresh tumor sample was dissociated mechanically, and the resulting cell suspension was either cultured to establish the in vitro tumor cell line or frozen. ADC-Coco cells were maintained in DMEM:Ham's F-12 medium supplemented with 10% FCS (Seromed). After thawing, viable TILs were cell-sorted by FACSVantage (BD Biosciences) using PE-labeled anti-CD3 mAb (Beckman Coulter) at one cell per microwell and stimulated by the addition of irradiated (10,000 rad) autologous tumor cells (3×10^3/well) and irradiated (10,000 rad) allogeneic EBV-transformed B cells (Laz509, 4×10^4/well) in RPMI 1640 medium supplemented with 10% human AB serum (Institut Jacques Boy), rIL-2 (200 U/ml; Roussel-Uclaf), and 8% of conditioned medium from PHA-activated lymphocytes. Cells were fed every 3 days with medium, IL-2, and conditioned medium from PHA-activated lymphocytes and were restimulated every other week with irradiated ADC-Coco cells and irradiated Laz509 cells. After 3 wk, the resulting cell clones (three clones per 96-well V-shaped microtiter plates; Nalge Nunc International) were screened for their capacity to kill the autologous tumor cell line and K562 and for NCR expression. Sixteen NK cell clones were obtained; three representative clones, NK2, NK3, and NK7, were retained for their capacity to proliferate, kill K562, and display distinct NCR phenotypes. These clones were restimulated every 3–4 wk with the same protocol.

Polyclonal NK cell cultures were derived from the $CD3^-$ fraction of autologous TIL (NK^{TIL}) and PBL isolated from blood samples collected at the time of surgery and 4 years later. The two PBL-derived NK cell cultures displayed a similar NCR phenotype and functional characteristics; therefore, only the NK cell culture (NK^{PBL}) derived 4 years after surgery was shown. Allogeneic healthy donor polyclonal NK cell cultures (NK^{HD}) were generated from PBL NK cells isolated by negative immunoselection (RosetteStep System; StemCell Technologies).

Allogeneic NSCLC tumor cell lines and cytotoxic assay

The IGR-Heu tumor cell line (HLA-A2/A68, B7/B35, and Cw4/Cw7) was established from patient Heu suffering from a large cell carcinoma (LCC) of the lung as described previously (25). LCC-B2 (A2/A68, B35/B38, and Cw4/Cw12) and IGR-Pub (HLA-A2/A68, B7/Bw6, and Cw7) tumor cell lines were established from a large cell carcinoma (LCC) and an ADC, respectively, as described previously (26). All tumor cell lines were cultured in DMEM:Ham's F-12 medium containing 10% heat-inactivated FCS, 1% Ultroser G (Invitrogen Life Technologies) and 1% penicillin-streptomycin, at 37°C in a humidified atmosphere with 5% CO_2.

K562 and Daudi tumor cell lines were used as targets in conventional NK and lymphokine-activated killer cytotoxic assays. Allogeneic NSCLC cell lines, including IGR-Pub, IGR-Heu, and LCC-B2, were used as targets in cytotoxicity assays. The cytotoxic activity of the polyclonal NK cell cultures and clones was measured by a conventional 4-h ^{51}Cr release assay using 3000 target cells/well as described previously (27). Functional effects of the mAb anti-NKp46 (KL247), anti-NKp30 (F252), anti-NKp44 (KS38), anti-NKG2D (Bat 221), anti-NKp46 plus anti-NKp30, anti-NKG2D plus anti-NKp30, or anti-LFA-1 mAb on effector cells were tested by incubating each of them for 15 min at room temperature before the assay. Values represent the mean of triplicate wells, with a SD of usually <10%.

FcR-positive P815 murine cells were used as targets in the redirected cytotoxicity assay. ^{51}Cr-labeled P815 target cells were preincubated for 45 min with anti-NKp46 (BAB281), anti-NKp30 (AZ20), anti-NKp44 (Z231), anti-NKG2D (ON72), anti-CD16 (3G8), or an isotypic control mAb, and then NK cells were added (E:T ratio of 2:1). All mAb, corresponding to ascitic fluid or hybridoma culture supernatants, were used at saturating concentrations predetermined by immunofluorescence titration curves on positive control cells.

Monoclonal Abs and immunofluorescence analyses

Anti-NKp46 (IgG1, BAB281), anti-NKp30 (IgG1, AZ20), anti-NKp44 (IgG1, Z231), anti-NKp80 (IgG1, MA152), anti-NKp46 (IgM, KL247), anti-NKp30 (IgM, F252), anti-NKp44 (IgM, KS38), and anti-NKG2D (IgG1, Bat 221 and ON72) mAb were produced in one of our laboratories. Anti-LFA-1 (IgG1), anti-LFA-3 (IgG2), PE-labeled anti-CD3 (IgG1), and FITC-conjugated goat anti-mouse IgG Abs were purchased from Beckman Coulter. W6/32 (anti-HLA-class I) and 9-49 (IgG2, anti-HLA-class II) mAb were reported previously (28, 29). Anti-MICA (IgG1, M673), anti-MICB (IgG1, M364), anti-ULBP1 (IgG1, M291), anti-ULBP2 (IgG1, M310), and anti-ULBP3 (IgG1, M551) were provided by Amgen. 25D7, 3G8, XC3, and B159.5.2 mAb recognize CD54 (ICAM-1), CD16, CD2, and CD56 molecules, respectively.

Phenotypic analyses of polyclonal NK cell cultures and clones were performed by direct or indirect immunofluorescence using a FACSCalibur flow cytometer (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences). Briefly, 3×10^5 cells were stained with mAb (diluted at saturating concentration in complete RPMI 1640 medium), washed with PBS (1×), fixed with formaldehyde (2% in PBS), and analyzed by flow cytometry.

MHC-I and ICAM-1 molecule expression on uncultured Coco tumor cells was tested by immunohistochemistry on paraffin tumor biopsies as described previously (30). Anti-β_2-microglobulin ((β_2m), IgG2, BBM1), anti-MHC-I H chain (IgG2, HC10), and anti-ICAM-1 (IgG1, MCA 1615; Serotec) mAb were included. Power Vision from Microm (Microtech) was used for visualization.

Results

Generation of a MHC-I-deficient lung ADC cell line

The ADC-Coco tumor cell line was generated from a human ADC of the lung (pT3, N1) as described in *Materials and Methods*. Cell surface immunofluorescence analysis indicated that ADC-Coco failed to express MHC-I and MHC-II molecules and the ICAM-1 (CD54) adhesion molecule. ICAM-2 and ICAM-3 were also not expressed (data not shown). In contrast, the CD2 ligand LFA-3 (CD58) adhesion molecule and Fas (Apo-1, CD95) receptor were expressed on virtually all cells (Table I and data not shown). Lack of MHC-I expression was due to a defect in expression of the β_2m L chain as indicated by intracytoplasmic immunofluorescence analysis performed using specific mAb (data not shown). This defect was confirmed by immunohistochemical staining of the corresponding tumor biopsy using the anti-MHC-I H chain (HC-10) mAb, which showed slight intracellular labeling of tumor cells, and anti-β_2m (BBM1) mAb, which failed to stain cancer cells, as opposed to the LCC-B2 tumor biopsy used as a positive control (Fig. 1). The ICAM-1 expression defect, which may influence tumor cell recognition by effector cells, was also confirmed in uncultured tumor cells by immunohistochemical analysis (Fig. 1).

Table I. *Phenotypic analysis of NSCLC tumor cell lines*[a]

	MHC-1	ICAM-1	LFA-3	MICA	MICB	ULBP1	ULBP2	ULBP3
ADC-Coco	0%[b]	0%	91% (57)	16% (1)	1%	0%	24% (7)	57% (10)
IGR-Heu	96% (40)	11% (5)	95% (25)	48% (7)	33% (6)	6%	0%	11% (5)
LCC-B2	97% (183)	97% (71)	97% (37)	3%	0%	0%	6%	8%

[a] Numbers in parentheses correspond to mean fluorescence intensity. Data shown are representative of five independent experiments.
[b] Percentages of positive cells are indicated.

NK cell clones isolated from autologous TILs display altered NCR expression

Mononuclear cells that infiltrated the ADC-Coco tumor biopsy were stained with PE-labeled anti-CD3 mAb, and negative cells (~38%) were sorted by FACS at one cell per microwell on an irradiated autologous tumor cell line and allogeneic EBV-transformed B cells and IL-2. Growing NK cell clones as well as polyclonal NK cell cultures, established from the $CD3^-$ fraction of patient TILs, and PBLs, hereafter termed NK^{TIL} and NK^{PBL}, respectively, were expanded with high doses (200 U/ml) of IL-2 on irradiated feeder cells as described in *Materials and Methods*. All NK cells were characterized phenotypically and compared with polyclonal NK cell cultures established from healthy donor PBL, hereafter termed NK^{HD}. Immunofluorescence analysis of 3 representative TIL-derived NK cell clones, NK2, NK3 and NK7, revealed a $CD3^-$, $CD16^+$, $CD56^+$, LFA-1^+ (CD11a/CD18), $2B4^+$ and $CD2^+$ phenotype (Fig. 2*A* and Table II). Regarding iNKR, NK2 clone expressed KIR2DL1 (p58.1/CD158a), KIR2DL2/3 (p58.2/CD158b), and KIR3DL2/p140 receptors, the NK7 clone expressed KIR3DL1/p70 and KIR3DL2/p140, and the NK3 clone was negative for all KIR expression. ILT2/LIR1 and CD94 were expressed on the three clones, and NKG2A was expressed on the NK3 clone only (Table II). With respect to activating receptors, NKG2D was highly expressed at the surface of the three NK cell clones, though at lower density (mean fluorescence intensity (MFI) between 20 and 27) than NK^{HD} (MFI 57) (Fig. 2). The NKG2D expression defect by a fraction of the NK^{TIL} cells may be associated with an alteration in ligand surface expression by tumor cells. In contrast, NCR expression varied from one clone to another and was rather low. Indeed, the NK2 clone expressed NKp46 and NKp30, although at lower levels as compared with NK^{HD}. Similar levels of NKp30 were expressed by NK3, while NKp46 was strongly reduced. Finally, regarding NK7 clone, it expressed very low levels of both NKp46 and NKp30 (Fig. 2*A*). Remarkably, NKp44 that was undetectable on all the clones could be very slightly detected on the NK3 clone only. Such a low undetectable expression of NKp44 is typical hallmark of NCR^{dull} clones (31). A similar NCR^{dull} phenotype was observed on polyclonal NK cell cultures isolated from the $CD3^-$ fraction of patient TILs (NK^{TIL}) and PBLs (NK^{PBL}) as compared with healthy donor NK cells (NK^{HD}) displaying a NCR^{bright} phenotype (32) (Fig. 2*B*). These results indicate that the three NK clones included in this study were representative, at least in terms of the NCR expression phenotype, of NK^{TIL} cells and that they displayed much lower NCR levels than NK^{HD} cells. Furthermore, immunofluorescence analysis of freshly isolated patient TILs, performed by three-color staining using anti-CD56, anti-CD3, and anti-NCR mAbs, indicated that subpopulations of uncultured tumor-infiltrating NK cells express a NCR^{dull} phenotype (data not shown). An alteration in the surface density of NCR may profoundly impact the NK cell-mediated cytolytic function against tumor cells as reported previously (31, 32).

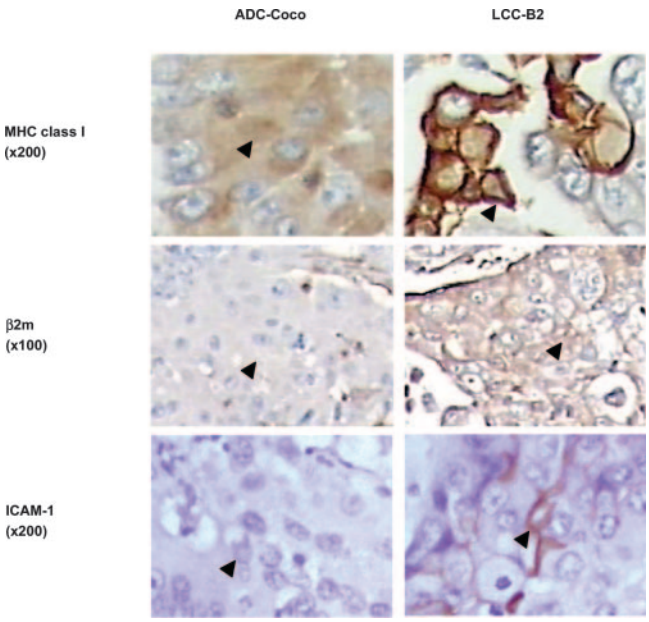

FIGURE 1. Immunohistochemical analysis of an ADC-Coco tumor biopsy. LCC-B2 tumor lesion was used as a positive control. Anti-MHC-I H chain (HC10), anti-β_2m (BBM1), and anti-ICAM-1 (MCA 1615) mAb were used. Arrows indicate tumor cell fields. Magnifications are ×100 or ×200.

NSCLC NK cell clones failed to lyse autologous MHC-I-deficient tumor cells

Additional experiments were performed to assess the cytotoxic activity of NK cell clones toward the autologous ADC-Coco tumor cell line. A panel of allogeneic lung carcinoma cell lines, lymphokine-activated killer (Daudi) cells, and NK (K562) target cells were included in cytotoxicity assays. As shown in Fig. 3*A*, all the clones failed to kill the ADC-Coco tumor cell line. Nor was lysis observed against the autologous PHA-activated blasts used as control (data not shown). Impaired cytotoxicity toward autologous tumor cells was not related to a defect in granzyme B or perforin expression because all the clones expressed high levels of the two proteases, as indicated by intracytoplasmic immunofluorescence analysis using a specific mAb (data not shown). Consistently, all clones were able to mediate strong cytotolytic activity against K562, Daudi, and allogeneic MHC-class I^+ lung carcinoma cell lines, except for IGR-Pub, which was resistant to NK2 clone lysis (Fig. 3*A*). Similar results were obtained with autologous NK^{TIL} and NK^{PBL} cell cultures (Fig. 3*B*). In contrast, allogeneic NK^{HD} polyclonal cell cultures were able to lyse ADC-Coco tumor cells (Fig. 3*B*). However, this lysis was weaker than that mediated by NK^{HD} toward Daudi, K562, and distinct NSCLC cell lines and

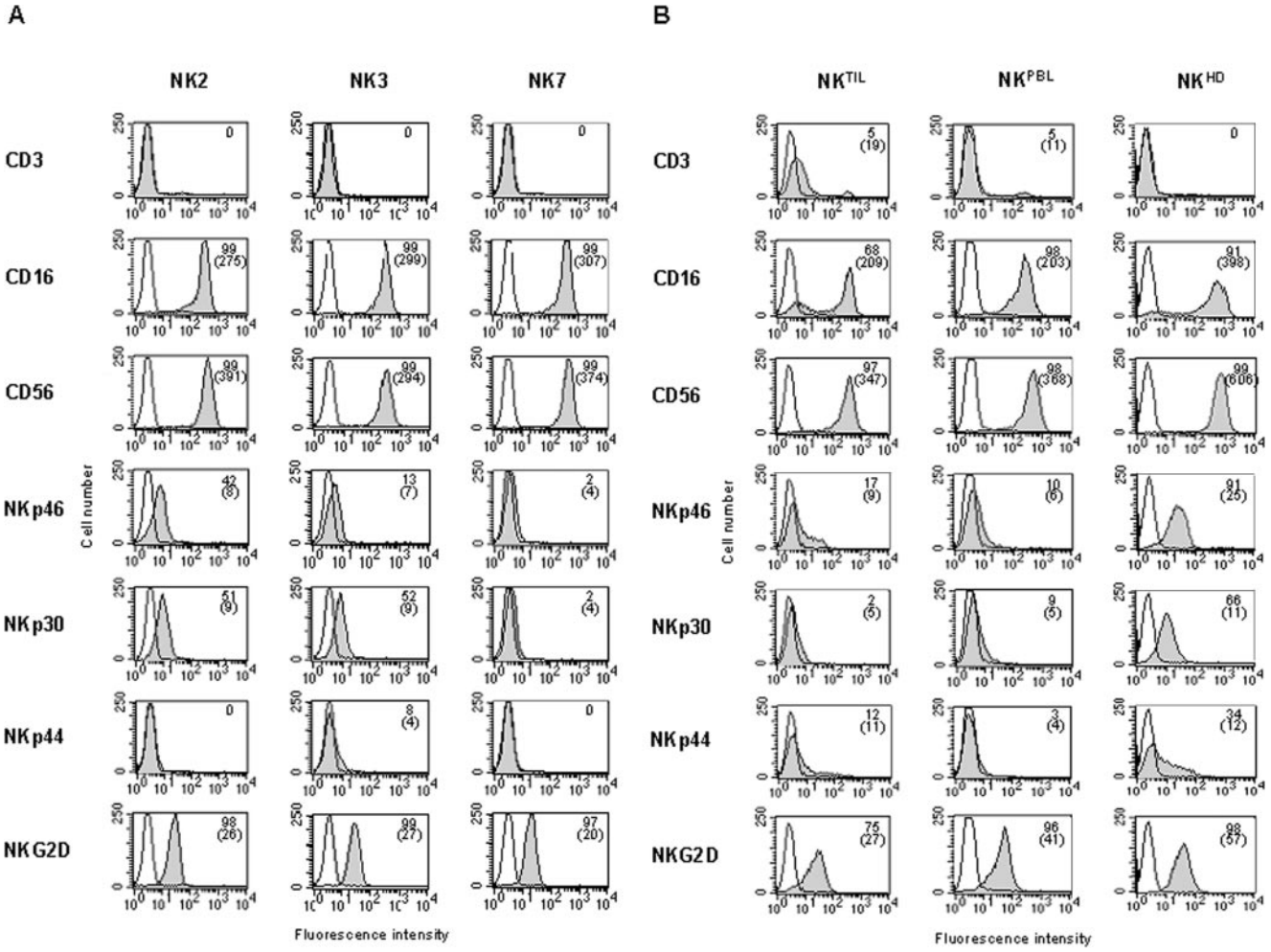

FIGURE 2. Flow cytometry analysis of polyclonal NK cell cultures and clones. *A*, Flow cytometry analysis of NK2, NK3, and NK7 TIL-derived NK cell clones. NK cells were stained with the indicated receptor-specific mAb (▨) or an isotype-matched control (□). Anti-CD3, -CD16, and -CD56 mAb used were also included. Data shown are representative of four independent experiments. *B*, Immunofluorescence analysis of NK^{TIL} and NK^{PBL} polyclonal NK cell cultures derived from ADC patient TILs and PBLs, respectively. The polyclonal NK cell culture, NK^{HD}, established from healthy donor PBLs, was used as control. Percentages of positive cells are indicated. Numbers in parentheses correspond to MFI. Data shown are representative of four independent experiments.

may have been related to an alteration in ICAM-1 and/or NKG2D ligand expression by ADC-Coco.

Next, experiments were performed to analyze whether the mAb-mediated engagement of NCR expressed by the NK cell clones could induce killing of murine ($Fc\gamma R^+$) P815 mastocytoma cells in a redirected cytotoxicity assay. Our results indicate that all NK clones displayed weak cytolytic activity toward P815 in the absence or in the presence of anti-NCR mAb (Fig. 4). This defect is likely consequent to the NCR^{dull} phenotype displayed by NK cell clones (31). The only clone that displayed some response to anti-NCR in this redirected assay was clone NK3 when tested in the presence of anti-NKp30 mAb. In contrast, anti-CD16 and

Table II. *Phenotypic analyses of NK cell clones and NK cell cultures*[a]

	CD94	NKG2A	KIR2DL1 (p58.1)	KIR2DL2/3 (p58.2)	KIR3DL1 (p70)	KIR3DL2 (p140)	ILT2/LIR1	LFA-1	2B4	CD2
NK2	99% (80)[b]	10% (6)	99% (30)	99% (589)	1%	92% (21)	93% (46)	98% (1180)	99% (91)	99% (1203)
NK3	98% (237)	99% (82)	1%	0%	0%	0%	67% (43)	99% (903)	100% (47)	99% (69)
NK7	98% (77)	15% (12)	0%	0%	98% (52)	73% (15)	84% (33)	100% (862)	100% (55)	91% (87)
NK^{PBL}	95% (255)	43% (78)	51% (86)	68% (579)	34% (168)	10% (40)	41% (22)	99% (1516)	99% (102)	99% (847)
NK^{TIL}	56% (122)	38% (47)	62% (532)	14% (418)	16% (139)	1%	11% (9)	92% (581)	97% (117)	98% (1085)
NK^{HD}	94% (76)	99% (424)	13% (223)	16% (729)	10% (148)	12% (48)	5% (53)	99% (757)	99% (144)	98% (836)

[a] Numbers in parentheses correspond to MFI. Data shown are representative of four independent experiments.
[b] Percentages of positive cells are indicated.

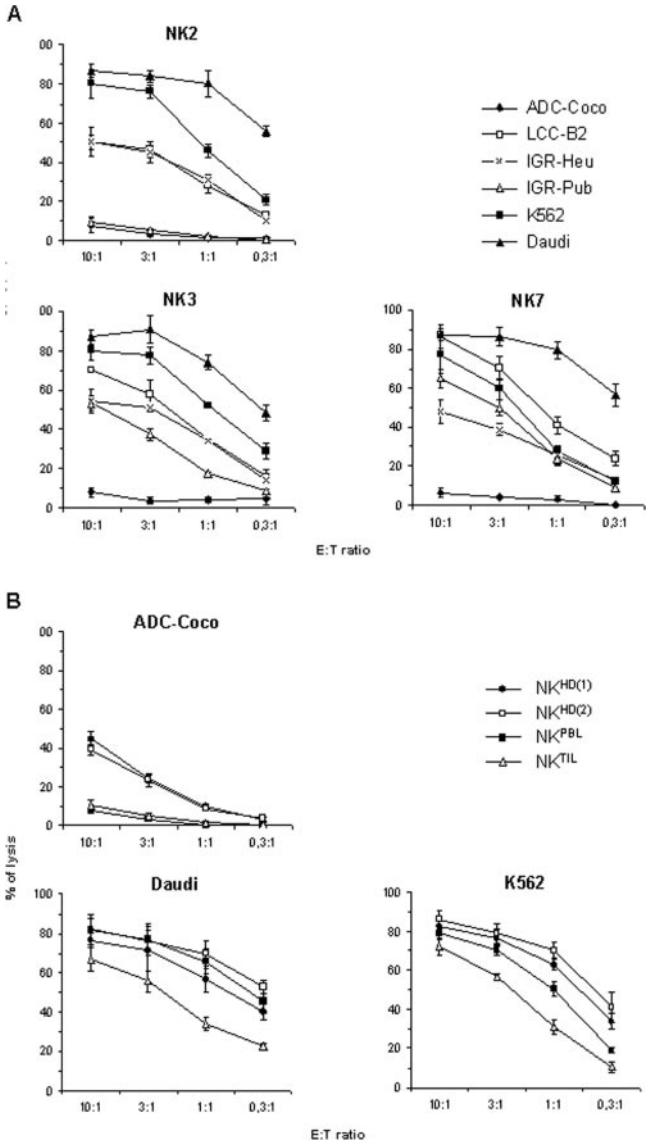

FIGURE 3. *A*, Cytotoxic activity of NK2, NK3, and NK7 NK cell clones. Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at indicated E:T ratios. The autologous MHC-I-deficient ADC-Coco tumor cell line and allogeneic NSCLC cell lines, LCC-B2, IGR-Heu, and IGR-Pub, as well as Daudi and K562 were used as targets. Results are expressed as means of triplicate culture ± SD. Data shown are representative of four independent experiments. *B*, Cytotoxic activity of polyclonal NK cell cultures derived from patient Coco TILs (NK^{TIL}) and PBLs (NK^{PBL}) and from healthy donor PBLs (NK^{HD}). ADC-Coco, Daudi, and K562 tumor cell lines were used as targets. The E:T ratios are indicated. Data shown are representative of four independent experiments.

anti-NKG2D were able to trigger a stronger cytolytic activity toward the P815 murine target, thus indicating that the cytolytic potential of these clones is preserved (Fig. 4). Enhancement of cytotoxicity was induced by anti-CD16, anti-NKG2D, as well as by anti-NCR mAb when the NK^{HD} polyclonal NK cell culture, displaying a NCR^{bright} phenotype, was used as a source of positive control effector cells (Fig. 4). Taken together, these data suggest that NK cells derived from lung cancer TILs are characterized by a normal cytolytic potential but that their NCRs are poorly or not at all functional.

Altered MICA, MICB, and ULBP1 ligand expression on the ADC-Coco tumor cell line

The above experiments indicated that NK2, NK3, and NK7 clones displayed altered NCR but functional NKG2D. To further assess the molecular basis of the NK cell defect in killing of autologous tumor cells, we tested the expression of the NKG2D ligands MICA, MICB, and ULBP1–3 on the ADC-Coco and the NK-sensitive IGR-Heu and LCC-B2 allogeneic tumor cell lines (33). We also tested the expression of MHC-class I, ICAM-1, CD48, and LFA-3 molecules on all target cells. Immunofluorescence analysis indicated that all target cells were $CD48^-$/LFA-3 $(CD58)^+$ (Table I and data not shown). The MHC-class I^-/$ICAM\text{-}1^-$ ADC-Coco expressed very low levels of MICA and failed to express MICB and ULBP1. Expression of ULBP2 and ULBP3 was moderate (Table I). Conversely, the MHC-class I^+/$ICAM\text{-}1^-$ IGR-Heu, used for comparison, more significantly expressed MICA and MICB but expressed ULBP1–3 very poorly, while the MHC-class I^+/$ICAM\text{-}1^+$ LCC-B2 failed to express all tested NKG2D ligands (Table I). These NKG2D ligands expression profiles were stable throughout a long follow-up period. Furthermore, immunofluorescence analysis of uncultured freshly resected tumor biopsies indicated that ADC-Coco tumor cells failed to express MIC and ULBP molecules (data not shown). These data suggest that partial or total resistance of the ADC-Coco tumor cell line to allogeneic or autologous NK cytolytic activity might be associated not only with alterations in NCR expression by NK cell clones but also with alterations in MICA, MICB, ULBP1, and ICAM-1 expression.

The role of NCR, NKG2D, and LFA-1 in tumor cell lysis

To further elucidate the role of NCR and NKG2D in NK clone-mediated cytotoxic activity toward allogeneic tumor cells, blocking experiments were performed using specific IgM mAb. Results in Fig. 5*A* indicate that, while anti-NKp46 was unable to block NK cell-mediated lysis toward the IGR-Heu tumor cell line, anti-NKp30 mAb blocked NK3 clone killing. In contrast, no inhibition was observed with the NK7 clone, in agreement with immunofluorescence analysis. Similar results were obtained with a combination of anti-NKp30 and anti-NKp46 mAb. With respect to NKG2D, specific mAb substantially inhibited the cytotoxicity of all NK cell clones toward allogeneic tumor cells. More significant inhibition was observed when anti-NKG2D was associated with anti-NKp30 (Fig. 5*A*). These results indicate that NKp30, even if expressed at low surface density on NK3 clone, can still play a role in allogeneic tumor cell-mediated killing. In contrast, in line with the absence of response to anti-NKp46 mAb in redirected killing assay, NKp46 did not appear to play a significant role in NK cell-mediated killing of this tumor cell line. Finally, we performed blocking experiments of NK cell clone cytotoxic activity toward the $ICAM\text{-}1^+$/MIC^-/$ULBP^-$ LCC-B2 allogeneic tumor cell line. Our results indicate that anti-LFA-1 (Fig. 5*B*) and to a lesser extent anti-ICAM-1 (data not shown) mAb strongly inhibit the cytotoxicity of all patient clones against these tumor cells. In contrast, anti-NCR mAb did not significantly inhibit lysis of these NCR^{dull} clones, while anti-NKG2D mAb were not or very slightly inhibitory. The low inhibition with anti-NKG2D mAb could be associated with expression of NKG2D ligands other than MICA, MICB, and ULBP1–3. These results underline a role of LFA-1 in NK cell function and support previous reports performed by exposing NK cells to human ICAM-1 expressed on *Drosophila* cells and demonstrating that expression of ICAM-1 on insect was sufficient to induce lysis by NK cells through LFA-1 (34–36).

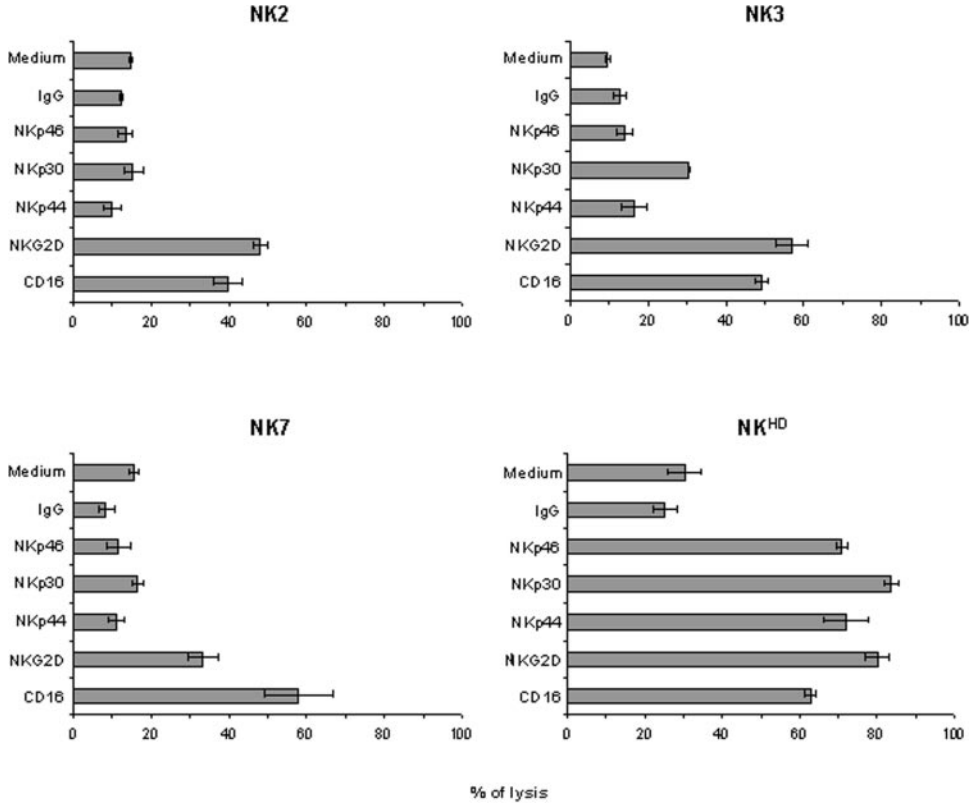

FIGURE 4. Redirected cytotoxic activity of NK2, NK3, and NK7 NK cell clones. The NK^{HD} polyclonal NK cell culture was included as control. FcR-positive P815 murine cells were used as targets at an E:T ratio of 2:1. Anti-NKp46, -NKp30, -NKp44, -NKG2D, and anti-CD16 or an isotypic control mAb were used. Data shown are representative of six independent experiments.

Finally, we investigated the receptors involved in healthy donor NK cell-mediated lysis toward the ADC-Coco tumor cell line. Cytotoxicity experiments indicated that anti-NKG2D mAb, or a combination of anti-NKp30 and anti-NKp46 IgM mAb, strongly inhibited the lytic activity of the NK^{HD} cell culture toward ADC-Coco (Fig. 5*C*). A similar magnitude of blocking was obtained when anti-NKG2D mAb was associated with either anti-NKp30 or anti-NKp46 mAb (Fig. 5*C* and data not shown). In contrast, less significant inhibition was observed when anti-NKp46 and anti-NKp30 mAb were used separately (Fig. 5*C*). These results suggest that engagement of both NCR and NKG2D triggering receptors is required for cytotoxicity mediated by normal donor NK cells against the ADC-Coco tumor cell line. Because this cell line was found to express both ULBP2 and ULBP3, it is likely that the observed involvement of NKG2D may reflect the engagement of this receptor by such ligands.

Discussion

NK cells correspond to the most efficient cellular mechanisms by which the immune system destroys abnormal cells that have lost HLA class I molecule expression during the tumor cell transformation process. Indeed, in the absence of inhibitory interactions between iNKR expressed on NK cells and MHC-I ligands expressed on normal target cells, tumor cells become susceptible to NK-mediated killing following interactions between activating receptors and their respective ligands. To analyze the magnitude of the antitumor NK response in a situation in which only triggering but not inhibitory NK receptors are engaged by their ligands, we established, from a lung ADC lacking β_2m expression, a MHC-I-deficient tumor cell line, and we isolated several NK cell clones from autologous TILs. Although the tumor lesion was infiltrated by NK cells, the derived MHC-I^- tumor cell line was completely resistant to autologous NK cell clone-mediated lysis. This defect is likely due to insufficient tumor cell/NK cell interactions primarily consequent to alterations in NCR expression at the NK cell surface associated with low numbers of NKG2D ligands and the absence of ICAM-1 expression at the tumor cell surface. Therefore, resistance of carcinoma cells to autologous NK cell-mediated lysis appears to result from several mechanisms of tumor escape from the immune response.

Although their tumor ligands are not yet identified, NCR have been described as being crucial receptors for target cell recognition and induction of NK cell-mediated cytotoxicity toward a wide range of cancer cells. A direct correlation has been established between NCR surface densities and the ability of NK cells to kill different tumor target cells (31). Along this line, it has been reported that down-regulation of NKp30 expression by exposure of NK cells to TGF-β1 resulted in markedly reduced NK-mediated cytotoxicity against several target cells (37). In the present study, we show that NK cell clones derived from NSCLC display altered NCR surface expression and/or functions correlated with a defect in lysis of autologous tumor cells. This defect cannot be consequent to a KIR or CD94/NKG2A interaction with HLA molecules because the tumor cells were negative for all MHC-I molecule expression; indeed, it was most likely due, at least in part, to insufficient numbers of NCR/ligand interactions. Accordingly, the ADC-Coco cell line was only partially sensitive to allogeneic healthy donor NK cells. This lysis was inhibited by a combination of anti-NKp30 and anti-NKp46 mAbs, indicating that NCR may play a major role in mediated cytotoxicity, and suggesting that tumor cells may express the corresponding ligands. Furthermore, although NSCLC-derived NK cell clones displayed the NCR^{dull} phenotype, they were able to kill allogeneic lung carcinoma cells. This lysis was inhibited by anti-NKp30 mainly when associated with anti-NKG2D mAb. Low NCR expression on the NK cell surface was also reported in HIV-infected individuals (38, 39) and

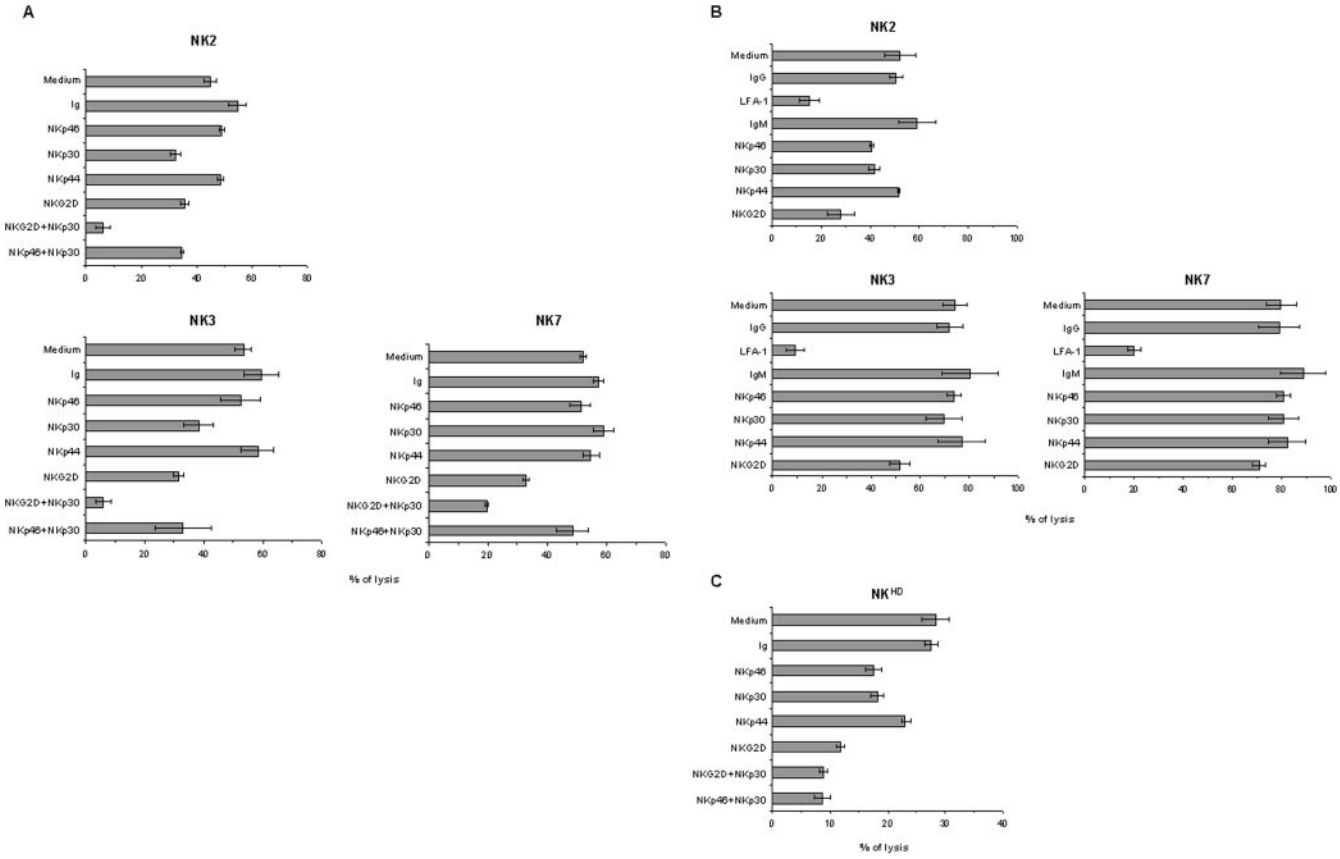

FIGURE 5. *A*, Role of NCR and NKG2D receptors in NK2, NK3, and NK7 clone-mediated lysis toward IGR-Heu allogeneic NSCLC tumor cell line. Cytolytic experiments were performed either in medium or in the presence of indicated mAbs at an E:T ratio of 10:1. NK cells were preincubated for 15 min with saturating concentrations of anti-NKp46 (KL247), anti-NKp30 (F252), anti-NKp44 (KS38), anti-NKG2D (Bat 221), anti-NKG2D plus anti-NKp30 or anti-NKp46 plus anti-NKp30 mAb, and then chromium-labeled target cells were added. Data shown are representative of three independent experiments. *B*, Role of LFA-1 in NK2, NK3, and NK7 clone-mediated lysis toward LCC-B2 allogeneic NSCLC tumor cell line. Cytolytic experiments were performed either in medium or in the presence of indicated mAbs at an E:T ratio of 10:1. NK cells were preincubated for 15 min with saturating concentrations of anti-LFA-1, anti-NKp46 (KL247), anti-NKp30 (F252), anti-NKp44 (KS38), and anti-NKG2D (Bat 221) mAb and then chromium-labeled target cells were added. Data shown are representative of two independent experiments. *C*, Role of NCR and NKG2D receptors in NK^{HD}-mediated cytotoxicity toward the ADC-Coco tumor cell line. Cytolytic experiments were performed either in medium or in the presence of the indicated mAbs. Healthy donor NK cells were preincubated for 15 min with saturating concentrations of anti-NKp46, anti-NKp30, anti-NKp44, anti-NKG2D, a combination of anti-NKG2D and anti-NKp30 or a combination of anti-NKp46 and anti-NKp30 mAbs, and then chromium-labeled allogeneic ADC-Coco target cells were added. The E:T ratio was 10:1. Data shown are representative of three independent experiments.

in patients with NK cell expansions (40), with colon adenocarcinoma (41) and with acute myeloid leukemia, which correlated with poor susceptibility of autologous leukemia cells to NK-mediated lysis (32). This phenotype is not associated with CD3-ζ or KARAP/DAP12 down-regulation, as shown by intracytoplasmic immunofluorescence analyses of NSCLC NK cell clones using specific mAb (data not shown). The decrease in NCR expression may be due to soluble factors secreted by NSCLC tumor cells. Indeed, coculture of ADC-Coco tumor cells with NK^{HD} cells, even when performed in Transwells, induced a strong decrease in NKp30 and NKp44 surface expression after 48 h incubation (data not shown). Experiments underway are seeking to identify such factors, their role in NCR down-regulation and whether the reduced expression of these receptors is stable or not. Alternatively, this down-regulation may be associated with alterations in genes regulating NCR expression. It should be noted that the NCR^{dull} phenotype of TIL-derived NK cell clones is stable and is not modified during in vitro culture, supporting previous results in acute myeloid leukemia patients (32).

Unlike NCR, which were expressed at different, generally low surface, densities by TIL-derived NK cell clones, the expression of NKG2D was high and was relatively homogeneous but somewhat weaker than NK^{HD} cells. This would imply that NK cell triggering via NKG2D may be dictated by expression of the appropriate ligands by the target cells. However, although the tumor cell line expressed significant levels of ULBP2 and ULBP3, it was completely resistant to autologous NK cells and only partially susceptible to allogeneic polyclonal NK cell cultures. The latter cytotoxicity was inhibited by anti-NCR and anti-NKG2D mAb, suggesting that ULBP2 and ULBP3 ligands are sufficient for NK cell triggering. These results also confirm in the case of this tumor cell line that the NK cell-mediated killing is dependent on both NCR and NKG2D (9). However, because the ADC-Coco tumor cell line was only partially killed by healthy donor NK cells (expressing a NCR^{bright} phenotype), it is possible that higher levels of both MIC and ULBP may be required for optimized lysis. Along this line, the combined use of anti-MIC and anti-ULBP mAb resulted in

stronger inhibition of NK-mediated cytotoxicity (42). The absence of (or low) surface expression of MIC and/or ULBP molecules has also been previously described in some melanomas, lymphoblastoid cell lines, and neuroblastomas (42).

It has been reported that MHC-I expression is frequently reduced or absent in NSCLC with tumor cell dissemination and is accompanied by loss of ICAM-1 expression (24). Accordingly, the tumor cell line used in this study is deficient for both MHC-I and ICAM-1 expression. ICAM-1 expression defect is most likely involved in poor NK cell lytic function. However, although the allogeneic MIC$^+$ IGR-Heu NSCLC cell line failed to express ICAM-1, it was sensitive to patient NK cells emphasizing a major role of NKG2D/NKG2D ligands interaction in NCR^{dull} NK cell triggering (9, 42). With regard to the ICAM-1$^+$/MIC$^-$/ULBP$^-$ LCC-B2 tumor cell line, our data suggest a crucial role of LFA-1/ICAM-1 interaction in patient NK cell triggering. The importance of LFA-1 in NK cell function is supported by recent reports demonstrating that binding of LFA-1 on NK cells to ICAM-1 on target cells induces signals that lead to cytotoxicity by NK cells and may initiate an early signaling cascade through activation of Vav1, leading to amplification of signals from other activation receptors (34–36).

In conclusion, we have identified, in a patient with metastatic ADC of the lung, several mechanisms of tumor escape from the immune system. One mechanism leading to tumor escape from the adaptive immune response is related to the lack of expression of the MHC-I molecule due to a β_2m deficiency. This expression loss, which may have rendered tumor cells more susceptible to NK-mediated lysis, was accompanied by at least three additional mechanisms of tumor escape from the innate immune response. The first mechanism is based on down-regulation of NCR expression (and/or function) by NK cells. The second mechanism appears to be associated with the low expression levels of NKG2D ligands by tumor cells and a last mechanism consists of the absence of ICAM-1 expression leading to a decrease in the effector cell/tumor cell interaction, as well as a decrease in the mechanism of the induction of cytotoxicity.

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Disclosures

The authors have no financial conflict of interest.

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