Up-regulation of Inhibitory Natural Killer Receptors CD94/NKG2A with Suppressed Intracellular Perforin Expression of Tumor-Infiltrating CD8$^+$ T Lymphocytes in Human Cervical Carcinoma

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Abstract

Inhibitory signals that govern the cytolytic functions of CD8$^+$ T lymphocytes have been linked to the expression of natural killer cell receptors (NKRs) on CTLs. There is limited knowledge about the induction of inhibitory NKR (iNKR) expression *in vivo*. Up-regulation of iNKRs has been linked to the modulation of the virus- and/or tumor-specific immune responses in animal models. In the present study, we directly examined the expression of various NKRs on tumor-infiltrating lymphocytes (TILs) derived from human cervical cancer. We found that in human cervical cancer, the percentage expression of immunoglobulin-like NKR$^+$CD8$^+$ T lymphocytes were similar in gated CD8$^+$-autologous TILs and peripheral blood mononuclear cells. On the contrary, cervical cancer–infiltrating CD8$^+$ T lymphocytes expressed up-regulated C-type lectin NKRs CD94/NKG2A compared with either peripheral blood CD8$^+$ T cells or normal cervix-infiltrating CD8$^+$ T lymphocytes. Dual NKR coexpression analyses showed that CD94 and NKG2A were mainly expressed on CD56$^-$CD161$^-$CD8$^+$ TILs within the cancer milieu. Immunohistochemical study showed that cervical cancer cells expressed abundant interleukin 15 (IL-15) and transforming growth factor-β (TGF-β). In kinetic coculture assay, cervical cancer cells can promote the expression of CD94/NKG2A on CD8$^+$ T lymphocytes. The cancer-derived effects can be reversed by addition of rIL-15Rα/Fc and anti–TGF-β antibody. Functional analyses illustrated that intracellular perforin expression of CD8$^+$ T cells was minimal upon up-regulation of CD94/NKG2A. Kinetic cytotoxicity assays showed that up-regulated expressions of CD94/NKG2A restrain CD8$^+$ T lymphocyte cytotoxicity. Our study strongly indicated that cervical cancer cells could promote the expression of iNKRs via an IL-15– and possibly TGF-β–mediated mechanism and abrogate the antitumor cytotoxicity of TILs. (Cancer Res 2005; 65(7): 2921-9)

Introduction

It has previously been found that natural killer (NK) cells bear functional NK cell receptors (NKRs), which regulate NK cell activity upon interaction with certain HLA class I molecules (1, 2). These MHC class I–specific NKRs were detected originally on NK cells and were later found on selected subpopulations of CD8$^+$ T lymphocytes, usually with activated (CD69$^+$)/memory (CD28$^-$CD45RO$^+$) phenotypes (1, 3–8). Expression of these inhibitory NKRs (iNKR) on cytolytic T lymphocytes is now known to be linked to inhibition of cytotoxic functions by recognizing either classic or nonclassic MHC molecules (1, 2). HLA-specific NKRs are broadly categorized into two subgroups: the immunoglobulin-like superfamily and the C-type lectin superfamily (9). The former group includes various members that specifically recognize groups of human leukocyte antigen HLA-C (*p58*), HLA-B (*p70*), and HLA-A (*p140*) alleles (4, 10–13). The latter group is a type II trans-membrane protein that contains a C-type lectin domain (14–17). This subgroup includes CD161 (NKR-P1A), NKG2D, and CD94/NKG2 heterodimers with activating (NKG2C, NKG2E/H) or inhibitory (NKG2A/B) isotypes (18–23). CD94, which is covalently associated with NKG2A (CD94/NKG2A), was originally thought to display less well-defined allele specificity, but recent reports indicate that the nonclassic HLA class I molecule HLA-E is its actual target (18–23).

CTLs may acquire NK-like activity and become cytolytic against certain HLA class I–negative as well as HLA class I–positive targets (1, 2, 9). The presence of NK-like cytotoxicity usually comes with the expression of certain types of NKR on CTLs. These NKR-expressing T-lymphocyte subpopulations have been identified as expressing various NK-cell markers, including CD56, CD161, and other triggering/inhibitory NKRs (5, 6). However, the mechanisms dictating the regulation of NKR expression on effector CTLs, especially those residing within the tumor milieu, remain to be clarified. Previously, we have shown that human cancer cells may alter the functional composition of antitumor effector cells, including CD8$^+$ cytotoxic T cells, within the tumor milieu (24). We defined predominant Th2/Tc2 patterns of cytokine expression in CD3$^+$CD8$^+$ T-cell subsets in the human cancer milieu (24). We have further illustrated that cancer-derived mediators such as metalloproteinase are responsible for the immunosuppressive conditions of tumor-infiltrating lymphocytes (TILs) in human cervical cancer (25). The regulation of cytolytic function, especially the NK-like cytotoxicity, of CD3$^+$CD8$^+$ CTLs in the human cancer milieu remains to be stratified. It is possible that premature or abnormal up-regulation of NKRs on TILs induced by cancer cells, if it exists, contributes to an effective way of paralyzing the antitumor immune defenses.

In the present study, we directly examined the expression of various NKRs on TILs (especially CD8$^+$ TILs) derived from

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human cervical cancer. We used an *in vitro* mixed lymphocyte and tumor-cell coculture (MLTC) model to evaluate the kinetic expression of NKRs on autologous cytotoxic T cells after they encountered cancer cells. Our data showed for the first time that $CD8^+$ TILs in the human cervical cancer milieu expressed up-regulated iNKRs, and that cancer cells could directly promote the expression of iNKRs on $CD8^+$ T lymphocytes and thus significantly alter their cytolytic function.

Materials and Methods

Sample collection and isolation of tumor-infiltrating lymphocytes and peripheral blood mononuclear cells. Tissue specimens from patients with stage Ib to IIa cervical cancer were aseptically excised as described before (24–27). Autologous noncancerous and cervical cancer tissue specimens were first washed with RPMI 1640 and then subjected to mechanical fragmentation by use of scalpels. The fragmented tissue mass was then sieved first through a 380-μm and then through a 45.7-μm sieve (Life Technologies, Grand Island, NY). The filtered cell suspension was subsequently centrifuged for 15 minutes at 400 × *g*, layered over a Percoll discontinuous gradient (three concentrations: 30%, 55%, and 100% on the bottom), and finally centrifuged at 800 × *g* for 30 minutes. After centrifugation, lymphocytes from noncancerous tissue-infiltrating lymphocytes (NCL) and TILs were isolated as a white layer between 55% and 100% Percoll layers. Cervical cancer cells were separated from the interface of 30% Percoll and the sieved solution. Autologous peripheral blood mononuclear cells (PBMCs) were isolated by use of Ficoll Hypaque (1.077 density) and resuspended at a cell concentration of 1×10^6 cells/mL in RPMI 1640.

Purification of $CD3^+$ T cells. A positive selection of $CD3^+$ T lymphocytes was achieved with a magnetic-activated cell sorter (MACS, Miltenyi Biotec, Gladbach, Germany) and anti-CD3 monoclonal antibody (mAb) conjugated to microbeads (Miltenyi Biotec). Freshly isolated PBMCs, NCLs, and TILs were cultured in FCS-free RPMI 1640 for 24 hours in a 37°C, 5% CO_2 humidified incubator before the enrichment process. Suspended lymphocytes were incubated in the presence of microbead-conjugated anti-CD3 mAb for 15 to 20 minutes at 4°C. After repeated washing, antibody-coated cells were passed through a MACS separator in a magnetic field. The effluent was collected as the CD3-negative fraction. The positively selected $CD3^+$ T lymphocyte fraction was washed repeatedly and flushed outside the magnetic field. The recovered cells were checked and counted for viability with the trypan blue staining method.

Immunophenotyping analysis of subpopulations of $CD3^+$ T cells by flow cytometry. Monoclonal antibodies labeled with FITC, phycoerythin, or peridinin chlorophyll protein were used for triple-color flow cytometry. Triple surface staining was done by a combination of one each of the FITC-conjugated antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD45, or anti-CD56), phycoerythin-conjugated antibodies (anti-CD69, anti-CD94, anti-CD158a, anti-CD158b, anti-CD161, anti-NKB1, or anti-NKG2A), and peridinin chlorophyll protein–conjugated antibodies (anti-CD3 or anti-CD8). All mAbs, except for anti-CD94 and -NKG2A, were purchased from Becton Dickinson Immunocytometry System (Becton Dickinson, San Jose, CA). Anti-CD94 and anti-NKG2A were obtained from Immunotech (Marseille, France). Anti-mouse-IgG1-FITC + -IgG2a-phycoerythin was used as a negative control. Triple-color flow cytometry was done on FACScalibur (BD Biosciences, Mountain View, CA). Data were acquired with CellQuest software (BD Biosciences) by use of forward-scatter/side-scatter thresholds with 200,000 gated events.

Analyses of the coexpressional status of various natural killer cell receptors on $CD8^+$ T lymphocytes. We used triple-fluorescence color staining (anti-CD56-FITC or anti-CD161-FITC + anti-CD94-phycoerythin or anti-NKG2A-phycoerythin + anti-CD8-peridinin chlorophyll protein) on $CD3^+$ T cell–enriched lymphocytes to determine the expression pattern of NKRs on $CD8^+$ T lymphocytes. $CD3^+$ T cells were enriched from freshly isolated TILs, NCLs, and autologous PBMCs by MACS as described above. The coexpressional status of CD56, CD161, and CD94/NKG2A by subpopulations of $CD8^+$ T lymphocytes was based on gated $CD8^+$ cells in the FL3 channel with at least 200,000 events.

Autologous mixed lymphocytes and tumor cells coculture assay. $CD3^+$ T cells were purified from PBMCs by MACS and activated with anti-CD3-Abs (1 μg/mL) + anti-CD28-Abs (1 μg/mL) and goat-anti-mouse cross-linkers (2 μg/mL) in a 37°C, 5% CO_2 humidified incubator before coculture (24). The activated cells were labeled with anti-CD69-phycoerythin and checked by flow cytometry. A constant number of 5×10^5 activated $CD3^+$ T cells were directly cultured with 1×10^6 autologous $CD3^+$ TIL-depleted cervical cancer cells (1:2) in 1.5 mL of culture medium. The MLTC coculture procedure was conducted by culturing of cervical cancer cells and $CD3^+$ T cells in 24-well culture plates. For exploring the possible cancer-derived mediators, additional sets of cocultures were done with $CD3^+$ T cells separated from cervical cancer cells by an inserted layer of 0.4 μmol/L permeabilizing membrane in each culture well (Culture Plate Insert, Millicell-CM, Millipore Co., Bedford, MA). The expression of NKRs on $CD3^+$ T cells after encountering cancer cells was analyzed at days 0, 1, 3, and 5 after coculture.

Expression of immunoregulatory cytokines in cervical cancer tissue. To explore the possibility that cervical cancer–derived mediators regulate NKR expression of T cells, we used an avidin-biotin-peroxidase complex immunohistochemical staining method for examining the expression pattern of selected immunoregulatory cytokines in formalin-fixed and paraffin-embedded tissue sections (24, 25). The anti-IL-15 antibody was obtained from R&D Systems, Inc. (Minneapolis, MN). The anti–transforming growth factor-β (TGF-β) antibody was obtained from Serotec, Inc. (Kidlington, Oxford, United Kingdom). The specificities of both antibodies were provided by the manufacturers.

Effects of rIL-15Rα/Fc and anti–transforming growth factor-β antibody on up-regulation of CD94/NKG2A. To clarify the functional role of cancer-derived IL-15 and TGF-β in up-regulation of CD94/NKG2A in T cells, we added neutralizing reagents specific to IL-15 [recombinant human IL-15Rα/Fc chimera protein (rIL-15Rα/Fc), R&D Systems] or anti–TGF-β antibody (Serotec) to the previously established MLTC (28). Cancer cells (1×10^6) were first cultured in 1 mL of culture medium containing 0.5 μg/mL of rIL-15Rα/Fc or 10 μg/mL of anti–TGF-β antibodies for 2 hours, and cocultured with activated T cells (5×10^5 cells). For matched comparison, a constant number (5×10^5) of activated T cells was cultured as the T-only group (without cancer cells). Mouse anti-human IgG1 and rat anti-human IgG (10 μg/mL) were used as isotype antibody controls.

Direct effects of recombinant interleukin 15 and transforming growth factor-β on up-regulation of CD94/NKG2A in T cells. We further directly explored the functional role of rIL-15 and rTGF-β on up-regulation of CD94/NKG2A in T cells. $CD3^+$ T cells were purified by MACS and activated as previously mentioned. Recombinant IL-15 and TGF-β (R&D Systems) were added to the cultures of activated T cells (5×10^5 cells/mL) at a final concentration of 20 ng/mL each, respectively. The CD94/NKG2A expression of gated $CD8^+$ T cells was measured on days 1, 3, and 5 after culture by flow cytometry as described in the previous sections.

Intracellular staining in natural killer cell receptor–expressing $CD8^+$ T lymphocytes. $CD3^+$ T cells from both PBMCs and TILs were enriched by MACS as described. For the detection of intracellular perforin levels, PBMC-derived T cells were stimulated as mentioned and analyzed both before and 5 days after coculture with cervical cancer cells. For IFN-γ/perforin detection within fresh $CD94^+$/$NKG2A^+CD3^+CD8^+$ TIL subsets, TIL-derived T cells were stimulated with cross-linked anti-CD3 mAb (1 μg/mL anti-CD3 and 1.8 μg/mL anti-mIgG) and brefeldin A (10 μg/mL) for 16 to 18 hours before intracellular staining. Addition of anti-CD8-peridinin chlorophyll protein and anti-CD94-phycoerythin/anti-NKG2A-phycoerythin mAbs was combined with intracellular staining of perforin or IFN-γ. For surface antigen staining, 20 μL of anti-CD8-peridinin chlorophyll protein and anti-CD94-phycoerythin (or anti-NKG2A-phycoerythin, anti-CD158b-phycoerythin) were used for every set of 1×10^6 T cells in 200 μL culture medium. After surface staining, cells were washed and fixed with 1 mL fixation buffer containing 1% paraformaldehyde for 10 minutes before

commencement of intracellular staining. For intracellular staining, cells were washed twice with 500 μL permeabilization buffer containing (0.5% saponin in 1× PBS with 10% fetal bovine serum), and subsequently stained with 20 or 0.5 μL anti-perforin-FITC or anti-IFN-γ-FITC (BD Biosciences), respectively, for an additional 30 minutes at room temperature. Data were acquired and analyzed as previously mentioned. Another set of anti-mouse cytosol-IgG1-FITC + cytosol-IgG2a-phycoerythin was used as negative control.

Analyzing the cytotoxicity of natural killer cell receptor–expressing CD8$^+$ T lymphocytes. To evaluate the possible inhibitory effect on cytotoxicity of CD94/NKG2A in CD8$^+$ T cells, we used a phiphilux-based cytotoxicity assay. This phiphilux-based cytotoxicity assay was based on the notion that phiphilux determined cell-mediated cytotoxicity levels through detection of caspase-3-like activities (29). Essentially, CTLs were enriched from PBMCs by direct CD3$^+$ MACS isolation as described. NK cells were deprived to exclude the possible interference with the cytotoxicity assay from NK cells. Enriched CTLs were activated for 6 to 8 days with the addition of cross-linked anti-CD3 mAb (1 μg/mL anti-CD3 and 1.8 μg/mL anti-mIgG) combined with rhIL-2 or rhIL-15 (20 or 10 ng/mL, respectively). Shortly before cytotoxicity assay, stimulated CD3$^+$ CTLs were further enriched with CD8$^+$ MACS isolation and the subsequent CD3$^+$CD8$^+$ CTLs were treated with anti-CD94 (HP-3B1), anti-NKG2A (Z199), or isotype control antibodies (Immunotech). For determining the NK-like cytotoxicity of T cells, K562 cells were used as the target cells in this assay and treated with 200 ng/mL rhIFN-γ for 2 days for the induction of HLA class I molecules. For the confirmation of HLA class I expression on K562 cells, cells treated with and without rhIFN-γ were stained with anti-HLA class I monomorphic determinant (NeoMarkers, Lab Vision Co., Westinghouse, CA) followed by anti-mIgG-FITC (BD Biosciences) before flow cytometric analysis. Shortly before coincubation with effector cells, target cells were labeled according to the manufacturer (red label, CytoxiLuxTM, Oncoimmunin, Inc., Gaithersburg, MD). Cytotoxic assays were done with different effector/target cell ratios (1:1, 3:1, and 9:1) in 200 μL of culture medium for 2 hours before treatment of Phiphilux (29). Phiphilux was treated at the end of the 2-hour coincubation according to the manufacturer (Oncoimmunin). The efficacy of cytotoxicity was assessed with dual-color flow cytometry on a FACScalibur (Becton Dickinson) as previously mentioned. Data collected with FACScalibur were analyzed with WinMDI software (Joseph Trotter, Scripps Institute, La Jolla, CA).

Statistical analysis. The percentage expression of a given marker was obtained for a subset of T lymphocytes. All data were expressed as median with intraquantile range (IQR) unless otherwise indicated. The Wilcoxon signed-ranks test was used for comparing paired samples of the same patients, and the Mann-Whitney *U* test was used for comparing variables between groups. Kinetic data of MLTC were analyzed by a generalized estimation equation for correlated data of repeated measurements and expressed as mean ± SE. Statistical significance was defined by a $P < 0.05$.

Results

High ratio of CD8$^+$ T lymphocytes in gated CD3$^+$ tumor-infiltrating lymphocytes. The viability of immunocytes used in the studies was above 95% at the completion of the isolation procedure in all cases. An enrichment rate of >98% purity in the positively selected CD3$^+$ pool was evaluated by flow cytometry after labeling with anti-CD3-FITC. A higher ratio of CD8$^+$ T lymphocytes was noted in gated CD3$^+$ TILs than that in autologous PBMCs/NCLs (53.60% [IQR = 11.95] versus 31.06% [IQR = 13.80], $n = 20$, $P < 0.001$), which was compatible with our previous data (27). The NK-T cell–associated marker CD56 was expressed in 4.26% [IQR = 5.98] of gated CD3$^+$ PBMCs/NCLs and in 6.01% [IQR = 11.27] of gated CD3$^+$ TILs ($n = 13$, no statistical significance).

Percentage expression of immunoglobulin-like natural killer cell receptors was not different on CD3$^+$CD8$^+$ tumor-infiltrating lymphocytes and peripheral blood mononuclear cells. The expression of immunoglobulin-like NKRs (CD158a, CD158b, and NKB1) was determined on either CD4$^+$ or CD8$^+$ T lymphocytes from peripheral blood, noncancerous, and cervical cancer tissues. These immunoglobulin-like NKRs could be detected at variable levels on CD8$^+$ T lymphocytes (Fig. 1). Triple-fluorescence flow-cytometric analyses showed that the percentage expressions of CD8$^+$ T lymphocytes positive for various immunoglobulin-like NKRs were similar in TILs and autologous PBMCs/NCLs (CD158a: 2.16% [IQR = 2.22] versus 2.44% [IQR = 2.72], $n = 13$; CD158b: 6.09% [IQR = 5.11] versus 3.89% [IQR = 4.82], $n = 14$; NKB1: 1.32% [IQR = 2.13] versus 1.56% [IQR = 5.20], $n = 14$, all without statistical significance; Fig. 1*B*). There was no or only minimal expression of immunoglobulin-like NKRs on CD4$^+$ T lymphocytes derived from PBMCs, NCLs, and TILs (Fig. 1*B*). The result showed that expression of immunoglobulin-like NKRs seemed not to be altered among TILs, NCLs, and autologous PBMCs.

Up-regulation of CD94 and NKG2A with down-regulation of CD161 by CD3$^+$CD8$^+$ tumor-infiltrating lymphocytes. The percentage expression of C-type lectin superfamily NKRs (CD94, NKG2A, and CD161) on T lymphocytes from peripheral blood, noncancerous, and cervical cancer was compared by

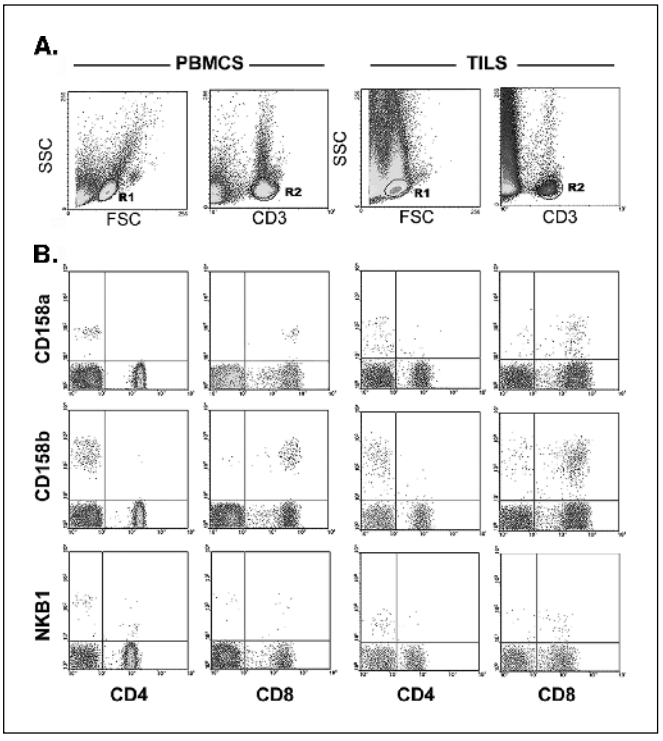

Figure 1. Representative dot plots of immunoglobulin-like NKR expression on CD4$^+$/CD8$^+$ subsets within gated CD3$^+$ T cells in TILs and PBMCs. Data collected from the fresh samples of one single patient. *A,* triple-fluorescence staining included a FITC-labeled anti-CD4 or anti-CD8 mAb, anti-CD3-peridinin chlorophyll protein, and PE-labeled mAbs against indicated NKRs. Data were acquired with forward scatter (*FSC*)/side scatter (*SSC*) thresholds with 200,000 gated events as *R1* to measure the proportion of lymphocytes in the sample being studied. Data were further gated on CD3$^+$ cells (*R2*) for evaluation of the expression of immunoglobulin-like NKR on CD4$^+$/CD8$^+$ T lymphocyte subsets. *B,* minimal expression of CD158a, CD158b, and NKB1 were noted in CD4$^+$ T lymphocyte fractions of either gated CD3$^+$ PBMCs or TILs. The expression of CD158a, CD158b, and NKB1 in gated CD3$^+$CD8$^+$ PBMCs were similar to those in TILs.

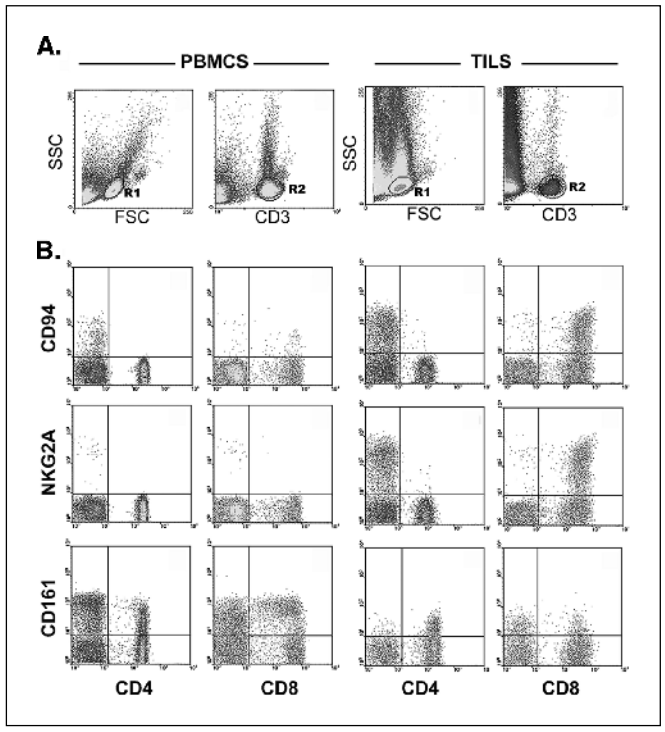

Figure 2. Representative dot plots of C-type lectin NKR expression on $CD4^+$/$CD8^+$ subsets within gated $CD3^+$ T cells in TILs and PBMCs. Data collected from the fresh samples of one single patient. *A,* triple-fluorescence staining included a FITC-labeled anti-CD4 or anti-CD8 mAb, anti-CD3-peridinin chlorophyll protein, and PE-labeled mAbs against indicated NKRs. Data were acquired with forward scatter (*FSC*)/side scatter (*SSC*) thresholds with 200,000 gated events as *R1* to measure the proportion of lymphocytes in the sample being studied. Data were further gated on $CD3^+$ cells (*R2*) for evaluation of the expression of NKR on $CD4^+$/$CD8^+$ T lymphocyte subsets. *B,* CD94 and NKG2A were not expression on the $CD4^+$ T-lymphocyte fractions of either gated $CD3^+$ PBMCs or TILs. The expression of CD94 and NKG2A on tumor-infiltrating $CD8^+$ T lymphocytes were higher than those in autologous peripheral blood $CD8^+$ T cells.

triple-fluorescence flow-cytometric analyses, and representative data are shown in Fig. 2. Analyzing the $CD4^+$ fractions of either gated $CD3^+$ PBMCs or $CD3^+$ TILs derived from cervical cancer tissue, we found no expression of CD94 and NKG2A (Fig. 2*B*). On the contrary, $CD3^+CD8^+$ TILs were found to express significantly more CD94 and NKG2A than did either $CD3^+CD8^+$ PBMCs or NCLs (Fig. 2*B*). For CD94, the percentage expression was 16.83% [IQR = 17.57] and 8.01% [IQR = 11.24] for $CD3^+CD8^+$ TILs and $CD3^+CD8^+$ PBMCs/NCLs (n = 16, P < 0.01), respectively. For NKG2A, the percentage expression was 13.11% [IQR = 16.23] and 3.02% [IQR = 3.60] for $CD3^+CD8^+$ TILs and $CD3^+CD8^+$ PBMCs/NCLs (n = 10, P < 0.01), respectively.

Unlike the expression of CD94 and NKG2A, CD161 expression was detected in both $CD4^+$ and $CD8^+$ T cells in variable proportions (Fig. 2*B*). A significantly lower percentage expression of CD161 was shown in $CD8^+CD3^+$ TILs compared with that in $CD8^+CD3^+$ PBMCs (7.64% [IQR = 15.79] versus 20.53% [IQR = 23.43], n = 12, P = 0.012). The results suggested up-regulation of CD94/NKG2A and down-regulation of CD161 on $CD3^+CD8^+$ TILs.

Absence of CD56 and CD161 on CD94/NKG2A$^+$CD3$^+$CD8$^+$ tumor-infiltrating lymphocytes. Dual NKR coexpression analyses showed the possible discordant expression of CD94/NKG2A with CD56 or CD161 on the same lymphocytes in the gated $CD8^+$ T cell population (Fig. 3). In PBMCs/NCLs, coexpression of CD94/NKG2A with CD56 was observed in about one third to more than one half of gated $CD8^+$ T cells (Fig. 3*B*). The same was true for CD94/NKG2A with CD161. On the contrary, CD94 and/or NKG2A were mainly detected on $CD56^-CD161^-CD8^+CD3^+$ TILs. The expressions of CD56/CD161 are extremely rare in TILs (Figs. 2 and 3*C*).

Cervical cancer–induced up-regulation of CD94/NKG2A on T cells. We used an MLTC *in vitro* kinetic assay (24, 25) to explore the possible cancer-derived effect on the expression of CD94/NKG2A in TILs. In six sets of repeated experiments, the mean expression percentage of CD94 on gated $CD8^{bright}$ T lymphocytes in MLTC increased >1- and 3-fold by days 3 and 5, respectively, when compared with that in controls (n = 6, P < 0.01; Fig. 4*A*). The mean

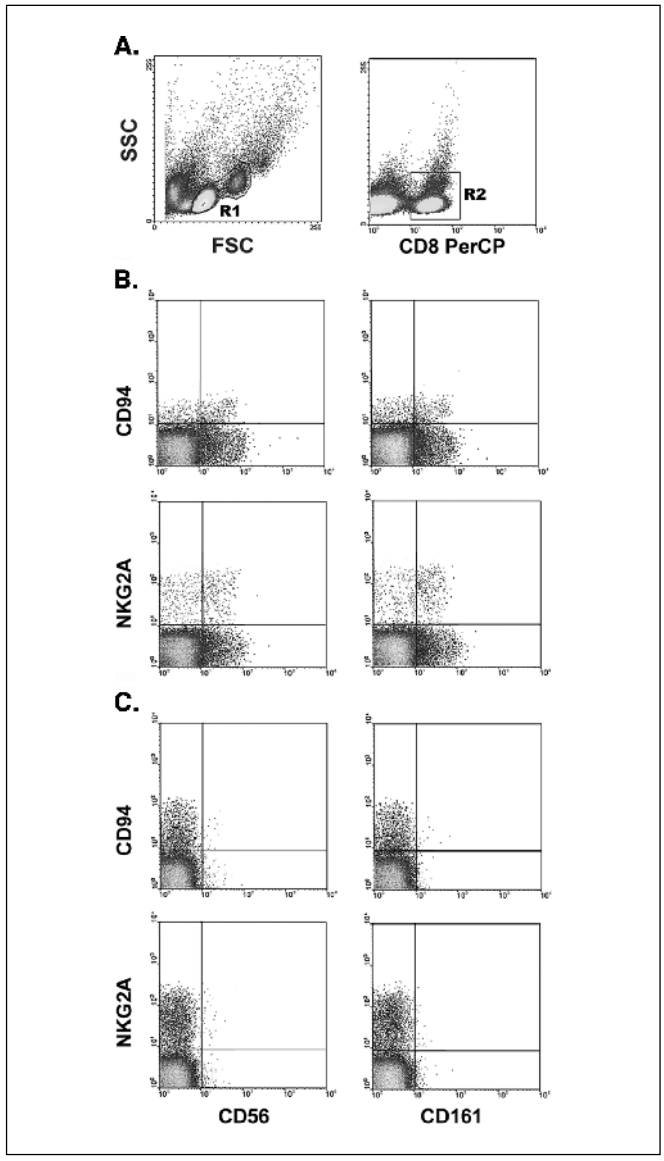

Figure 3. Representative dot plots of dual NKR coexpression analyses in gated $CD8^+$ T lymphocytes. *A,* data were acquired with forward scatter (*FSC*)/side scatter (*SSC*) thresholds with 200,000 gated events (*R1*) to measure the proportion of lymphocytes in the sample being studied. Data were further gated on $FL3^+$ cells with a CD8 trigger (*R2*) for evaluation of the $CD8^+$ T-cell subsets. *B,* in PBMCs, concordant expression of CD94/NKG2A with CD56 and/or CD161 was observed in gated $CD8^+$ T lymphocytes. *C,* $CD8^+$ T lymphocytes derived from TILs showed significantly discordant coexpressions of CD94/NKG2A and CD56 and/or CD161.

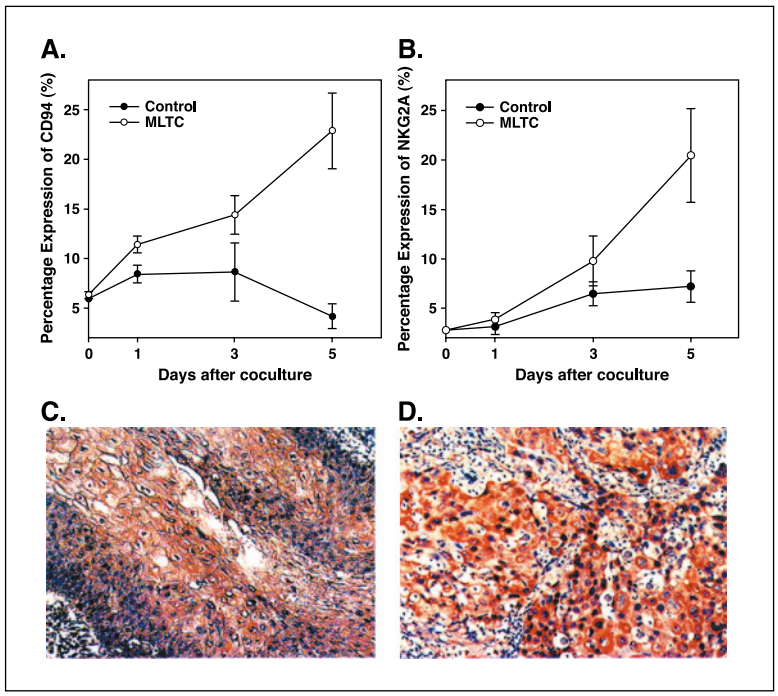

Figure 4. *In vitro* kinetic MLTC assays of cancer-derived effect on the expression of CD94/NKG2A. The expression percentages of CD94 and NKG2A were measured on gated $CD8^+$ T cells after encountering cancer cells. *A,* in six sets of repeated experiments after 5 days of coculture, the mean expression percentage of CD94 on gated $CD8^{bright}$ T lymphocytes in MLTC increased >1- and 3-fold by days 3 and 5, respectively, which was significantly higher than that of activated PBMCs ($n = 6$, $P < 0.01$). *B,* mean expression percentage of NKG2A on gated $CD8^{bright}$ T lymphocytes in MLTC was also significantly higher than that in controls, after 3 and 5 days of coculture (increase >3- and 6-fold, respectively; $n = 6$, $P = 0.013$). *C,* immunohistochemical staining showed abundant IL-15 expression in cancer cells. *D,* abundant expression of TGF-β was noted in cancer cells but minimal in stromal cells.

expression percentage of $NKG2A^+$ on gated $CD8^{bright}$ T lymphocytes in autologous MLTC was also significantly increased >3- to 6-fold compared with that in controls during these 3- to 5-day cocultures ($n = 6$, $P = 0.013$; Fig. 4*B*). The cervical cancer–derived effects were not changed after insertion of a layer of 0.4-μm permeabilizing membrane in the culture well. The data showed that cancer cells could promote the expression of CD94 and NKG2A by $CD8^+$ T lymphocytes through certain cancer-derived mediators rather than by direct cell contact.

Cancer cells expressed immunoregulatory cytokines interleukin 15 and transforming growth factor-β. To examine the cancer-derived mediators that possibly regulate the expression of iNKRs by T cells, we examined the expression of IL-15 and TGF-β by immunohistochemical staining of cervical cancer tissues from 40 patients. IL-15 and TGF-β were abundantly expressed in most, if not all cancer cells, but were not or only very weakly expressed in normal cervical epithelial or stromal cells (Fig. 4*C* and *D*). Our data were compatible with those in previous reports (24, 30).

Cancer-derived interleukin 15 and transforming growth factor-β up-regulate expression of inhibitory natural killer cell receptors on T cells. To characterize the possible role of IL-15 and TGF-β in regulating the expression of iNKRs on T cells after encountering cancer cells further, we added rIL-15Rα/Fc and anti–TGF-β antibody in the autologous MLTC to determine whether these specific neutralizing reagents could reverse or limit the cancer-induced effects. By addition of rIL-15Rα/Fc, a significant decrease (53% and 82%) in the mean percentage expression of CD94 on gated $CD8^{bright}$ T lymphocytes in the MLTC was observed in day 3 and 5 cocultures, respectively ($n = 10$, $P < 0.01$; Fig. 5*A*). The mean percentage expression of NKG2A in rIL-15Rα/Fc-treated MLTC was synchronously reduced on day 3 and 5 cocultures (42% and 79% reduction, respectively; $n = 10$, $P < 0.01$; Fig. 5*B*). The effect of anti–TGF-β antibody on cancer-induced expression of CD94/NKG2A was also observed but to a lesser extent than with that of the rIL-15Rα/Fc, with 27% and 33% decreases in day 3 and 5 cocultures, respectively (Fig. 5*A* and *B*). A maximal decrease (76% and 97%, $n = 10$, $P < 0.01$) in the mean percentage expression of CD94 and NKG2A on gated $CD8^{bright}$ T lymphocytes was observed when both rIL-15Rα/Fc and anti–TGF-β antibody were added in the MLTC (MLTC + rIL-15Rα/Fc + anti–TGF-β group). The isotype controls did not alter the mean percentage expression of CD94/NKG2A in the MLTC. Our data illustrated that cancer cells can promote the expression of CD94/NKG2A in T cells. These cancer-derived effects can be reversed by rIL-15Rα/Fc and/or anti–TGF-β antibody.

Up-regulation of CD94/NKG2A on $CD8^+$ T lymphocytes by recombinant interleukin 15 and transforming growth factor-β. To clarify the possible additive effects of cancer-derived IL-15 and/or TGF-β on the expression of CD94/NKG2A in T cells, we further incubated activated T cells with recombinant human interleukin 15 (rhIL-15), TGF-β, and IL-2 (control group; Fig. 5*C* and *D*). In rhIL-15-induced up-regulation, CD94 was induced within 24 hours after cytokine treatment, whereas NKG2A expression reached a comparative level in day 3 (Fig. 5*C* and *D*). Both the up-regulation of CD94 and NKG2A could reach a consistent level with those induced by cervical cancer cells within 5 days of culture (Fig. 5, for rhIL-15-induced expression, $n = 10$, $P = 0.008$ and $P < 0.001$ for CD94 and NKG2A, respectively). rhTGF-β-induced up-regulation pattern followed that of rhIL-15, only to a lesser degree (Fig. 5; for rhTGF-β-induced expression, $n = 10$, $P = 0.02$ and $P = 0.005$ for CD94 and NKG2A, respectively). The rhIL-2-induced expression of both CD94 and NKG2A was insignificant. It became evident that recombinant IL-15 and TGF-β recapitulated the effect of cervical cancer–derived IL-15 and/or TGF-β in the induction of the expression of CD94/NKG2A on T cells.

Minimal expression of perforin in $CD94^+$/$NKG2A^+CD8^+$ T cells. To clarify the functional roles of discordantly expressed NKRs on subpopulations of $CD8^+$ T lymphocytes after encountering cancer cells, we examined the expression of perforin by

triple-fluorescence intracellular staining in TILs and in the MLTC model. Low expression of IFN-γ in gated $CD3^+CD8^+$ TILs (Tc1 subset, 27.42% [IQR = 17.26]) derived from cervical cancer tissue, which was compatible with our previous study (24). NKG2A was synchronously expressed with intracellular IFN-γ in gated $CD3^+CD8^+$ TILs, with more than half of the gated $CD3^+CD8^+NKG2A^+$ population stained positive with IFN-γ (Fig. 6*A*). The intracellular perforin expression in these gated $CD94^+/NKG2A^+CD8^+$ TILs was minimal (Fig. 6*B*). In the kinetic model of MLTC, high expression of perforin was observed in activated $CD8^+$ T cells before coculture (Fig. 6*C*, *top*). After 3 days of coculture, the intracellular perforin expression in $CD8^+$ T cells was minimal upon up-regulation of CD94/NKG2A expression (Fig. 6*C*, *bottom*). Our data showed that the expression of CD94/NKG2A by CTLs is legitimate to abrogate their cytotoxic ability.

CD94/NKG2A expression significantly thwarted CTL cytotoxic capability. Furthermore, we tested the effect of rIL-15-induced CD94/NKG2A on the NK-like cytotoxicity of $CD8^+$ T lymphocytes. CTLs were enriched from PBMCs to a final purity of >99.7%. NK cells were deprived to <0.3% to exclude the possible interference with the cytotoxicity assay from NK cells. Phiphilux cytotoxic assays were done with different effector/target cell ratios (1:1, 3:1, and 9:1; Fig. 7*A*). The expression of HLA class I molecules on the target cells was induced by the treatment of rhIFN-γ (Fig. 7*B*). The mean killing efficacy of the rIL-15-treated CTLs was lower than that of the rIL-2-treated CTLs (Fig. 7*C*). Upon the blockade of CD94-associated NKG2A by anti-CD94 (HP-3B1) or anti-NKG2A mAb (Z199), the cytotoxicity level of rIL-15-treated group was restrained to that of rIL-2-treated group. In rIL-2-treated group, the blockade of both anti-CD94/anti-NKG2A did not alter the killing level of CTLs (Fig. 7*C*). Our data showed that cervical cancer cells could promote the expression of CD94/NKG2A and abrogate the cytotoxicity of $CD8^+$ T cells through an IL-15- and possibly TGF-β–mediated mechanism.

Discussion

There is limited knowledge about the induction of NKR expression *in vivo*. iNKRs are proposed not only to restrain T cell receptor (TCR)–mediated cytolysis but also to hold back the NK-like cytotoxicity implemented by activated $CD8^+$ CTLs in both *in vitro* human and mouse models (1, 2, 31–34). The acquisition of both NK-like activity and up-regulation of iNKRs CD94/NKG2A by $CD8^+$ T cells is parallel to prevent damage to normal host cells (35), reminiscent of the safety mechanism involved with CTL functions and NKRs (34, 36). Up-regulation of iNKRs has been linked to the modulation of the virus- and/or tumor-specific immune responses (37, 38). In mice, the immediate expression of an iNKR heterodimer (CD94/NKG2A) by virus-specific CTLs upon clearance of the virus infection is considered to be a protective mechanism from potential autoimmunity before a complete discharge of the CTLs (39). In experimental tumor models, CTLs that exhibit a low level of lytic activity against autologous tumor cells can be dramatically reversed upon iNKR blockade (32, 33).

Both $TCR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ lymphocyte subpopulations have been identified as expressing various NK cell markers (5, 6, 40, 41). CD56 is thought to be a general cell marker correlating the cytolytic (procytotoxic) function of NK-like $CD8^+$ T cells (42, 43). CD161 mediates costimulatory roles assisting CD1d-dependent activation in T cells (40). Circulatory T cells have been shown to exhibit both NK-like (TCR independent) and TCR-dependent cytotoxic activities *in vitro* (42, 43). The actual mechanisms that regulate the *in vivo* expression of NKRs on these NK-like T lymphocytes remain to be stratified, although cytokines could have significant roles in the regulation of NKRs (44–46).

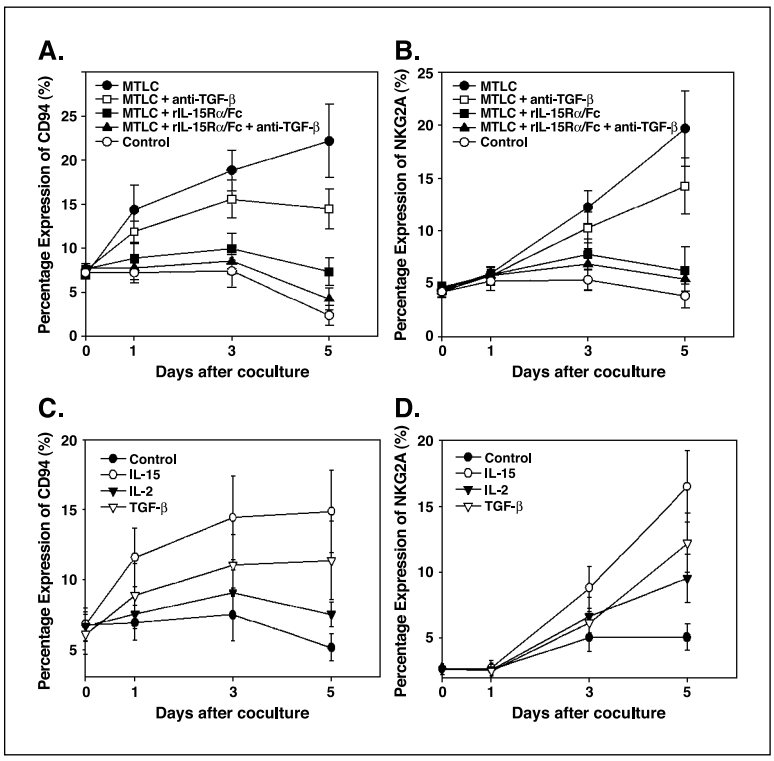

Figure 5. Cancer-derived IL-15 and/or TGF-β upregulate the expression of CD94/NKG2A in T cells. *A,* by addition of rIL-15Rα/Fc and/or anti–TGF-β antibody, reversed cancer-derived effect with decreases in the mean percentage expression of CD94 on gated $CD8^{bright}$ T lymphocytes in the MLTC were observed in day 3 and 5 cocultures ($n = 10$, $P < 0.01$). *B,* mean percentage expression of NKG2A in MLTC was synchronously reduced on day 3 and 5 cocultures after addition of rIL-15Rα/Fc and/or anti–TGF-β antibody ($n = 10$, $P < 0.01$). *C,* up-regulation of CD94 on $CD8^+$ T lymphocytes induced by recombinant IL-15 and TGF-β. *D,* recombinant IL-15 and TGF-β up-regulated the expression of NKG2A on $CD8^+$ T lymphocytes. $CD3^+$ T cells were activated with cross-linked anti-CD3 and anti-CD28 in the presence of rIL-15 (5-20 ng/mL), IL-2 (10-20 ng/mL), or TGF-β (10-20 ng/mL). The percentage expression was measured as the percentage of CD94- or NKG2A-positive cells within all gated $CD8^+$ T lymphocytes. *Points,* mean from 10 independent experiments of kinetic cultures in triplicate; *bars,* ±SE.

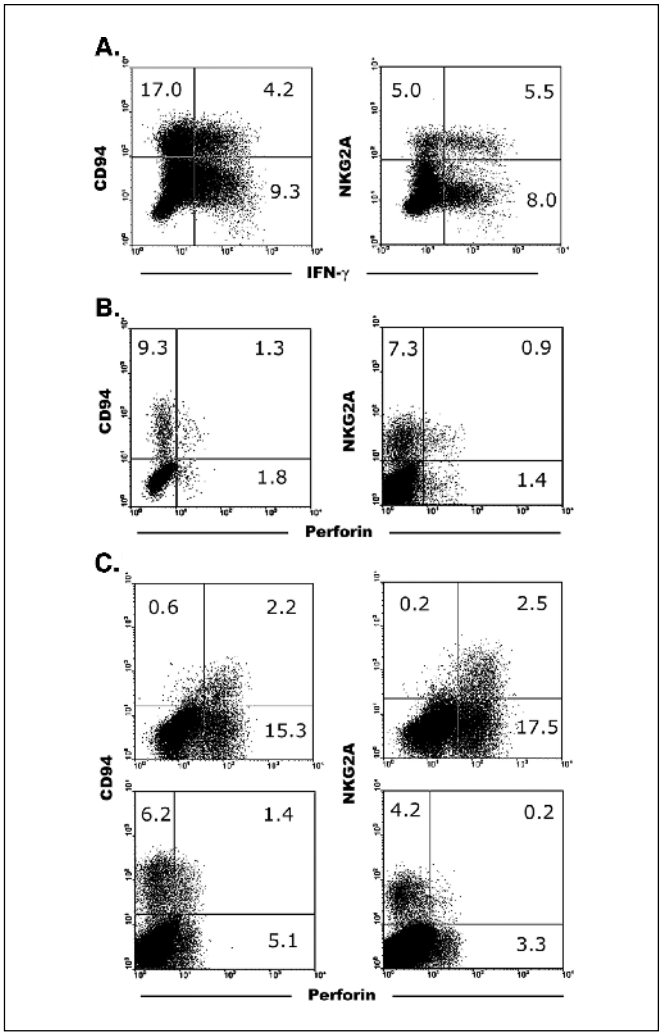

Figure 6. Reduced expression of perforin in CD94/NKG2A$^+$CD8$^+$ T cells. *A* and *B,* expression of CD94/NKG2A associated intracellular IFN-γ and minimum perforin expression by CD8$^+$CD3$^+$ TILs. For IFN-γ staining, re-stimulated CD3$^+$ TILs were stained with anti-CD8-peridinin chlorophyll protein, anti-IFN-γ-FITC, and anti-CD94-PE/anti-NKG2A-PE as indicated and gated on CD8$^+$ population. *A,* each dot plot was revealed with at least 50,000 gated events. *B,* freshly isolated CD3$^+$ TILs were directly stained with anti-CD94-PE/anti-NKG2A-PE, anti-CD8-peridinin chlorophyll protein, and anti-perforin-FITC as indicated and gated on CD8$^+$ population. *C,* expression of CD94/NKG2A on CD8$^+$CD3$^+$ CTLs with down-regulated expression of intracellular perforin after MLTC. CD3$^+$ CTLs before (*top*) and after (*bottom*) MLTC were stained with anti-CD8-peridinin chlorophyll protein, anti-perforin-FITC, and anti-CD94-PE/anti-NKG2A-PE as indicated with at least 20,000 gated events on CD8$^+$ population.

In the present study, we have shown a reduced proportion of CD161$^+$CD8$^+$ T cells in cervical cancer tissues. We showed that CD8$^+$ TILs expressed up-regulated CD94/NKG2A, as a direct result of tumor cell and T cell interaction. CD94 and NKG2A were mainly expressed on CD56$^-$CD161$^-$CD8$^+$ TILs within the cancer milieu, excluding the possibility of the up-regulated CD94/NKG2A$^+$ T lymphocytes being the NK-T lineage lymphocytes. In addition, CD94/NKG2A was synchronously expressed with intracellular IFN-γ in gated CD3$^+$CD8$^+$ TILs (Fig. 6), which illustrated a predominant expression of iNKRs in Tc1 subset. Moreover, we showed that up-regulated expression of CD94/NKG2A was associated with minimal intracellular expression of perforin, indicating a possible restricted cytolytic function of TILs within the tumor milieu. The expression of immunoglobulin-like NKRs (i.e., CD158a, CD158b, and NKB1) was, however, not affected in TILs in cervical cancer.

The finding of an up-regulation of CD94/NKG2A in CD8$^+$ TILs is not entirely unexpected. Both the CD94/NKG2A heterodimer and KIR2DL3 have been described on human melanoma–specific CTLs derived from *in vitro* activated autologous T cells (32, 33). Expression of iNKRs and their *in vitro* conjugation with ligands on effector CTLs have been linked to the reduction of tumor necrosis factor secretion and inhibition of specific cytolysis against melanoma peptide-coated targets (32–34, 47). The increased expression of CD158b was linked to a diminished cytolytic function of tumor-specific TILs against renal cell carcinoma (48).

Expression of CD94/NKG2A inhibitory receptor may be restricted to a clonal attribute of CD8$^+$ T-cell subsets and may shape the cytotoxic responses (49). Our study showed a predominant expression of CD94/NKG2A in Tc1 subset, which was about 27.42% of total CD3$^+$CD8$^+$ TILs (24). Stimulatory signaling of TCR in the presence of certain cytokines, including

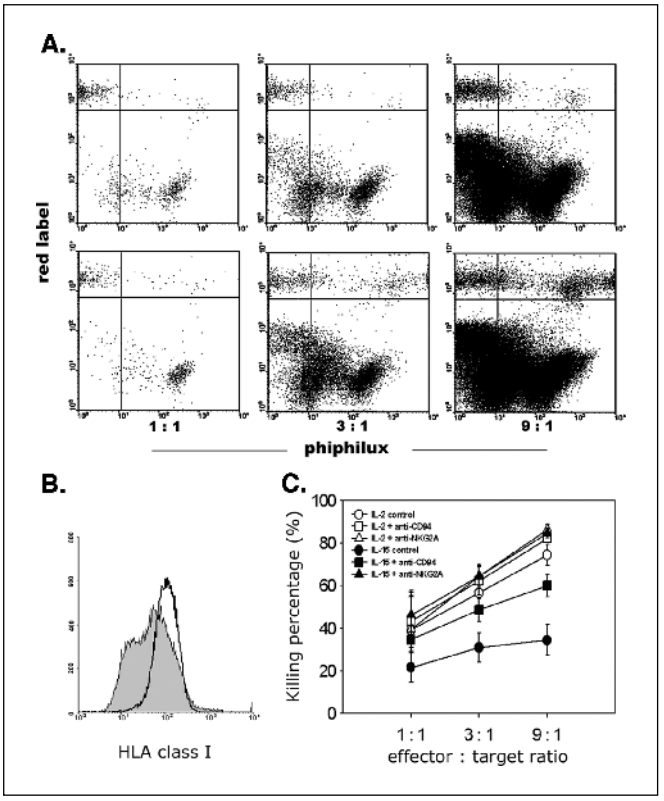

Figure 7. Expression of CD94/NKG2A on CD8$^+$CD3$^+$ CTLs significantly thwarted the NK-like cytotoxicity. *A,* representative dot plots showed kinetic phiphilux cytotoxic assays in CTLs. Target cells were treated with rhIFN-γ for HLA class I induction and properly labeled with red label. Effector cells (rIL-15-induced CD8$^+$CD3$^+$ CTLs) treated with isotype (*top*) or anti-NKG2A mAbs (Z199, *bottom*) were incubated with labeled target cells at indicated effector/target ratios (1:1, 3:1, and 9:1) for 2 hours before assessment of cytotoxicity. Kinetic phiphilux cytotoxic assays were measured by the percentage of phiphilux-positive cells within all red label-positive cells. *B,* induction of HLA class I molecule expression on target cells with rhIFN-γ. *C,* kinetic data of cytotoxicity assay was compared between groups of different treatment as indicated. Various combinations of cytokines and antibodies added to the assays include rIL-15, rIL-2, anti-CD94, and anti-NKG2A. Activated CTLs treated with rIL-15 only (●) or rIL-2 only (○) were used as controls. CTLs treated with rIL-15 or rIL-2 were further incubated with anti-CD94 (■ and □) or anti-NKG2A (▲ and △) before cytotoxicity assays. By addition of anti-NKG2A and anti-CD94 mAbs, the mean killing percentage of CTLs in the rIL-15-treated group was restored to the level of rIL-2-treated group. *Points,* mean from six independent experiments in triplicate; *bars,* ±SE.

IL-12, IL-15, and TGF-β, has been linked to the expression and maintenance of immunoglobulin-like and, perhaps, other types of NKRs on $CD8^+$ T cells (50–53). Local abundance of IL-10, IL-12, IL-15, and TGF-β was also reported in cervical cancer (24, 30, 54). Our data directly illustrated that cervical cancer–derived IL-15 and/or TGF-β may drive the up-regulation of $CD94^+/NKG2A^+$ cells within the $CD8^+$ CTLs and thus diminish their antitumor effect in the cancer milieu.

In the cytotoxicity assay, we observed that the expression of CD94/NKG2A heterodimer on CTLs blocks the cytotoxicity against HLA class I^+ target cells. CTLs treated with rIL-15 had up-regulated expression of CD94/NKG2A with decreased intracellular perforin expression (Figs. 5 and 6). The cytolytic effect of the rIL-15-treated CTLs was lower than that of the rIL-2-treated CTLs (Fig. 7). Upon the blockade of CD94-associated NKG2A by anti-CD94 (HP-3B1) or anti-NKG2A mAb (Z199), the cytotoxicity level of rIL-15-treated group was restrained to that of rIL-2-treated group (Fig. 7). Our study confirms that cancer cells can directly promote the expression of iNKRs on TILs/CTLs and thus abrogate the antitumor cytotoxicity through an IL-15- and possibly TGF-β–mediated mechanism.

In summary, we showed for the first time that $CD8^+$ T cells in the human cervical cancer milieu expressed up-regulated iNKRs, CD94, and NKG2A. The present study directly illustrated two possible pathways of cancer immune escape: down-regulation of perforin and direct blockade of cytotoxicity through CD94/NKG2A engagement through its ligands. Our study on these iNKR-expressing TILs may provide important insights into an understanding of the interaction between cancer cells and the immune system and the development of anticancer strategies.

Acknowledgments

Received 6/18/2004; revised 1/3/2005; accepted 1/26/2005.

Grant support: National Science Council grants NSC93-2314-B002-164, NSC93-2314-B002-065, NSC92-2314-B002-159, and NSC92-2314-B002-307 and National Taiwan University Hospital grants NTUH.92A10-4, NTUH.93S017, and NTUH.94S45.

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