Selection of tumor-resistant variants following sustained natural killer cell-mediated immune stress

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Abstract. Resistance of tumor cells to cell-mediated cytotoxicity remains an obstacle to the immunotherapy of cancer and its molecular basis is poorly understood. To investigate the acquisition of tumor resistance to cell-mediated cytotoxicity, resistant variants were selected following long-term natural killer (NK) cell selection pressure. It was observed that these variants were resistant to NK cell-mediated lysis, but were sensitive to autologous cytotoxic T lymphocytes or cytotoxic drugs. This resistance appeared to be dependent, at least partly, on an alteration of target cell recognition by NK effector cells, but did not appear to involve any alterations in the expression of KIR, DNAM1 or NKG2D ligands on resistant cells, nor the induction of protective autophagy. In the present study, in order to gain further insight into the molecular mechanisms underlying the acquired tumor resistance to NK cell-mediated cytotoxicity, a comprehensive analysis of the variant transcriptome was conducted. Comparative analysis identified an expression profile of genes that best distinguished resistant variants from parental sensitive cancer cells, with candidate genes putatively involved in NK cell-mediated lysis resistance, but also in adhesion, migration and invasiveness, including upregulated genes, such as POT1, L1CAM or ECM1, and downregulated genes, such as B7-H6 or UCHL1. Consequently, the selected variants were not only resistant to NK cell-mediated lysis, but also displayed more aggressive properties. The findings of the present study emphasized that the role of NK cells may span far beyond the mere killing of malignant cells, and NK cells may be important effectors during cancer immunoediting.

Introduction

The majority of the current cancer immunotherapy treatments involve the generation and activation of antigen-specific and non-specific killer cells. However, successful induction of tumor-specific immune responses is not always followed by tumor rejection in patients. Indeed, several mechanisms have been associated with the acquisition of tumor resistance to cell-mediated cytotoxicity. Tumor cells can evade adaptive immunity through the selection of variants that are resistant to specific cytotoxic T lymphocyte (CTL) pressure. Indeed, the interaction between immune and tumor cells can either eliminate the developing tumor or generate a tumor cell repertoire that is able to survive in immunocompetent hosts (1-3). In this regard, Dunn *et al* reported that tumor specific T-cell responses can select tumor-associated antigen-negative cells and variants resistant to CTLs *in vivo* (4,5). We previously reported that the reorganization of the actin cytoskeleton may be used by tumor cells as a strategy to promote their resistance to CTL-mediated lysis (6). Therefore, it is likely that tumor variants resistant to T cells will emerge, most frequently in the context of effective immunotherapies (7). Consequently, even if a strong and sustained cytotoxic response is induced, there remain complex issues, such as tumor evasion and selection of tumor-resistant variants. Natural killer (NK) cells are also involved in the control of tumor progression (8,9) and several reports have indicated that solid tumor infiltration

by NK cells is a favorable prognostic marker (10-12). NK cell-mediated cytotoxic activity can eliminate tumor cells that have evaded CD8+ T cells through loss of antigen or MHC class I molecules. Moreover, through their secretion of cytokines or by mutual activation links established with dendritic cells (DCs) (13-15), NK cells can positively modulate adaptive immune responses against tumor cells and their susceptibility to CD8+ T cells (16). However, it should be noted that *in vitro* and *in vivo* studies demonstrated a poor or defective activity of NK cells in cancer, as well as resistance of tumor cells to NK cell-mediated lysis. Mechanistically, the imbalance between activating and inhibitory signals, which may be caused by low expression of ligands to activating receptors and high expression of ligands to inhibitory receptors of NK cells on tumor cells, was suggested as being one of the major mechanisms underlying the poor anticancer activity of NK cells (17).

Although NK cells have been established as the main effectors of antitumor immune response, their putative role on the emergence of tumor cytotoxic resistant variants remains poorly understood. Evidence of NK cell immunoediting was reported by studies using NK-deficient models, and demonstrated how exposure to NK cells causes modification of cancer immunogenicity to allow survival and progression of the tumor clone in an immunocompetent environment. In addition, Moretta *et al* reported that NK cells play an important regulatory role by selectively editing DCs during the course of the immune response, and that NK cell-mediated killing of immature DCs results in selection of immunogenic DCs during the initiation of anticancer immune responses (18). While several studies have revealed the contribution of adaptive and innate immunity to cancer immunoediting (1,5,19-25), whether the innate immune system can suppress tumor formation without adaptive immunity remains elusive. We previously used a lung cancer model to study the emergence of tumor resistance following specific cytotoxic T lymphocyte (CTL) selection pressure (6). We also described various mechanisms of resistance to NK cells that may differ between tumor types, tumor aggressiveness and environmental contexts (26-28). The focus of the present study was to investigate the consequences of sustained exposure of melanoma cells to NK cell-mediated immune stress, in order to determine whether sustained pressure on tumor cells drives the selection of variants resistant to NK cell-mediated lysis and acquisition of aggressive properties, and to assess the role of the innate immunity in tumor editing.

Materials and methods

Cell lines. The T1 melanoma cell line was derived from a primary lesion, as previously described (29). T1 cells and their derivatives were grown in RPMI-1640/Glutamax™ (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% heat-inactivated fetal calf serum (FCS), 1 mM sodium pyruvate and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO$_2$. Autologous LT2 CTL clone, specific for the peptide Melan-A25-36, was isolated from tumor-infiltrating lymphocytes as previously described (29), and grown in RPMI-1640/Glutamax™ supplemented with

8% AB human serum (Institut Jacques Boy), 1 mM sodium pyruvate, 150 U/ml recombinant IL-2 and in the presence of the irradiated autologous tumor cell line T1, lymphoblastoid LAZ cells (B cells transformed by Epstein-Barr virus) and allogeneic peripheral blood mononuclear cells (PBMCs). NK cells were isolated from human healthy donor blood using a human NK Cell Isolation Kit (Miltenyi Biotec), grown in RPMI-1640/Glutamax™ supplemented with 8% AB human serum, 1 mM sodium and 300 U/ml recombinant IL-2 in the presence of irradiated lymphoblastoid LAZ cells and allogeneic PBMCs. The purity of CD56+ CD3- NK cells was >95%, as determined by flow cytometry.

Selection of NK cell-resistant variants. The three T1 cell lines corresponded to three independent batches of T1 reference cells. The three NK cell-resistant T1 cell lines (T1R) were obtained following several months of co-culture with NK cells. A total of 10^5 tumor cells/well were plated in 6-well plates. On the following day, NK cells were added in RPMI-1640 medium supplemented with 5% heat-inactivated FCS, 1 mM sodium pyruvate and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.) and 300 U/ml of IL-2. Surviving tumor cells were amplified with regular addition of NK cells in order to obtain a sustained selective pressure.

Cell morphology and actin cytoskeleton staining. Actin and nuclear staining were performed using Alexa Fluor 488-coupled Phalloidin and DAPI (4',6-diamidino-2-phenylindole, dihydrochloride; Thermo Fisher Scientific, Inc.), respectively. Cells were cultured overnight on glass slides and fixed with 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature. Cells were permeabilized with 0.1% Triton X100 for 10 min at room temperature, stained with Rhodamin-Phalloidin R415 and To-PRO3 iodide (Invitrogen; Thermo Fisher Scientific, Inc.) for 45 min at 4°C, mounted in Fluoromount-G (Southern Biotech) and analyzed with a Zeiss laser scanning confocal microscope (LSM-510 Meta; Carl Zeiss AG) using an objective of x40 oil immersion lens. Images were later analyzed by LSM Image Examiner software, version 4.2.0.121 (Carl Zeiss AG).

Cytotoxicity experiments. NK cell and LT12 cytotoxic activity was measured by a 4 h ^{51}Cr release assay by using triplicate co-cultures in U-bottomed 96-well plates. Various effector-to-target (E:T) ratios were used, with 2,000 target cells per well. Following co-culture, the supernatants were transferred to LumaPlate-96 wells (PerkinElmer, Inc.), dried down, and ^{51}Cr release was measured on a Packard TopCount NXT. Data are expressed as the percentage of specific ^{51}Cr release from target cells, calculated as (experimental release-spontaneous release)/(maximum release-spontaneous release) x100.

Confocal microscopy analysis of immunological synapse assembly. Tumor cells and NK cells were co-cultured for 30 min at a 1:2 ratio on poly-L-lysin slides (MatTek Corporation) at 37°C. The cells were then fixed with 4% (w/v) paraformaldehyde/PBS (Sigma-Aldrich; Merck KGaA) for 30 min at room temperature and permeabilized with 0.1% (w/v) SDS solution in PBS for 10 min at room temperature, followed by blocking with

10% FCS (v/v) solution in PBS for 20 min at room temperature. The cells were stained at 4˚C with anti-phosphotyrosine mAb (clone 4G10, cat. no. 05-321, Upstate Biotechnology) diluted at 1:400 or mAb mouse anti-human granzyme B (GzmB), clone GB11 (cat. no. MA1-80734, Caltag Laboratories) diluted at 1:50, followed by a secondary mAb conjugated to Alexa Fluor 488 (cat. no. A-11094, Thermo Fisher Scientific, Inc.) diluted at 1:200 for 30 min at 4˚C combined with nuclear staining with TO-Pro 3 iodide (Invitrogen; Thermo Fisher Scientific, Inc.). Fluoromount G (Southern Biotech) was added to each slide and analysis was performed with a Zeiss LSM-510 Meta Laser Scanning Confocal microscope. Images were analyzed using LSM Image Examiner software, version 4.2.0.121 (Carl Zeiss AG).

Analysis of autophagosome formation. T1 cells were transfected with green fluorescent protein (GFP)-light chain 3 (LC3) encoding vector or its corresponding empty vector using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's recommendations. The presence of autophagosomes was assessed at 24 h post-transfection by monitoring the formation of dot-like structures by confocal microscopy (LSM-510 Meta Laser Scanning Confocal microscope; Carl Zeiss AG) using an objective of x40 oil immersion lens.

Western blot analysis of autophagy markers. The expression of the autophagy markers LC3-I and LC3-II (Cell Signaling Technology, Inc., cat. no. 2775), SQSTM1/p62 (Cell Signaling Technology, Inc., cat. no. 8025), ATG5 (Cell Signaling Technology, Inc., cat. no. 2630) and Beclin-1 (Cell Signaling Technology, Inc., cat. no. 3738) was assessed using total cell extract. Proteins (30 μg) were resolved on 10% SDS-PAGE, blotted on nitrocellulose membranes and then incubated with the appropriate primary antibodies and secondary antibody, Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch, cat. no. 111-035-003).

siRNA targeting of autophagy markers. Autophagy-defective cells were generated by transfection with ATG5 siRNA. Briefly, cells were transfected by electroporation of 50 nmol/l of siRNA targeting human ATG5 (Qiagen APG5L-6 FlexiTube siRNA SI02655310). Luciferase siRNA was used as a negative control. The silencing of ATG5 was assessed by western blotting 48 h after transfection using appropriate Abs.

Microarray. RNA was extracted with TRIzol and purified on RNeasy Micro Kit spin columns (Qiagen GmbH). The RNA amount, purity and integrity were evaluated by NanoDrop and Bioanalyser (Agilent Technologies, Inc.). For array hybridization, RNA samples (100 ng) were amplified and stained with fluorophores using the Low RNA Input Linear Amplification Labeling kit (Agilent Technologies, Inc.). Cy3-stained cRNA were hybridized on Agilent Human Whole Genome Oligo Microarray format 8x60K, design 028004 (Agilent Technologies, Inc.), then washed and scanned. Data were extracted by Feature Extraction software (v10.5.1.1; Agilent Technologies, Inc.) and analysis was performed by the limma package (30) of the Bioconductor Project. An intra-array normalization was performed, followed by a quantile inter-array normalization. The median of all probes for a given transcript was then recovered. Differential expression level analysis was performed using the following criteria: Absolute fold change >2 and corrected P-value [false discovery rate (FDR)] <0.05. The microarray data and protocols are available at the European Molecular Biology Laboratory European Bioinformatics Institute database (https://www.ebi. ac.uk/arrayexpress/) under accession no. E-MTAB-8777.

Anchorage-independent growth. A total of 500 and 1,000 cells were incubated in 1% (w/v) methylcellulose (Methocell MC4000, Fluka) culture medium in 35-mm non-culture-treated Petri dishes. After 21 days of incubation, cells able to grow in an anchorage-independent manner gave rise to visible colonies that were then counted.

Tumor cell migration ability. A total of $10x10^6$ cells/ml were plated in the channels of 24-well BioFlux plates (Labtech) precoated with fibronectin (20 μg/ml). After 48 h, the micro-fluidic device enables to submit only half of the channel to a 5 dyn/cm^2 trypsin laminar flow for removing cells only on half of the channel. Then, the channel was washed by a 5 dyn/cm^2 culture medium flow and cell migration was observed under a 2 dyn/cm^2 passive culture medium flow by video microscopy with a Zeiss AxioVert 200 fluorescence inverted video microscope and analyzed with AxioVision software, version 4.8.2 (Carl Zeiss AG).

Tumor cell adhesion on extracellular matrix. Flat-bottomed 96-well plates were coated overnight at 4˚C with 100 μl of matrix composed of fibronectin (5 μg/ml), collagen-I (5 or 200 μg/ml), osteopontin (1 μg/ml), thrombospondin (1 μg/ml), laminin (1 μg/ml) and Matrigel (1:10 dilution). After washing and blocking with 1% PBS-BSA solution for 1 h at 37˚C (Sigma-Aldrich; Merck KGaA), 10,000 EDTA-detached cells were added in serum-free RPMI-1640 and plated for 2 h. PBS washing removed unattached cells and a mixture of medium and MTT (10% v/v) was added. After 4 h, formazan crystals were lysed with 10% SDS (w/v, Bio-Rad Laboratories, Inc.) and 50% dimethyl formamide (Sigma-Aldrich; Merck KGaA) solution (pH 4.7). The optical density at 570 and 630 nm was measured to estimate the attached cell number.

Flow cytometry analysis of main KIR ligand expression. A total of $2x10^5$ cells were washed in PBS and incubated at 4˚C for 20 min with the specified mAbs. Following incubation and washing, the samples were analyzed on LSR Fortessa or FACS Canto II (Becton, Dickinson and Company) using DIVA software, version 6.1.3 (BD Biosciences). The following Abs were used: PE-conjugated anti-CD112 mAb (cat. no. IM3452; Beckman Coulter, Inc.); PE-conjugated anti-CD155 mAb (cat. no. 337610; BioLegend, Inc.); PE-conjugated anti-MICA/B mAb (cat. no. 558352; BD Biosciences); PE-conjugated anti-ULBP1 mAb (cat. no. IC1380P; R&D Systems, Inc.); PE-conjugated anti-ULBP2 mAb (cat. no. FAB1298P; R&D Systems, Inc.); PE-conjugated anti-ULBP3 mAb (cat. no. FAB1517P; R&D Systems, Inc.); PE-conjugated anti-HLA-E mAb (cat. no. 12-9953-42; eBioscience); PE-conjugated anti-HLA-G mAb (cat. no. ab24384; Abcam); and FITC-conjugated anti-HLA ABC (cat. no. IM1838U; Beckman Coulter, Inc.).

Flow cytometry conjugate formation. The NK cells and tumor cells were stained with CellTracker Green 5-chloromethylfluoresceindiacetate (CMFDA) and Orange [5-(and-6)-(((4-chloromethyl)benzoyl)amino) tetramethylrhodamine] (CMTMR) (Thermo Fisher Scientific, Inc.) for 30 min at 37˚C. On the following day, the cells were co-cultured for 45 min, fixed for at least 30 min in 4% (w/v) paraformaldehyde/PBS (Sigma-Aldrich; Merck KGaA) at 4˚C and analyzed on FACScalibur (BD Biosciences) or AccuriC6 (BD Biosciences) and the data were entered into the respective CellQuest (version 4.0) or C6 Flow (version 227.4) software (both from BD Biosciences). Double-stained events, corresponding to the interaction between a tumor cell and at least one NK cell, were compared to the whole stained tumor cell population.

CD107a externalization assay. NK cells were stained with CMFDA and then co-cultured with tumor cells for 90 or 180 min in the presence of APC-conjugated anti-CD107a antibody (clone H4A3, cat. no. 560664, BD Pharmingen; BD Biosciences). The cells were then fixed in 4 (w/v) paraformaldehyde/PBS (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature and analyzed with AccuriC6 flow cytometer (BD Biosciences). Data were processed with C6 Flow software, version 227.4 (BD Biosciences).

Statistical analysis. Data are expressed as mean ± standard deviation. P-values were determined by unpaired two-tailed Student's t-tests. *P<0.05, **P<0.01 and ***P<0.001 were considered to indicate statistically significant differences and error bars were used to indicate standard deviation.

Results

NK cell pressure induced the selection of tumor variants resistant to NK cell-mediated lysis and displaying a differential susceptibility to other cell death inducers. For this study, the T1 melanoma cell line and NK cells isolated from a healthy donor were used. T1 cells were continuously co-cultured with NK cells during several months or left untreated during the same period to serve as control. Three independent batches of T1 cells were co-cultured with healthy donor NK cells in parallel. The sensitivity of control or NK-treated cells to NK cell-mediated lysis was then assessed. The data depicted in Fig. 1A demonstrated that T1 cells isolated following sustained NK cell pressure (T1R) exhibited a strong decrease in their susceptibility to NK cell-mediated lysis compared with the parental cell line (T1). Of note, NK cell-mediated lysis of both T1 and T1R cells was completely inhibited by concanamycin A (a Ca²⁺ chelator that inhibits cytotoxic granule exocytosis), indicating that their killing by NK cells is mostly dependent on the perforin (PFN)/GzmB pathway (data not shown). In parallel, we examined the putative cross-resistance of T1R cells to additional cell death inducers, including an autologous CTL clone (LT12) and dacarbazine (DTIC), which are commonly used in melanoma chemotherapy. No significant differences were observed between T1 and T1R cells regarding their sensitivity to LT12-mediated lysis or to DTIC-induced cell death (Fig. 1B and C). It should be noted that the LT12 CTL clone also destroys T1 cells trough the PFN/GzmB pathway (26),

suggesting that T1R resistance to NK cell-mediated lysis is not associated with a resistance to GzmB-mediated cell death. However, a slight but significant decrease in T1R cell susceptibility to tumor necrosis factor (TNF)-α as compared to the parental T1 cell line was observed (Fig. 1D), which was associated with a decrease in TNF-R1 expression (Fig. 1E). Taken together, these results indicated that NK cell pressure can select variants that are resistant to NK cell-mediated lysis by a probable multiparametric mechanism.

T1R cell resistance to NK cell-mediated lysis is independent of autophagy induction. We have previously reported that autophagy can protect tumor cells against NK cell- and CTL-mediated cytotoxicity (27,28). Therefore, we sought to determine whether the selection of resistant cells following NK cell pressure involves activation of autophagy in these cells. The autophagy level was first evaluated by following the distribution pattern of the microtubule-associated protein-LC3 (referred to as LC3 hereafter) fused with a GFP tag (LC3-GFP). T1 and T1R cells were transfected with a vector encoding the LC3-GFP fusion protein before visualizing the autophagosomes. Confocal microscopy data revealed a significant basal level of autophagy in T1 cells, with punctate LC3-GFP staining, but no significant increase was observed in T1R cells (Fig. 2A). This was also supported by assessing the expression of the phosphatidylethanolamine-conjugated form of LC3 (or LC3-II), which is similar between T1 and T1R cells. Moreover, no difference in the expression of other main autophagy-related proteins, such as p62, ATG5 and Beclin-1 (involved in autophagosome formation and the autophagy process) was observed between T1 and T1R cells (Fig. 2B). Finally, silencing of ATG5 using siRNAs (Fig. 2C) had no impact on T1R cell susceptibility to NK cell-mediated lysis (Fig. 2D). Taken together, these results clearly indicated that, in our model, autophagy was not implicated in T1R cell resistance to NK cell-mediated lysis.

T1R cell resistance to NK cell-mediated lysis is associated with an alteration of the effector/target interaction. It was next investigated whether T1R cell resistance to NK cell-mediated lysis was associated with alteration in the target cell recognition process. For this purpose, the immune synapse formation between NK and T1 or T1R cells was analyzed using confocal microscopy. The percentage of tumor cells interacting with NK cells, and the establishment of active immune synapses, evaluated by both phosphotyrosine staining and GzmB relocalization at the contact zone, was measured in at least 200 target cells from each of the three independent batches of T1 and T1R cells. The data revealed a mild decrease in the percentage of NK cells in contact with T1R compared to T1 cells. More interestingly, a strong decrease was observed in active immune synapse formation between T1R and NK cells, compared with T1 control cells, as shown by the decrease in phosphotyrosine staining and GzmB relocalization at the immune synapse (Fig. 3A-C). To corroborate those results, flow cytometry analysis was performed to quantify the percentage of conjugate formation between NK and T1 or T1R cells. The data depicted in Fig. 3D revealed a mild but non-statistically significant reduction of contacts between T1R and NK cells
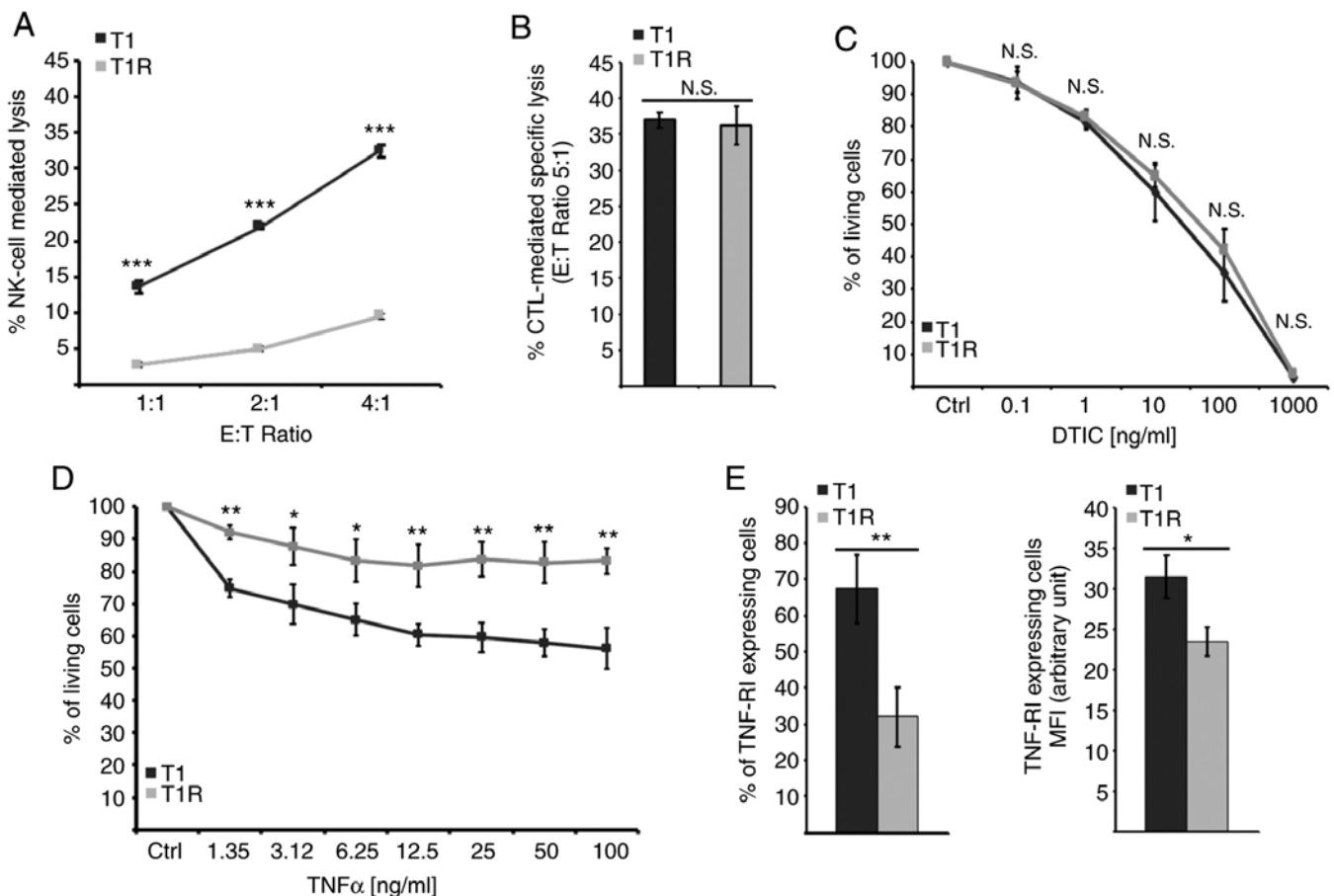
Figure 1. The acquisition of resistance to NK cell-mediated lysis induced by sustained NK cell pressure does not confer resistance to autologous CTL-mediated specific lysis or to DTIC, but does attenuate TNF response in association with a decreased membrane expression of TNF-R1 receptor. (A) Susceptibility to NK cell-mediated lysis for cocultured cells (T1R) in comparison to reference cells (T1) was analyzed by a conventional 4-h ^{51}Cr release test. Data of a representative experiment are presented as means of the three independent batches of T1 and the three independent batches of T1R cells, each performed in triplicate ± standard deviation. (B) Susceptibility to autologous CTL-mediated specific lysis was observed by a conventional 4-h ^{51}Cr release test. Data of a representative experiment are presented as means of the three independent batches of T1 and the three independent batches of T1R cells, each performed in triplicate ± standard deviation. (C) Susceptibility to DTIC was evaluated by MTT assay following a 24-h treatment by 0.1-1,000 μg/ml of DTIC. Data represent the means of three experiments for the three independent batches of T1 and the three independent batches of T1R cells, each performed in triplicate±standard deviation. (D) MTT assay evaluation of susceptibility to a 72-h TNF-α treatment at a concentration ranging between 1.55 and 100 ng/ml. Data of a representative experiment are presented as the means of the three independent batches of T1 and the three independent batches of T1R cells, each performed in duplicate ± standard deviation. (E) Percentage of positive cells by flow cytometry for evaluation of TNF-R1 membrane expression and TNF-R1-expressing cells MFI. Data of a representative experiment are presented as the means of the three independent batches of T1 and the three independent batches of T1R cells ± standard deviation. NK, natural killer; CTL, cytotoxic T lymphocyte; DTIC, dacarbazine; TNF, tumor necrosis factor; MFI, mean fluorescence intensity; E:T, effector:target; N.S., not significant. *P<0.05, **P<0.01, ***P<0.001.

as compared to T1 control cells. In addition, using a degranulation assay based on CD107 externalization following target cell recognition leading to cytotoxic granule exocytosis, it was demonstrated that T1R triggering of CD107 externalization by NK cells was significantly reduced in comparison to T1 parental cells (Fig. 3E). Finally, it was observed that this alteration of the T1R cell recognition by NK cells occurred in a KIR-, DNAM1- and NKG2D-independent manner, as the expression of the ligands for these receptors (CD112, CD155, MICA/B, ULBP1-3, HLA-E and HLA-G) was similar between the parental cells and their resistant counterparts (Fig. 3F). Taken together, these results indicated that T1R cell resistance to NK cell-mediated lysis is, as least partly, dependent on defective immune synapse signaling following effector/target conjugation, as evidenced by the alteration of GzmB relocalization, phosphotyrosine signaling and CD107 staining in NK cells interacting with T1R cells.

Impact of sustained NK cell pressure on tumor cell transcriptional signature. A global transcriptional analysis comparing T1 and T1R cells was next performed in order to evaluate the impact of NK cell pressure on tumor cell behavior and to elucidate the putative molecular basis of the NK cell-mediated lysis resistance mechanisms. Transcriptional profiles of the three independent T1 and T1R series were compared by DNA microarray, performed in duplicate. Dendrogram representation of the unsupervised analysis of the whole data set revealed a significant discrimination between the T1 and T1R cell populations (Fig. 4A). These data, confirmed by principal component analysis (Fig. 4B), enabled us to define a particular genomic signature of tumor cells subjected to sustained NK cell pressure with robust statistical significance. A supervised analysis was then performed to select differentially expressed genes [absolute fold-change (FC) >2 and a corrected P-value (FDR) <0.05]. The heatmap in Fig. 4C represents the 99
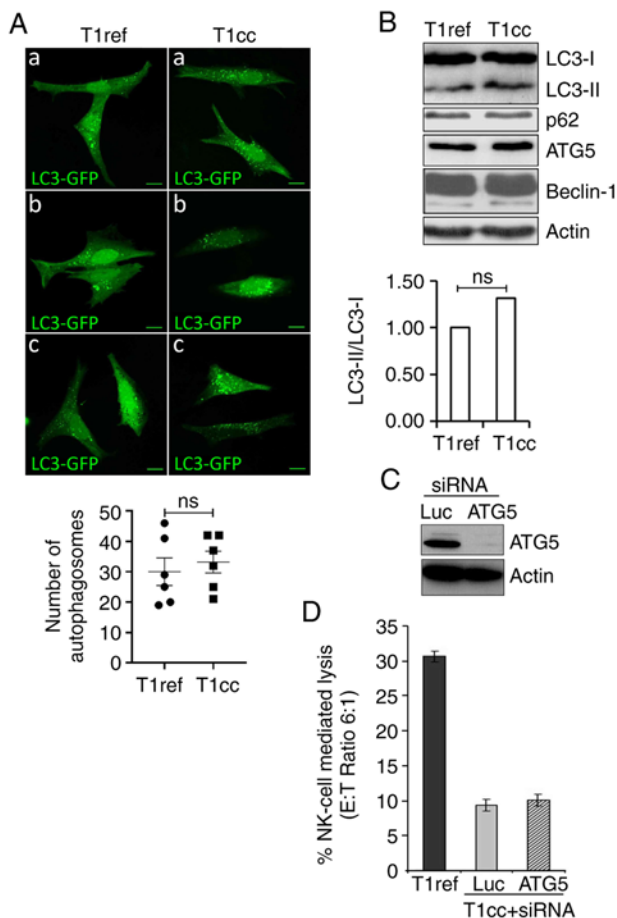
Figure 2. The resistance of T1R cells to NK cell-mediated lysis is independent of autophagy. (A) Confocal microscopy analysis of autophagosome formation in the three independent batches of T1 and the three independent batches of T1R cells transfected with LC3-GFP. Lower panel: Graph showing the quantification of green dot-like structures (autophagosomes) in each cell. The results represent the mean of autophagosome number in six T1ref and T1cc cells. The results are shown as mean ± standard error of the mean (error bars). Statistically significant differences were calculated compared with control conditions (T1ref cells) using an unpaired two-tailed Student's t-test (ns, not significant). (B) Western blotting for main autophagy-related protein expression. (C) Western blotting for ATG5 protein expression after siRNA treatment. Lower panel: Western blot densitometry quantification of the autophagy marker LC3-II/LC3-I ratio in T1ref and T1cc cells. The results are reported as fold change (FC). (D) Susceptibility to NK cell-mediated lysis of cocultured cells (T1R) after ATG5 siRNA treatment in comparison to reference cells (T1) was analyzed by a conventional 4-h ^{51}Cr release test. NK, natural killer; LC3, light chain 3; GFP, green fluorescent protein; E:T, effector:target; N.S., not significant.

identified genes, including 26 overexpressed and 73 downregulated genes in T1R compared with T1 cells. As three independent biological replicates (all performed in technical duplicates) were used for each condition, the impact of variability in basal gene expression due to cell line heterogeneity was reduced to a minimum. Therefore, the number of genes in this signature is relatively limited. However, no main modifications of functional gene groups or signaling pathways were significantly detectable with DAVID or INGENUITY analysis tools (data not shown). Similarly, no particular pathways were found to be significantly enriched on Gene Set Enrichment Analysis (data not shown).

Nevertheless, among the overexpressed or downregulated genes in T1R cells, several may be of interest, either regarding the description of newly acquired characteristics of migration and invasiveness in the T1R cell model, or regarding their resistance to NK cell-mediated lysis. These overexpressed genes include matrix metallopeptidase-1 (FC +7.561) involved in extracellular matrix reorganization, in metastatic process and in resistance to NK cell-mediated lysis (31). Trefoil factor 2 (FC +2.631) is involved in cell migration and apoptosis regulation in gastrointestinal mucosa. *HLA-DRB5* (FC +2.381), *-DRB4* (FC +2.287) and *-DRB1* (FC +2.211) may be implicated in resistance to NK cells, as previously described (32). More importantly, inhibition of protection of telomeres protein 1 (*POT1*; FC +2.381) has been demonstrated to increase apoptosis and to limit gastric cancer cell proliferation (33). In our model, *POT1* overexpression, following NK cell pressure, may have induced the emergence of cells more resistant to apoptosis. Moreover, given that *POT1* has been identified in a high-scale screening of molecules affecting NK cell-mediated lysis susceptibility (34), it may be a potential candidate associated with resistance in our T1R model. *L1CAM* (FC +2.321) encodes L1 cell adhesion molecule, which is overexpressed in several types of cancer and contributes to invasiveness, metastasis (35,36) and apoptosis resistance (37). *S100B* (FC +2.315) encodes *S100* calcium-binding protein B, which is involved in the regulation of various cellular processes, such as cell cycle regulation or differentiation. Its alteration has been implicated in melanoma cell proliferation and metastatic progression (38). *ECM1* (FC +2.109) encodes extracellular matrix protein 1, the overexpression of which has been shown to contribute to cancer cell invasiveness (39). Finally, *CEACAM19* (FC +2.063), encoding carcinoembryonic antigen-related cell adhesion molecule 19, may regulate NK cell-mediated lysis, similar to CEACAM1 (40).

Regarding the downregulated genes in T1R cells, ubiquitin carboxy-terminal hydrolase-L1 (*UCHL1*; FC -9.011) was identified. *UCHL1* encodes a deubiquitinating enzyme that may play a proapoptotic role, and its downregulation may be associated with an increase in cell survival (41). *SERPINF1/PEDF* (FC -3.313) encodes pigment epithelium-derived factor, which is involved in anti-angiogenic and anti-tumor activities. Therefore, its downregulation in T1R cells may be associated with tumor-promoting characteristics (42). Finally, our data indicated downregulation of B7-H6 (FC -2,839), a NKp30-activating ligand that may contribute to T1R cell resistance to NK cell-mediated lysis. Thus, the present analysis revealed putative contributors to T1R cell resistance to NK cell-mediated lysis, as well as several genes involved in cell adhesion or migration and invasiveness, strongly suggesting that NK cell pressure may lead to the selection of more aggressive tumor cells.

Effect of sustained NK cell pressure on tumor cell phenotype. Tumor immunoediting is a phenomenon that may also lead to tumor cell shaping through alteration of phenotypical characteristics. As our transcriptomic analysis revealed putative alteration of T1R cell adhesion, migration and invasiveness, their migration ability was examined. First, no morphological changes were observed by actin staining (Fig. 5A) that could contribute to migration or to an alteration of target recognition and resistance to NK cell-mediated lysis (6). As shown in Fig. 5B, T1R cells exhibited increased migration ability compared with the T1 control cells,
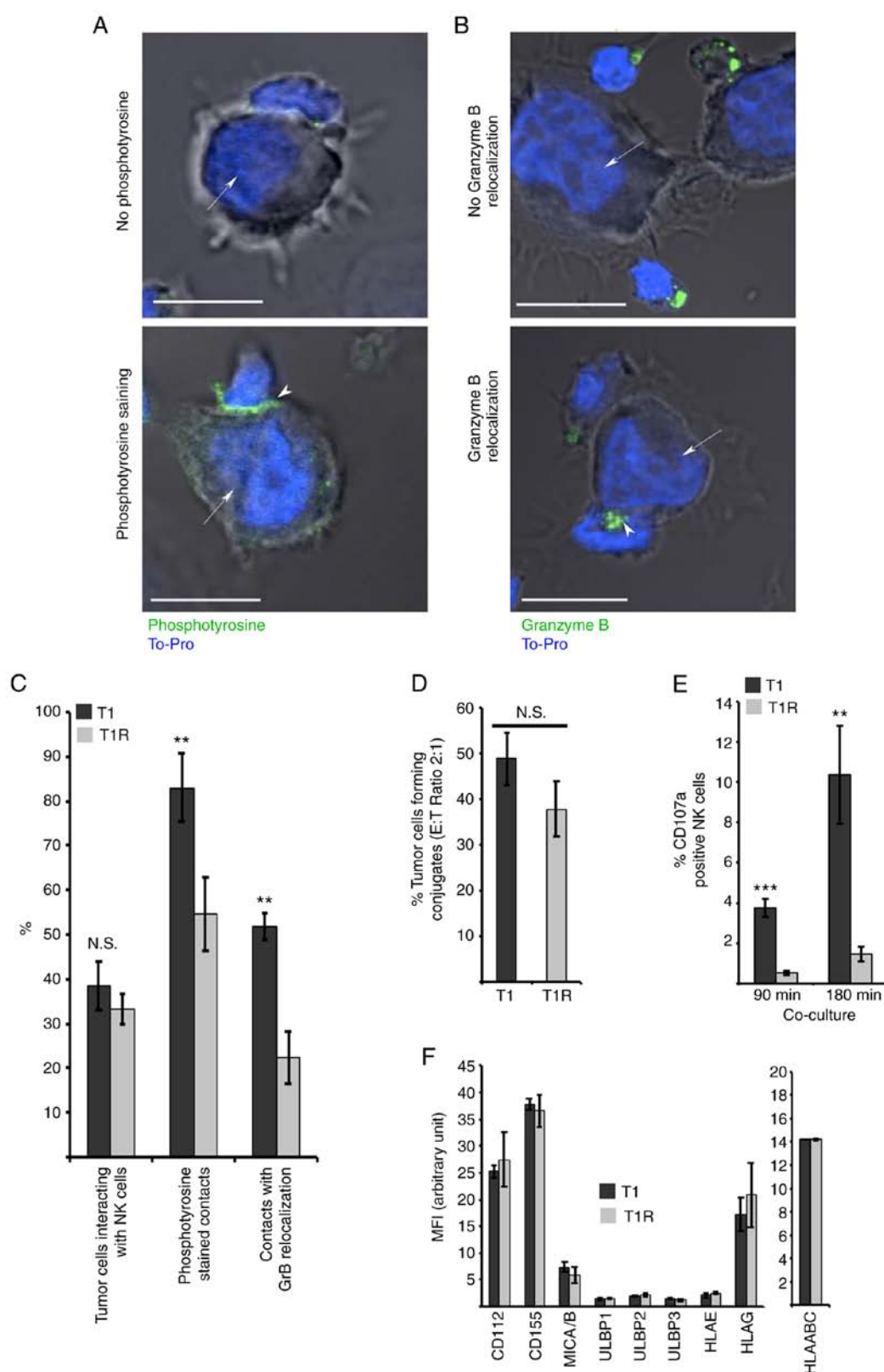
Figure 3. The acquisition of resistance to NK cell-mediated lysis correlates with an effector/target interaction default. (A) Confocal microscopy staining of phosphotyrosine (arrowhead, accumulated at the contact zone) on co-harvested tumor (arrow) and NK cells. (B) Confocal microscopy staining of Granzyme B (arrowhead, relocalized at the contact zone) on co-harvested tumor (arrow) and NK cells. Scale bar, 10 μm. (C) Statistical analysis of confocal microscopy evaluation of tumor and NK cell contacts, of the establishment of active immune synapses (by phosphotyrosine staining) and of Granzyme B relocalization toward the contact zone. At least 200 tumor cells in 10 different fields were counted for each three independent batches of T1 and each three independent batches of T1R cells. Data represent mean percentage ± standard deviation. (D) Flow cytometry evaluation of conjugate formation between NK cells and tumor cells. Data represent the means of three experiments for the three independent batches of T1 and the three independent batches of T1R cells ± standard deviation. (E) Flow cytometry evaluation of CD107a externalization on NK cell membrane in response to cocultured cells (T1R) in comparison to reference cells (T1). Data represent the means of the three independent batches of T1 and the three independent batches of T1R cells ± standard deviation. (F) Flow cytometry evaluation of main KIR, DNAM1 and NKG2D ligand expression on cocultured cells (T1R) in comparison to reference cells (T1). Data represent the means of the three independent batches of T1 and the three independent batches of T1R cells ± standard deviation. NK, natural killer; N.S., not significant. [**]P<0.01, [***]P<0.001.
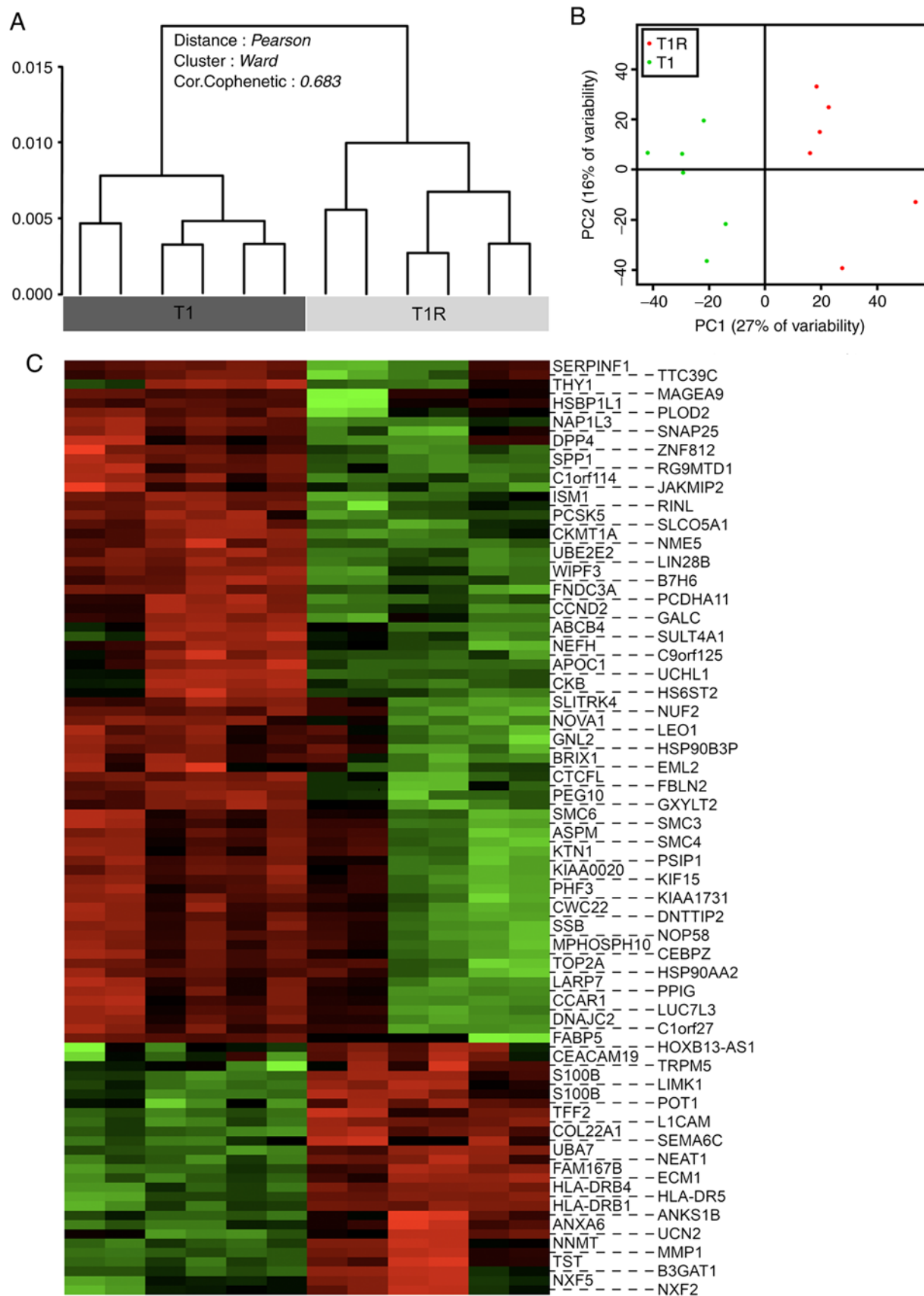
Figure 4. Transcriptional signature associated with resistance to NK cell-mediated lysis acquired after sustained NK cell pressure on tumor cells. (A) Unsupervised clustering generated on all the data. Pearson's correlation coefficient and Ward's linkage algorithm were used. (B) PC analysis of expression data in cocultured (T1R) and reference (T1) cell samples. The first two PC are shown. The first component captures the variability between the two types of samples. (C) Heatmap representation of genes differentially expressed between T1 and T1R cells. Heatmap represents genes comparatively upregulated (red) or downregulated (green) in cocultured cells (T1R) and in reference cells (T1). The method used for calculating the distance between genes or samples was Pearson's correlation coefficient, and Ward's method was used for clustering. All data correspond to the analysis of technical duplicate for each three independent batches of T1 and each three independent batches of T1R cells. NK, natural killer; PC, principal components.
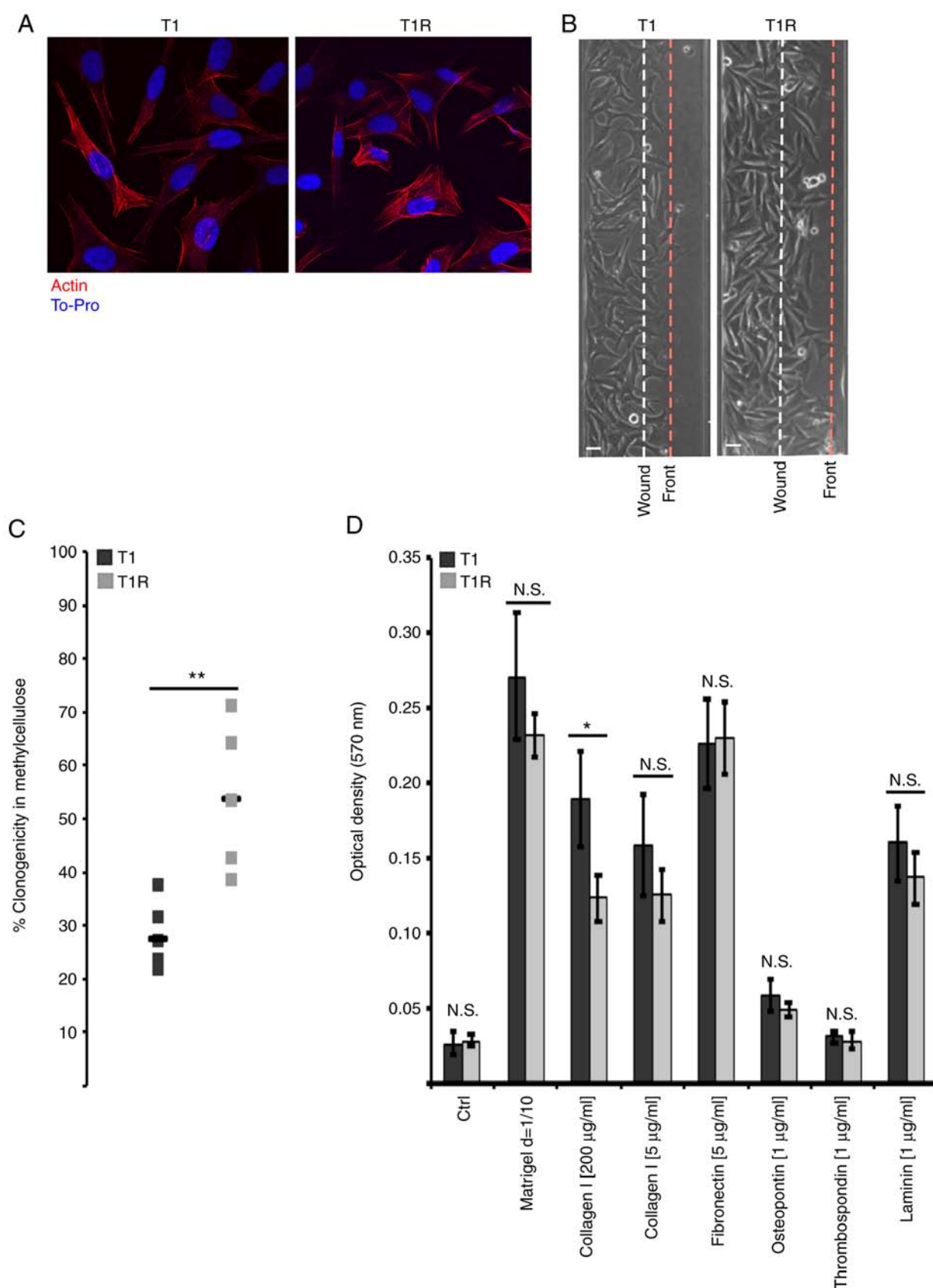
Figure 5. Phenotypical characteristics of T1R tumor cells selected by sustained NK cell pressure. (A) Confocal microscopy analysis of cocultured (T1R) and reference (T1) cell morphology by rhodamin-phalloidin staining (red). Scale bar, 10 μm. (B) Evaluation of migration properties of cocultured cells (T1R) and reference cells (T1) during 40 h in a laminar flux tubular system. The white hatched line corresponds to initial border of the cell population termed 'wound'. The red hatched line corresponds to the migration 'front' after 40 h. (C) Anchor-independent growth evaluation in methylcellulose. After 21 days of culture, clonogenicity was evaluated by counting. Data represent values of duplicates for each three independent batches of T1 and each three independent batches of T1R cells. Black bar, mean. (D) Evaluation of adhesion properties of cocultured cells (T1R) and reference cells (T1) on several extracellular matrices. Data represent the means of the three independent batches of T1 and the three independent batches of T1R cells, each performed in triplicate ± standard deviation. NK, natural killer; N.S., not significant. *P<0.05, **P<0.01.

whereas the proliferation rate was comparable between T1 and T1R cells (data not shown). Anchorage-independent growth in methylcellulose was also evaluated, and a significant increase in the clonogenic ability of T1R cells was observed in comparison to T1 cells (Fig. 5C). On the contrary, adhesion assay on several matrices indicated a significant decrease of T1R adhesion only to collagen at high concentration, whereas no statistically significant differences were observed for other proteins. Taken together, these results suggested that NK cell pressure may lead to the selection of tumor cells exhibiting phenotypical characteristics associated with a more aggressive behavior.

Discussion

It has become clear that the host immune system is involved in eliminating tumors, as well as in shaping the immunogenic phenotypes of tumors that eventually form in immunocompetent hosts, indicating that immunity plays a dual role in the complex interactions between tumors and the host. Despite some success with recent cancer immunotherapy approaches, the majority of the patients do not respond to this type of treatment, which is likely due to intrinsic tumor resistance that involves the innate molecular qualities of the tumor inhibiting the antitumor immune response. Several mechanisms have been proposed, including the reduction in antigenic expression and the alteration of the quality and number of immune effector cells in the tumor microenvironment. The other major mechanism involves an acquired resistance associated with several mechanisms by which tumor cells develop resistance over the course of treatment, resulting in cancer progression despite an initial response to immunotherapy. This includes loss of T-cell function, lack of T-cell recognition due to immunoediting, and the development of escape mutation variant tumor cells.

Blurring the boundary between innate and adaptive immune system, NK cells, a key component of innate immunity, are recognized as potent anticancer mediators. Tumor cells may develop several strategies to evade NK cell-mediated killing. In this regard, the involvement of NK cells in immune editing has been studied in relation to NKG2D and DNAM1 (21,43). Guillerey and Smyth demonstrated the NK cell activity in the cancer immune editing process, with particular emphasis on the elimination and escape phases (44). NK cells have been also shown to kill immature DCs due to their low amount of surface HLA class I molecules (45) and, therefore, impact the quality of adaptive immune response. These previous studies were undertaken in an attempt to further unravel the involvement of NK cells in tumor immunoediting and the emergence of tumor-resistant variants using a melanoma model. It should be noted that tumor cells evading NK cell-mediated lysis are well characterized, but few data are available regarding the implication of NK cells in the selection of such mechanisms.

The present study investigated the consequences of sustained NK cell-mediated immune stress and demonstrated that NK cells can contribute to immunoediting of the tumor, leading to emergence of cytotoxic resistant variants by a selection process or induction of tumor cell characteristics alteration. It was observed that the established resistant variant T1R did not display cross-resistance to autologous specific CTLs, suggesting that the resistant cells may use different mechanisms to escape cell-mediated cytotoxicity. In addition, no cross-resistance to dacarbazin was observed, which emphasizes that NK cells do not select tumor-resistant variants with an altered apoptotic signaling pathway. A decrease was observed in the susceptibility to TNF-α associated with a reduced TNF-R1 expression, which confirms the putative broad effect of long-term sustained NK-cell selective pressure, during which lysis pathways and the secreted molecules may differ from ^{51}Cr release standardized conditions. In contrast to short-term ^{51}Cr release, the death domain receptor pathway may be used by NK cells in sustained co-culture conditions, which may explain the decreased susceptibility to TNF-α. Furthermore, it should be noted that, under ^{51}Cr release conditions, T1 and T1R NK cell-mediated lysis was completely inhibited by concanamycin A (data not shown), indicating that their lysis in such an assay is fully dependent on the PFN/GzmB pathway.

Importantly, the data of the present study indicated that the resistance of T1R cells to NK cell-mediated lysis was associated with an alteration in immune synapse signaling. The reduction of immune synapse signaling following effector/target conjugation may be due to the loss in the expression of one of the main KIR and NKG2D ligands (46), as reported in the short 15 days without renewal of NK cell population co-culture model described by Balsamo *et al* (47). These investigators demonstrated that melanoma cells co-cultured with NK cells could induce, via IFN-γ, an increase of classical and non-classical MHC I molecules and a decrease of NKG2D ligands. However, in our model, no clear variation of these ligands, or MHC I and main KIR ligands, was observed.

An interesting question raised by the present study is whether the resistant variants pre-exist and/or adapt to NK cell-mediated immune stress. It was hypothesized that both selection and adaptation may be involved. However, further genetic, transcriptomic and cell tracking analysis of the clones will be required to address this question.

The transcriptomic analysis was performed using three independent biological replicates for each condition, all performed in technical duplicates. As evidenced by the relatively low number of genes differentially expressed between the parental T1 and T1R cells, this experimental design likely reduced much of the basal variability in gene expression due to cell line heterogeneity. Indeed, 99 genes were found to be differentially expressed between the parental T1 and T1R cells, and no particular pathways were found to be significantly enriched in subsequent analyses (GSEA, DAVID or INGENUITY). Among these genes, reduced expression of B7-H6 activator ligand of NKp30 was observed, which may play a key role in the activation of NK cells by tumor cells (46,48,49). To the best of our knowledge, such an event in NK cell-mediated selection has never been described to date, as the majority of studies report NKG2D ligand reduction (47). Future studies should investigate this possibility, as well as other putative mechanisms involved in inhibiting immune synapse formation and signaling.

In the context of melanoma, NKG2D does not appear to play a key role, and cytotoxic NK cell activity appears to be preferentially triggered by DNAM1 and NCRs (50). T1R cell resistance may also involve other molecules, and transcriptomic signature raises several possibilities. For example, POT1 overexpression constitutes an interesting candidate due to the

fact that it has been identified in a high-scale screening of molecules affecting NK cell-mediated lysis susceptibility (34). Importantly, the transcriptomic analysis also revealed the acquisition of genes associated with pro-metastatic and pro-invasive characteristics, which may reflect the aggressiveness of tumor cells selected by NK cells. Measurements of anchorage-independent growth ability as well as 2D migration capacity tend to confirm this hypothesis.

Collectively, the results of the present study demonstrated that NK cells are able to select tumor cells resistant to their cytotoxic activity that exhibit enhanced tumor aggressiveness characteristics. This should be considered in the current immunotherapeutic strategies. Indeed, as regards melanoma, the existence of numerous specific antigens has led to the development of several immunotherapeutic strategies, but clinical efficacy remains limited (51). As melanoma cells often express low levels of MHC class I molecules and a broad panel of NK receptor-activating ligands (52), NK cells represent a major alternative strategy (50) and are considered as key cytotoxic cells in adoptive antitumor immunotherapies (53), but their clinical benefits remain limited (52). Low level of tumor sites targeted by adoptive transfer NK cells may explain the lack of efficiency. Some targeting strategies may represent an interesting alternative (54). In this context, metalloproteinases have been reported to contribute to the ability of NK cells to reach tumor sites (55,56). Another obstacle in the efficiency of adoptive NK cell immunotherapies is the immune escape mechanisms developed by tumor cells. These processes may result from microenvironmental modulations leading to immunosuppression disturbing NK cell antitumor immune response through modification of activator and inhibitor receptor expression levels (57-59). In melanoma, indoleamine 2,3-dioxygenase and prostaglandin E2 have been shown to target NKp30, NKp44 and NKG2D on NK cells, causing loss of their activity (60). Blockade of these pathways may increase the antitumor efficiency of NK cells.

Tumor cell immune escape may also be due to intrinsic resistance mechanisms, such as the loss of NK cell activator ligands on tumor cells. Some treatments are being developed to increase the expression of those ligands on tumor cells in order to overcome this immune escape process (61-64). All these data regarding NK cell homing and increased tumor cell susceptibility through activator ligand re-expression or direct stimulation of NK cells (65,66) provide a rationale for the development of NK cell-based immunotherapeutic strategies (67). However, the sustained immune pressure of NK cells does not only induce loss of expression of activating ligands, but may also induce selection of tumor variants exhibiting aggressive properties. This should be considered as a putative side effect of such immunotherapies. We believe that the establishment of combinatory strategies integrating NK cells together with other cellular components and compounds, such as CD8^{+} T cells, DCs, monoclonal antibodies or chemotherapeutics drugs, is essential (65,68) for preventing tumor immunoediting and the emergence of resistant aggressive variants.

The findings of the present study may provide mechanistic insight into how NK cell pressure may lead to the emergence of resistant variants. Future studies aimed at examining whether such resistance occurs following NK-based cell therapy may be key to the design of NK-based innovative cancer treatments.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request. The microarray data and protocols are available at the European Molecular Biology Laboratory European Bioinformatics Institute database (https://www.ebi.ac.uk/arrayexpress/) under accession no. E-MTAB-8777.

Authors' contributions

Design of the study: TC and SC. Acquisition of data: TC, BJ, GG, GM and DO. Data analysis and interpretation: TC, JT, BJ, ST, GG, GM, CK, DO and SC. TC, JT, ST and SC drafted and critically revised the article. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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