Ex Vivo Expansion of CD8\(^+\)CD56\(^+\) and CD8\(^+\)CD56\(^-\) Natural Killer T Cells Specific for MUC1 Mucin

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INTRODUCTION

Tumor cells also present a unique challenge to the adaptive immune response by evading immune detection. Diverse mechanisms that include inappropriate activation of antigen presenting cells (APCs; Ref. 5), antigen shedding, and down-regulation of HLA class I molecules have been described (5–7). Under these circumstances, CTLs, which recognize antigen in context of HLA class I molecules, have distinct limitations in their ability to kill tumor cells.

Where the adaptive CTL immune response is limited, the innate immune response of natural killer (NK) cells can play a critical role in guarding the body against malignancies. The advantage that NK cells provide to immune surveillance is that they recognize and spontaneously lyse tumor cells without the need for prior sensitization or the involvement of HLA class I molecules (8). Thus, the synergy between innate and adaptive immunity can play a key role in antitumor responses.

Even with both arms of the immune system in place, it is often the case that many tumors fail to elicit a strong in vivo antitumor immune response. Thus, targeting tumor-specific antigens by vaccination or the adoptive transfer of CTL and NK cells (9, 10) is a promising means of specifically eradicating tumor cells to overcome a weak in vivo antitumor immune response.

CD8\(^+\) CTLs that are specific for the core protein of MUC1 mucin expressed by tumor cells have been expanded ex vivo from peripheral blood- and tumor-infiltrating lymphocytes (TILs) of patients with breast cancer (11, 12), ovarian cancer (12, 13), pancreatic cancer (14), or MUC1-mucin expressing myeloma (15). Although synthetic MUC1 peptides (12), autologous APCs transfected with MUC1 mucin cDNA plus inhibitors of glycosylation to ensure exposure of tumor-specific epitopes (16, 17), and allogeneic tumor cells (15) have been used to stimulate the mucin-specific CTLs, each of these antigenic forms of mucin has yielded similar results. Namely, the mucin-stimulated CTLs express the \(\alpha\beta\) T-cell receptor (TCR). In addition, although the cytolytic activity against MUC1-bearing target cells has been variable, and oftentimes weak, the mucin-stimulated CTLs are specific for the mucin core protein and generally recognize and lyse HLA-matched and unmatched MUC1-expressing target cell lines irrespective of tissue. These CTL cell lines generally lack NK cytolytic activity and, therefore, may be limited in their ability to kill tumor cells that fail to express HLA class I molecules.

The expansion of tumor-specific CTLs ex vivo from peripheral blood mononuclear cells (PBMCs) and TILs is evidence that such T cells were present because of prior in vivo exposure to tumor (12). In this communication, we show that PBMCs from patients with a MUC1-expressing adenocarcinoma of the prostate, as well as from men and women who had no history of cancer or immunosuppressive disorders (i.e., healthy), were stimulated with synthetic mucin peptides in the presence of interleukin 2 (IL-2) and interleukin 12 (IL-12), type 1 cytokines that direct strong Th1 cellular immune response. The resulting T-cell lines displayed cytolytic activity against both mucin-bearing and NK target cells and comprised CD3\(^+\)CD8\(^+\) natural killer T (NKT) cells; no classical CD3\(^+\)CD56\(^-\) NK cells were present.

Two major subsets of NKT cells have been described (18). The more widely studied subset is the NKT cell that is dependent on the
MATERIALS AND METHODS

Materials. RPMI 1640, penicillin, streptomycin, L-glutamine, and 1 mM sodium pyruvate were purchased from Mediatech (Washington, DC). FCS was purchased from Hyclone (Logan, UT). Oxaloacetate/pyruvate/insulin was purchased from Sigma (St. Louis, MO). Recombinant human IL-2 was a generous gift from Genetics Institute (Cambridge, MA). Recombinant human IL-2 was obtained from the National Cancer Institute (Frederick, MD). The 40-aminoc acid MUC1-mtr peptide [(PDPTRAPGSTAPPAGHVTSA]) was synthesized by the Emory University Microchemical Facility (Atlanta, GA). All other chemicals were of reagent grade or tissue-culture grade purity.

Cell Lines. Tumor target cell lines, MCF-7 (breast), CaOV3 (ovary), and LN-CaP (prostate) were purchased from American Type Culture Collection (Rockville, MD). These cell lines were maintained in medium recommended by the American Type Culture Collection, which was supplemented with 10% FCS, oxaloacetate/pyruvate/insulin (Sigma), and 2 mM L-glutamine (Mediatech). K562, a NK target cell, and Raji, a lymphoma-activated killer (LAK) target cell, were purchased from American Type Culture Collection. Both K562 and Raji cells were cultured in RPMI 1640 containing 10% FCS and 2 mM L-glutamine. The EBV-transformed B cell line 32993 was cultured and maintained as described previously (19). In addition, B cells from each donor were immortalized by transformation with EBV as described previously (19) for use as APCs. All cultures contained penicillin and streptomycin and were treated with fungazone (Mediatech) as needed.

Primary MUC1-Specific CTL and Long-Term CTL Cell Lines. PBMCs were isolated from whole blood donated by men and women without any history of cancer or immunological disorders (i.e., healthy individuals) and nine men with diagnosed prostate cancer (CaP) after obtaining informed consent. PBMCs from two CaP patients were used to develop and characterize a CaP-specific cell line. PBMC line CP000-0705 was derived from a 44-year-old Caucasian male with clinical stage T3N0N0, adenocarcinoma of the prostate, Gleason grade 3 + 4 = 7. The cancer involved both lobes of the prostate with predominance in the left lobe (80% of the biopsy cores). Serum prostate specific antigen was 37 ng/ml. Bone scintigraphy and computerized tomography of the abdomen and pelvis failed to document metastatic spread. There was a suggestion of increased uptake on tracer in the region of the right iliac vessels on 111In-prostascintigraphy. However, subsequent bilateral pelvic lymph node dissection proved negative for metastases. This donor was otherwise healthy and was taking no medication. Patient was treated with total androgen blockade for three months followed by radical retropubic prostatectomy. Blood samples from this donor for this study were drawn during preoperative work up.

The CaP00-0720 cell line was derived from the PBMCs of a 61-year-old African-American male presenting with elevated prostate specific antigen who underwent radical retropubic prostatectomy with bilateral pelvic lymphadenectomy. Histological examination revealed clinical stage T2, adenocarcinoma of the prostate, Gleason grade 2 + 3 + 5, involving 10% of the right lobe. The prostastic capsule, seminal vesicles, surgical margins, and lymph nodes were free of disease. Other medical problems included hyperlipidemia, obesity, hypertension, alcohol abuse, and degenerative joint disease. There was no history of immune disease or disorders. The patient took no medication. Blood from this donor was drawn for this study during preoperative work up.

The HXY00-0103 and HXX98-0408 cell lines were derived from a 37-year-old male and 31-year-old female, respectively, with no history of cancer or immunosuppressive disorders. These donors were considered to be "healthy." To generate cell lines, blood was collected from the healthy and CaP donors in vacutainer tubes containing heparin sulfate or ACD solution E tubes. Blood was diluted 1:2 with Dulbecco’s PBS (calcium and magnesium free), and PBMCs were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. Purified PBMCs were washed three times with PBS, aliquoted, and stored at −70°C in Origen DMSO freeze medium (Igen International, Inc, Gaithersburg, MD). An aliquot of freshly isolated PBMCs was resuspended in RPMI 1640 containing 10% FCS and seeded at 1 × 10^6 PBMCs/well in a Linbro 24-well plate (ICN, Costa Mesa, CA) with 1 × 10^5 γ-irradiated (3000 rads) autologous PBMCs as APCs in a final volume of 1 ml of complete medium containing 10% FCS, 1% oxaloacetate/pyruvate/insulin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 10–50 IU/mL rIL-2, 2 μg of the 40-amino acid mucin peptide MUC1-mtr, and 2 ng of rIL-12. The cells were cultured in an atmosphere of 5% CO₂ in air at 37°C. For short-term experiments, cells were harvested at 6–8 days and used as effector cells in 31Cr-release cytotoxicity assays. To generate cell lines, cultures were fed every 3–5 days with complete medium [supplemented with either 10–50 units/ml human rIL-2 or 10–20% lymphophcto-T (Biotech, Denville, NJ) and restimulated every 7–14 days with 25 μg of MUC1-mtr peptide, irradiated autologous PBMCs, and 2 ng/ml human rIL-12. Alternatively, PBMCs were replaced with autologous, irradiated EBV-transformed B cells pulsed with the peptide. Lymphocytes were maintained at ~60% confluence in wells. Debris and dead cells were removed by harvesting and pooling wells, followed by a Ficoll-Paque gradient. Viable cells were then returned to culture in 24-well plates with fresh complete medium at 1 × 10^6 cells/well and restimulated as described above. Established cell lines were maintained in 24-well plates and expanded in 25-cm² or 75-cm² flasks (Falcon) with continued feeding and stimulation as before, but using 10 μg of the MUC1-mtr peptide.

Cytotoxicity Assays. Target cells were loaded with radioactivity by incubating 5 × 10^5 cells with 100 μCi of Na^24/CrO_4 (NEN, Boston, MA) in 200 μl of RPMI 1640 containing 10% FCS for 1 h at 37°C. Loaded cells were washed three times with RPMI 1640 containing 2% FCS and plated at 1 × 10^4 cells/well in 96-well U-bottomed plates (Costar) containing effector cells at various concentrations to obtain the desired E:T ratios in a final volume of 200 μl. Effector cells were routinely >90% viable by trypan blue exclusion. All data points were performed in triplicate and are reported as the mean ± SD. Spontaneous release was determined by incubation of target cells in assay medium alone. Maximum release was determined by addition of 100 μl of 2% Triton X-100. Plates were incubated for 4 h in 5% CO₂ atmosphere at 37°C. Supernatants were harvested using a Skatron supernatant collection system (Skatron Instruments, Sterling, VA) and counted in a Packard Cobra II gamma counter. Specific cytotoxic activity was determined by the following equation: (experimental release – spontaneous release)/maximum release – spontaneous release) × 100.

Flow Cytometric Analysis. The phenotype of cell lines were determined using monoclonal antibodies (mAbs) conjugated with FITC, PE, PerCP, or APC [all mAbs were purchased from Caltag (Burlingame, CA) unless otherwise noted]. Cells were stained with mAbs directed against CD3-FITC (MEM-57, Ig-Ga2a), CD4-FITC (S3.5, Ig-Ga2a), CD8-FITC (3B5, Ig-Ga2a), CD8-PerCP, CD56-PE, CD56-APC (NK1-nbl-1, Ig-G1), CD45 RO-PE (UCHL1, Ig-Ga2a), α/β TCR-PE (BMA 031, Ig-G2b; T1089.1A-31, IgM; [PharMingen]), γδ TCR-PE (B1.1, IgG1; PharMingen), Vα24-2FITC, and CD161-PE. Briefly, 4 × 10^6 cells were stained in "V"-bottom 96-well plates (Nunc) by incubating with the respective mAb for 30 min on ice. Plates were spun to collect cells and washed three times with PBS containing 3% FCS and 0.05% NaN₃. Labeled cells were analyzed by a FACScalibur (Becton Dickinson) using Cellquest and FloJo data analysis software.

TCR Vα and Vβ Expression. Total mRNA was prepared from 5 × 10⁶ NKT cells from the male donor using the Qiagen RNA miniprep kit according to the instructions of the manufacturer. cDNA was prepared by transcribing 2 μg of total mRNA using the TaqMan reverse transcriptase kit (PE Biosystems) using an oligodeoxynucleotidic acid primer in a final reaction volume of 100 μl. Reaction conditions were 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. The TCR Vα and Vβ repertoire was determined by relative real-time PCR using the Bio-Rad iCycler (Hercules, CA). Each PCR reaction contained 5 μl of a 1:5 dilution of the cDNA and 1 μM of the Vα or Vβ chain primers.
with their respective Ca and Cβ primers as previously described (20–26) in a volume of 12.5 μl plus an equal volume of SyberGreen 2× PCR MasterMix (PE Biosystems). cDNA was amplified for 40 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Immediately after the last cycle, a melt-curve was performed to detect the purity of the PCR products. Product size was confirmed by electrophoresis using a 1.5% agarose gel. The amount of each PCR product was quantitated by normalization against a 5-fold dilution series of the Vε1.5 product from Jurkat T cells.

RESULTS

Cytolytic Activity and Phenotype of Primary Cultures Stimulated with Tumor-Specific Antigen, IL-2, and IL-12. Nonstimulated PBMCs from CaP patient CaP00–0705 displayed cytolytic activity against MUC1 mucin-expressing target cells (~30% at an E:T ratio of 40:1) and NK target cells, K562 (~50% at an E:T ratio of 40:1), but no lysis of the LAK target cell line Raji (Fig. 1A). To test whether these levels of cytolytic activity were representative of nonstimulated PBMCs from CaP patients, PBMCs from eight additional CaP donors were assayed. These PBMCs displayed a mean cytolytic activity at an E:T ratio of 40:1 of 9.4% against MCF-7 (range 2.7–18.8%), 26.3% against K562 (range 2.5–50.4%), and 10% against Raji cells (range, 2.2–11.8%). Unstimulated PBMCs from the healthy male donor HXY00–0103 also displayed innate cytolytic activity against the NK target cell line, K562, with 80% specific target lysis at an E:T ratio of 40:1, which titered to 0% with halving dilutions (Fig. 1B). The unstimulated PBMCs also displayed ~40% specific cytolytic activity against MCF-7 cells at an E:T ratio of 40:1, which also titered to 0% lysis (Fig. 1B). These cells did not display significant levels of cytolytic activity against the LAK target cell line, Raji (Fig. 1B). Thus, nonstimulated PBMCs from both healthy and CaP donors displayed variable levels of endogenous cytotoxicity against MUC1-expressing and NK target cells, but little to no endogenous LAK activity.

IL-2 and IL-12 have strong stimulatory effects on NK cells, as well as directing a type 1 effector cells (i.e., CTL). Therefore, the influence that these cytokines might have on the generation of CTL cell lines was examined. Because PBMCs from CaP patients were of limited availability, as well as the fact that those men underwent chemotherapy and androgen therapy after prostatectomy, which may alter their immune response, the role of tumor-specific peptide, IL-2, and IL-12 in stimulating tumor-specific T cell responses was tested using PBMCs from the healthy male donor (HXY00–0103). After 7 days in primary culture, cells stimulated with mucin peptide alone lost much of the innate cytolytic activity with <20% specific target lysis of MCF-7 and K562 cells, but still did not display any significant LAK activity (Fig. 1C). Thus, mucin peptide alone is not sufficient to stimulate the expansion of CTL. However, PBMCs stimulated for 7 days with mucin peptide plus low-dose IL-2 displayed a 43% specific lysis against MCF-7 at an E:T ratio of 40:1 (Fig. 1D). The mucin-stimulated primary cultures also displayed a level of cytolytic activity against K562 cells that was approximately equal to the lysis of MCF-7, but had little to no LAK activity (Fig. 1D). By contrast, mucin peptide plus IL-12 stimulated effector cells having ~90% specific lysis against MCF-7 at an E:T ratio of 40:1 (Fig. 1E), which was >2-fold greater than cells stimulated with IL-2 and tumor antigen (P < 0.001; Fig. 1D). These mucin-stimulated lymphocytes also displayed ~110% lysis of K562, which was slightly greater than the lysis of MCF-7 (Fig. 1E). This high cytolytic activity against K562 also suggests that the lysis of these target cells by the mucin and IL-2-stimulated cell lines is more efficient than the “maximum release” of the 53Cr by Triton X-100. These lymphocytes also had a high level of LAK activity against Raji target cells (Fig. 1F). When both IL-2 and IL-12 were present with the tumor-specific mucin peptide, the cytolytic activity against MCF-7 and K562 cells remained high, yet the LAK activity against Raji cells remained low (Fig. 1F). This suggests that mucin peptide, in the presence of IL-2 and IL-12, acts synergistically to direct the cytolytic activity of primary cultures away from nonspecific LAK activity. In addition, the effector cells and target cells were not HLA-matched. These results were repeated four times with this same healthy male donor (HXY00–0103) and three times with a female donor (HXX98–0408) with no history of cancer or immunosuppressive disorders, with nearly identical results (not shown).

Because IL-12 stimulates Th1 responses and nonrestricted NK effector cells, it would be reasonable to expect that the NK-like cytolytic activity described in Fig. 1F could be accounted for by the presence of both CTL and NK cells. However, we observed that MUC1-expressing MCF-7 cells are not lysed by the human NK cell line NK3.3 at an E:T ratio of 40:1 (data not shown). Because MCF-7 is not a target for classical NK cells, it did not seem likely that the cytolytic activity against this target cell could be attributable to classical NK cells. Nonetheless, the phenotype of this primary culture was determined to rule-out the possibility that the lysis of K562 was attributable to classical NK cells. The 7-day primary culture stimu-
labeled with MUC1 peptide, IL-2, and IL-12 (Fig. 1F) contained >90% CD3+ T cells (not shown). Two-color flow cytometric analysis showed that ~53% of the CD3+ T cells expressed CD4+ (Fig. 2A), but <3% the cells were CD4−CD56+ (Fig. 2A), a value that was not distinguishable from background staining with the isotype control (not shown). The culture also contained ~43% CD8+ T cells, ~7% of which coexpressed CD56 (Fig. 2B), suggesting the presence of NKT cells. No significant numbers of cells displayed the CD3−CD56+ phenotype characteristic of classical NK cells (not shown). Thus, the NK-like activity cannot be accounted for by classical NK cells, but possibly by “promiscuous” CTL or NKT cells, both of which would display cytolytic activity of CTL and NK cells.

**Cytotoxic T-Cell Lines from CaP Patients.** T-cell lines were established from the PBMCs of CaP patients. PBMCs (CaP00–0720) were stimulated ex vivo four times (~2 months in culture) with MUC1 mucin peptide, IL-2, and IL-12 and assayed for cytolytic activity 20 days after the fourth stimulation. The cell lines displayed equally strong cytolytic activity against the MUC1 mucin-expressing target, MCF-7, and the NK target, K562, with nearly 80% specific lysis at an E:T ratio of 80:1 (Fig. 3A). This cell line also contained a significant amount of cytolytic activity against the LAK target cell line Raji with ~30% cytolytic activity at an E:T ratio of 80:1 (Fig. 3A). Although the displayed a 35% specific lysis against both MCF-7 and K562 at an E:T ratio of 10:1 (Fig. 3A), continued stimulation of this cell line an additional four times (for a total of eight stimulations and nearly 4 months in culture) resulted in an 85% increase of the cytolytic activity against MCF-7 cells at an E:T ratio of 10:1 (Fig. 3B). The maximum specific target lysis also increased from ~80% (Fig. 3A) to ~110% (Fig. 3B) with continued stimulation. Unlike the cytolytic activity of the cell lines after four stimulations (Fig. 3A), which titered down immediately with halving dilutions (Fig. 3A), cytolytic activity of the cell lines stimulated eight times did not begin to titter down until an E:T ratio of 10:1 (Fig. 3B). In contrast to the increase in cytolytic activity against MCF-7 cells, the NK cytolytic activity decreased from 80% (Fig. 3A) to 60% (Fig. 3B), and LAK activity was nearly eliminated with continued stimulation (Fig. 3B). Because LAK cells can often lyse NK target cells, the decrease in lysis of the NK target K562 may be attributable to the elimination of the LAK cells. The generation of cell lines having high cytolytic activity against MUC1 and NK target cell lines, but not against LAK target cells, was not donor specific because similar results were obtained by generating a cell line from donor CaP00–0720 (data not shown). These results suggest that continued stimulation of the cell line results in the expansion of cells with cytolytic activity against mucin-expressing and NK target cells and away from nonspecific LAK activity.

**Cytotoxic T-Cell Lines Having Mucin-Specific and NK-Like Cytolytic Activities from Healthy Individuals.** PBMCs from healthy male and female donors were isolated and stimulated in parallel with mucin peptide in the presence of autologous γ-irradiated PBMCs as APCs, IL-2, and IL-12 to develop cell lines. The mucin-stimulated F2 cell line from the healthy male donor displayed high cytolytic activity against MCF-7 cells and K562 cells (Fig. 4A), in a pattern that was similar to that displayed by the CaP patient cell lines (Fig. 4). The mucin-expressing ovarian cancer cell line CaOV3 was lysed to approximately the same extent as MCF-7 (Fig. 4A). In addition, this cell line lysed the CaP cell line, LN-CaP, albeit weaker than MCF-7 and CaOV3 (Fig. 4A). The reason for the weak lysis of LN-CaP cells is not known. LAK activity in this mucin-stimulated cell line was negligible (Fig. 4A).

The E8 cell line developed from the PBMC of the female donor was similar to that of the cell line from the healthy male donor in that displayed strong cytolytic activity against MCF-7 cells and K562 cells (Fig. 4B). This cell line also had strong cytolytic activity against CaOV3, weak cytolytic activity against LN-CaP, and low levels of LAK activity (Fig. 4B).

The observations that multiple mucin-expressing tumor cell lines derived from different tissues are lysed by the mucin-stimulated T cells support the idea that the cytolytic activity is directed against mucin and not tissue-specific determinants. Nevertheless, the mucin...
specificity of the cell lines was also assayed to further test the specificity of the cell lines for mucin, against the HLA-positive cell lines MS and MS-MUC1, the latter of which was stably transfected with MUC1 mucin. The mucin-expressing MS-MUC1 cell line, but not the non-mucin-expressing MS cell line, was lysed by the mucin-stimulated T cell line (Fig. 5A). In addition, F2 (Fig. 5B) and E8 (Fig. 5C) cell lines lysed the allogeneic EBV-transformed B cell line 32993 pulsed with the 40-amino acid mucin peptide MUC1-mtr₂, but did not lyse the non-peptide-pulsed EBV-transformed B cells. Although the specific target lysis against the MUC1-expressing MS-MUC1 cell line and the MUC1-pulsed EBV-transformed B cells was lower than that against the tumor cell lines (e.g., MCF-7, Ca-OV3), a range of 10–30% specific target lysis by mucin-stimulated T cells against MUC1-transfected cell lines and target cell loaded with exogenous MUC1 peptide is not an uncommon observation (12, 27–30). Taken together, the results presented above are consistent with the idea that the mucin-stimulated T-cell lines are specific for endogenously synthesized and exogenously loaded mucin.

The cell lines derived from the healthy male and female donors described above have been established on at least three different occasions and showed little variation in cytolytic activity described after mucin stimulation with IL-2 and IL-12. Furthermore, the similarity in cytolytic activity between male and female donors, as well as CaP patients, suggests that gender and tumor burden may not be important factors that influence the ex vivo generation of cell lines having cytolytic activity against mucin-bearing targets and NK targets.

**Mucin-Specific T-Cell Lines Display the Phenotype of NKT Cells.** Our results have reproducibly shown that the T-cell lines established by stimulating PBMCs with mucin peptide, IL-2, and IL-12 display both mucin-specific and NK-like cytolytic activities. The primary culture displayed the phenotype of CD8⁺ CTL with a minor population of CD8⁺CD56⁺ NKT cells, but no classical CD3⁺CD8⁺CD56⁺ NK cells (Fig. 2). Nevertheless, a detailed phenotypic analysis of the mucin-specific T cell lines described above (see Figs. 3–5) was performed to determine the identity of the cytolytic lymphocytes.

The E8 cell line generated from the PBMC of the healthy female donor is representative of our observations. This cell line was >99% CD3⁺ (Fig. 6A), and nearly half expressed CD56 (Fig. 6B). The lack of CD3⁻ cells suggested that classical CD3⁺CD56⁺ NKT cells were not present, but rather that the cell line contained CD3⁺CD56⁻ T cells and CD3⁻CD56⁺ NKT cells. This cell line did not contain CD4⁺CD56⁺ NKT cells (Fig. 6C), but rather approximately 50% of the T cells were CD8⁺CD56⁺ NKT cells (Fig. 6D). In addition, the CD8⁺ T cells expressed the α/βTCR (Fig. 6E), but not the γδ TCR (not shown), with staining ranging from high to low. Furthermore, the CD8⁺ T cells were virtually all 100% memory/effector cells based on the expression of CD45RO (Fig. 6F).

A similar analysis was also performed with the F2, HXY00–0103, and CaP00–0705 cell lines established after ~4 months of culture. These cell lines also expressed CD3, CD45RO, and the α/βTCR, but not CD4 (not shown). Like the E8 cell line, the F2 (not shown) and the HXY00–0103 cell lines from the healthy male donor also contained ~50% CD8⁺CD56⁻ CTL and CD8⁺CD56⁺ NKT cells (Fig. 7A). The CaP00–0705 cell line also contained CD8⁺CD56⁻ T cells, but nearly a 50% higher amount of CD8⁺CD56⁺ NKT cells (Fig. 7B) than either of the HXX98–0408 (Fig. 6D) or HXY00–0103 (Fig. 7A) cell lines. The reason for the different levels of NKT cells in cell lines derived from different donors, but developed and maintained under identical conditions, is not known. The cell lines from both the healthy male and female donors and CaP patients have been in continuous culture for >1.5 year, with no changes in the cytolytic activity.

**IL-12 Contributes to the Expression of CD56.** The continued stimulation of PBMCs with tumor-specific peptide, IL-2, and IL-12 to develop a cell line resulted in an increase in the number of CD8⁺CD56⁻ T cells. Two possible explanations could account for this increase: (a) the outgrowth of the CD8⁺CD56⁻ NKT cells over CD8⁺CD56⁻ T cells or (b) the up-regulation of CD56. Because IL-12 is thought to participate in the generation of NKT cells (31–35), the effect that this cytokine has in the expression of CD56 was tested. Equal aliquots of the HXY00–0103 cell line were stimulated in parallel with MUC1 peptide, APCs, and IL-2 in the presence or absence of exogenous IL-12 condition. The cells stimulated with each condition culture remained CD3⁺, CD8⁺, and αβTCR⁺ (Fig. 8). However, the cells stimulated with IL-12 expressed nearly a 2-fold higher amount of CD56 compared with the cells stimulated without this cytokine (Fig. 8). These results suggest that IL-12 is necessary for the expression of CD56 on NKT cells.

To test the hypothesis that CD56 is up-regulated on the CD8⁺ T cells to yield the CD8⁺CD56⁺ NKT cell phenotype, the CD8⁺CD56⁻ and the CD8⁺CD56⁺ T cells were separated. A repre-

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Fig. 4. Mucin-stimulated T cell lines recognize mucin-bearing target cells. The F2 cell line from the healthy male donor (A) and the E8 cell line from the healthy female donor (B) were assayed for cytolytic activity 5 days poststimulation by ⁵¹Cr-release assay as described in "Materials and Methods" against the MUC1-expressing MCF-7 (●), the mucin-expressing ovarian adenocarcinoma cell line CaOV3 (○), the NK target K562 (▲), the prostate cancer cell line LN-CalP (□), and the lymphokine-activated killer target Raji (■).
sentative experiment that tests this hypothesis is discussed. The un-
separated HXY00–0103 cell line contained 60% CD8+CD56− NKT cells and 40% CD8+CD56+ T cells (Fig. 9A). These cells
lysed MCF-7 and K562 cells equally, but did not lyse Raji cells (Fig.
9B). The CD8+CD56+ and CD8+CD56− cells were separated and
immediately assayed for phenotype and cytolytic activity. A contour
plot shows that the CD8+CD56+ NKT cells were a single population
after separation using anti-CD56 magnetic beads (Fig. 9C): these
lymphocytes displayed equal lysis of MCF-7 and K562 cells, but no
lysis of Raji cells was observed (Fig. 9D). The T cells not bound to
the anti-CD56 magnetic beads were CD8−CD56− (Fig. 9E). The
CD8−CD56− T cells also displayed equal lysis of both MCF-7 and
K562 cells, but little to no lysis of Raji cells (Fig. 9F). However, the
lytic activity of the CD8−CD56− NKT cells was 2-fold lower than
that of the CD8−CD56− T cells. The specificity of the separated
populations for MUC1 mucin was also tested. Both the CD8+CD56+
NKT cells (Fig. 9G) and CD8−CD56− T cells (Fig. 9H) lysed the
MUC1-expressing cell line MS-MUC1, but not the non-mucin ex-
pressing MS cell line. The unseparated HXY00–0103 cell line and
each of the separated subsets were stimulated in parallel one time with
MUC1 peptide, IL-2, and IL-12, and the phenotype was determined
14 days poststimulation. The amount of CD8+CD56+ NKT cells
increased in the unseparated cell line from ~58% to ~77% (Fig. 9I),
but only a modest increase in the amount of CD8+CD56− NKT cells
was observed in the CD56− subset [i.e., ~82% (Fig. 9C) to ~87%
(Fig. 9J)]. However, the expression of CD56 dramatically increased
from ~5% (Fig. 9E) to ~69% (Fig. 9K) in the CD56− T-cell subset.
This increase could not be accounted for by the addition of the

Fig. 6. Phenotype of mucin-stimulated T-cell line. Flow cytometric analysis was
performed on the HXX98–0408 cell line as described in “Materials and Methods”
using the antibodies indicated in each panel. Inset, values listed are the percentage of cells in
each respective quadrant. Background isotype staining using anti-IgG1-FITC and anti-
IgG2a-PE was <1%. TCR, T-cell receptor.

Fig. 7. Phenotype of mucin-stimulated T-cell lines from a healthy donor and a prostate
cancer patient. Flow cytometric analysis was performed on the T-cell lines from the
healthy male donor HXY00–0103 (A) and prostate cancer patient CaP00–0705 (B) using
anti-CD8 and anti-CD56 as described in “Materials and Methods.” Inset, values listed are
the percentage of cells in each respective quadrant. Background isotype staining using
anti-IgG2a-PE was <1%.

Fig. 8. Interleukin 12 (IL-12) contributes to the expression of CD56 on natural killer
T cells. The mucin-stimulated T-cell line HXY00–0103 from the healthy male donor after
6 months in culture was stimulated for 1 week with MUC1 peptide and interleukin 2 in
the absence (~IL-12, left column) or presence (+ IL-12, right column) of interleukin 12
as indicated. The phenotype of the cell cultures was measured 6 days poststimulation.
Values listed are the percentage of cells in each respective quadrant. TCR, T-cell receptor.
Fig. 9. CD56− T cells having antigen-specific and natural killer-like cytolytic activities give rise to natural killer T cells. The phenotype (A) and cytolytic activity (B) of the HXY00–0103 MUC1-stimulated cell line were determined as described in "Materials and Methods." Cytolytic activity was measured by 51Cr-release assay using the MCF-7 (C), K562 (D), and Raji (E) cell lines. The CD56+ T cells were selected by magnetic bead separation using anti-CD56 (Miltenyi) according to the instructions of the manufacturer. The phenotype (C) and cytolytic activity (D) of the CD56− T-cell subset was determined as described for A and B, respectively. Similarly, the phenotype (E) and cytolytic (F) activity of the CD56− T cells that did not bind to the anti-CD56 magnetic beads were also determined as described for A and B, respectively. G and H, the cytolytic activity of the CD56+ and CD56− populations, respectively, was measured by 51Cr-release assay using the MS (G), MS-MUC1 (H), and Raji (I) cell lines. 1–K, the unseparated HXY00–0103 cell line and the CD56+ and CD56− populations were stimulated one time with MUC1 peptide, interleukin 2, and interleukin 12. Flow cytometric analysis was performed on the respective populations 14 days poststimulation by two-color flow cytometry using anti-CD8-APC and anti-CD56-PE. Values in each flow cytometric analysis plot indicate the percentage of cells in that quadrant.

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γ-irradiated APC, because these cells died within 1 week (not shown). In addition, the cytolytic activity of the subset in which CD56 was up-regulated was restored to that observed with the unseparated and CD8+CD56+ NKT cell subsets. This up-regulation of CD56 on CD8+CD56− T cells was repeated three times and suggests that the CD56−CD56+ T-cell subset having both mucin-specific and NK-like cytolytic activities were NKT cells. We also observed that the CD56+ NKT cells proliferated more slowly than CD56− NKT cells or cell lines containing both CD56+ and CD56− NKT cells and that continued stimulation of the separated CD56+ NKT cell population results in eventual death of these lymphocytes. By contrast, NKT cell lines that contain a significant amount of CD56− NKT cells have been maintained in culture for >1.5 year as described above. This would suggest that the cell lines may reach a "steady state" of CD56+ and CD56− NKT cells, the latter of which replenishes the CD56+ population. Although the detailed interaction and relationship between the CD56+ and CD56− NKT cells is not known, the up-regulation of CD56 does not appear to be attributable to the removal of a potential soluble inhibitory factor released from the NKT subset, because medium from the separated NKT subset did not suppress up-regulation of CD56 on isolated CD56+ NKT cells (not shown). Nevertheless, these results are consistent with the hypothesis that the stimulation of PBMCs with MUC1 peptide, IL-2, and IL12 results in the generation of CD56+ and CD56− NKT cells and that this "NK" marker is regulatable.

Involvement of HLA and CD1 Molecules in the Cytolytic Activity of Mucin-Stimulated NKT Cell Lines. Classical CTLs recognize antigens in the context of HLA class I molecules, and NKT cells recognize nonpeptide antigens in the context of the nonclassical CD1 family of antigen-presenting molecules. We, therefore, asked whether either of these classes of antigen-presenting molecules had a role in directing the cytolytic activity of mucin-stimulated NKT cell lines. To address whether target recognition and lysis was dependent on HLA class I molecules, but nonrestricted to a single haplotype, blocking assays were performed using the pan-anti-HLA mAb, clone W6/32, at concentrations that inhibited HLA-restricted cytolytic activity of mucin-specific CTLs (27). These NKT cell lines had a lysed MCF-7 and K562 cells equally, but not Raji cells, as described above (Fig. 10A). No inhibition of cytolytic activity was observed against MCF-7 cells with an anti-isotype antibody or K562 cells using the W6/32 antibody as a negative control (Fig. 10A). This suggests that target recognition by these mucin-stimulated lymphocyte cell lines was independent of HLA class I molecules.

The involvement of CD1 subtypes on target recognition was also tested using anti-CD1 antibodies reported previously to block NKT cytolytic activity (36). Cytolytic activity of the mucin-stimulated cell lines was determined against the MCF-7 target cell line in the presence or absence of antibodies against the CD1a, CD1b, CD1c, and CD1d subtypes. None of these antibodies blocked cytolytic activity against this mucin-bearing target cell line (Fig. 10B). This result suggests that target recognition by these mucin-stimulated NKT cell lines was independent of CD1 molecules.

MUC1-Stimulated NKT Cell Lines Do Not Express CD161 or the Vo24Vβ11 TCR. In addition to the expression of CD56, the expression of CD161 and the Vo24Vβ11 TCR have also generally been regarded as markers that define these lymphocytes. However, <8% of the CD8+CD56+ NKT cells from the HXY00–0103 (Fig. 11A), CaP00–0705 (Fig. 11B), and HXX98–0408 (not shown) cell lines expressed CD161. This low level of CD161 expression was not considered significant because subsequent flow cytometric analysis of these cell lines did not display any staining with anti-CD161 (not shown). The expression of the Vo24 TCR on the mucin-specific αβ TCR NKT cell lines was also tested. However, flow cytometric analysis using anti-Vo24 mAb showed that the HXY00–0103 (Fig.
as CD4+, CD8+, or CD4−CD8− T cells that express a biased Vα24Vβ11 TCR (18, 37–39), as well as markers usually associated with NK cells, such as CD56 and the killer inhibitory receptor NKR-P1A (i.e., CD161; Ref. 40, 41). These NKT cells are dependent on the presentation of the glycolipid antigen α-GalCer through CD1d (18). NKT cells stimulated by α-GalCer display variable NK-like cytolytic activity and generally high LAK activity, but the antigen responsible for target recognition is not known. Nonetheless, NKT cells have been shown to play a role in antitumor immunity, and IL-12 has been a key factor in directing the phenotype and cytolytic activity.

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Fig. 10. A, cytolytic activity of MUC1-specific natural killer T cells is independent of HLA class I molecules. The HXY00–0103 cell line was assayed for cytolytic activity by 51Cr-release assay as described in “Materials and Methods” against the MCF-7 cells (●) or MCF-7 cells preincubated with 10 μg/ml W6/32 (○), 20 μg/ml W6/32 (△), 10 μg/ml anti-IgG2α isotype monoclonal antibody (mAb; ×). The cytolytic activity against K562 cells preincubated without (□) or with (■) 20 μg/ml W6/32 and Raji cells (◆) was also measured. B, cytolytic activity of mucin-specific natural killer T-cell lines is independent of CD1 molecules. The HXX98–0408 cell line was assayed for cytolytic activity by 51Cr-release assay as described in “Materials and Methods” against MCF-7 cells alone (●) and MCF-7 cells preincubated for 1 h at 37°C with 10 μg/ml anti-isotype mAb of anti-isotype mAb (○), anti-CD1a mAb (■), anti-CD1b mAb (△), anti-CD1c mAb (◆), or anti-CD1d mAb (□). The negative control against Raji cells is also shown (▼). All target cells were preincubated for 1 h at 37°C with or without the antibodies as indicated.

Fig. 11. NKT cells stimulated by MUC1 mucin peptide, interleukin 2 and interleukin 12 do not express CD161 or Vα24. Representative two-color flow cytometric analysis of the established natural killer T-cell lines from the healthy male donor HXY00–0103 (A) and the prostate cancer patient CaP00–0705 (B) using anti-CD8-PerCP and anti-Vα24-FITC. Representative two-color flow cytometric analysis was also performed on the HXY00–0103 (C) and CaP00–0705 (D) cell lines using anti-CD8-PerCP and anti-CD161-PE. Samples were prepared and analyzed as described in “Materials and Methods.” Inset, values listed in each quadrant indicate the percentage of positive-staining cells.

Fig. 12. T-cell receptor (TCR) Vα and Vβ expression of mucin-specific natural killer T-cell lines. Relative real-time reverse-transcription PCR was performed using mRNA from the mucin-stimulated natural killer T-cell lines to test for the expression of the Vα (A) and Vβ (B) TCR repertoire for the HXY00–0103 cell line and the Vα (C) and Vβ (D) TCR repertoire for the CaP00–0705 cell line. Dotted lines, relative expression levels of Vα TCR <2 and Vβ <4 were considered to be background based on melt-curve analysis, which was confirmed to be primer-dimers by agarose gel electrophoresis.

DISCUSSION

NKT cells are a lymphocyte subset that is distinct from conventional T cells and NK cells. NKT cells have generally been described

11C), CaP00–0705 (Fig. 11D), and HXX98–0408 (not shown) cell lines did not express this Vα chain.

Because the MUC1-stimulated NKT cell lines differed from Vα24 NKT cells, reverse-transcription PCR was performed using mRNA from the NKT cell lines from the healthy male donor and a CaP patient to test for the expression of the Vα and Vβ repertoire. Melt-curve analysis of the PCR products showed that the HXY00–0103 cell line did not express Vα24 mRNA, rather the predominant Vα chain was Vα12 (Fig. 12A). This cell line was oligoclonal, also expressing a high level of Vα10, as well as minor amounts of Vα27 and Vα29 (Fig. 12A). The Vβ expression for the HXY00–0103 cell line was also determined by reverse-transcription PCR. No Vβ11 was expressed by this cell line (Fig. 12B), but rather the predominant Vβ chain expressed was Vβ17 (Fig. 12B). Vβ9 was also expressed, but at a level that was 4-fold less than Vβ17 (Fig. 12B). Similar analysis was performed on the mRNA from the CaP00–0705 cell line. Like the cell line from the healthy male donor, the CaP-derived NKT cell line was oligoclonal with the predominant Vα chain being Vα29, with minor amounts of Vα6 and Vα14, but no Vα24 (Fig. 12C). The Vβ TCR usage was largely Vβ3 with Vβ2, Vβ6, and Vβ14 present in minor amounts, but no Vβ11 (Fig. 12D). Although the TCR repertoire varied with the cell line, the mucin-specific NKT cell lines are distinct from the Vα24Vβ11 NKT cells.

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profiles of these lymphocytes. In murine systems, NKT cells that were stimulated with IL-12 (32–34) or α-GalCer (33–35) mediated tumor rejection through an NK-like effector mechanism. The antitumor activity produced by α-GalCer is mediated through the production of IL-12 by dendritic cells. In humans, Vα24 + NKT cells stimulated with α-GalCer exhibited perforin-mediated cytotoxicity against allogeneic mismatched hematopoietic malignancies, whereas the NK target, K562, and the LAK target Daudi showed little sensitivity (42). Exogenous IL-2 and IL-12, but without a defined protein antigen, have also been used to stimulate CD8+ CD56+ NKT cells having high LAK activity, but no activity against NK target cells (31, 43). By contrast, we observed that exogenous IL-2 and IL-12 plus a defined tumor-specific peptide antigen from the tandem repeat region of MUC1 mucin acted synergistically to generate CD8+ CD56+ NKT cells that displayed mucin-specific and NK-like cytolytic activities and generally no LAK activity. Thus, it would appear that the mechanism by which mucin-stimulated NKT cells recognizes target cells is different from NK cells stimulated with α-GalCer.

NKT cells that express a nonbiased TCRαβ repertoire have also been reported (31). Although the target specificity of these NKT cells is not known, they are activated independent of CD1 molecules. Herein, we described the ex vivo expansion of CD8+ CD56+ CD161− NKT cells from the PBMCs of CaP patients and healthy male and female donors. The Vα and Vβ TCR repertoire of the NKT cell lines varied among individuals, but none of the cell lines expressed Vα24 or Vβ11. The CD8+ CD56+ CD161− NKT cells lysed target cells that express MUC1 mucin or were pulsed with mucin peptide regardless of the tissue of origin. These results suggest that the NKT cells are specific for MUC1 mucin. In addition, the mucin-stimulated NKT cells lysed NK target cells to the same extent as mucin-bearing target cells, but the mechanism by which the NK target cells are recognized is not yet known. However, unlike the Vα24+ Vβ11+ NKT cells that often display high levels of LAK activity, the mucin-specific Vα24 Vβ11+ NKT cells displayed little to no LAK activity. This suggests that the peptide antigen might play a key role in directing antigen specificity of NKT cells and away from nonspecific LAK activity.

In addition to the CD8+ CD56+ NKT cells described here, CD8+ CD56+ mucin-specific T cells were also expanded. Like the CD8+ CD56+ NKT cells, the CD8+ CD56+ T cells also had mucin-specific and NK-like cytolytic activities, but no LAK activity. However, the cytolytic activity of the CD8+ CD56+ T cells was 2-fold lower than that of the CD8+ CD56+ NKT cells. Although CTLs have been reported to display “promiscuous” cytolytic activity against both MCF-7 and K562 (44), we concluded that the CD8+ CD56+ T cells described in this communication were a unique population of NKT cells based, in part, on their cytolytic activity profile. Moreover, CD56 was up-regulated on the CD56+ population to yield the CD8+ CD56+ NKT cell phenotype, as well as the enhanced cytolytic activity in response to IL-12. The role that CD56 plays in the enhanced cytolytic activity is being investigated. Thus, these results suggest that mucin peptide, in combination with IL-2 and IL-12, resulted in the ex vivo expansion of CD8+ CD56+ and CD8+ CD56+ NKT cells, the latter of which gives rise to the CD8+ CD56+ NKT cell phenotype.

Mucin-specific CD8+ Vα24+ Vβ11+ NKT cells were generated from cancer patients, as well as healthy men and women. These observations suggest that parameters such as gender and in vivo exposure to tumor may not have a large impact on the ex vivo expansion of these cells for adoptive immunotherapy of cancer. In addition, the generation of lymphocytes having antimucin cytolytic activity also argues against the assumption that an individual needs to be presensitized by the tumor in vivo for successful ex vivo expansion (12). Cell lines generated from the CaP patients also had nearly 50% higher levels of CD8+ CD56+ NKT cells than cell lines generated from healthy individuals. The reason for this difference is not known. However, we observed that the CD56+ NKT cell population responded poorly to continued antigenic stimulation, whereas the CD56+ NKT cells and cell lines containing both CD56+ and CD56+ NKT cells were able to be maintained in long-term culture. This may suggest that CD56+ NKT cells are terminal and that a steady state between CD56+ and CD56+ NKT cells is maintained by the up-regulation of CD56 on the CD56+ NKT cells. Although multiple mechanisms contribute to a weak in vivo antitumor immune response, we speculate that cancer patients can generate an in vivo antitumor NKT cell response, but these cells may not adequately proliferate in response to the tumor burden.

The mucin-specific NKT cells described herein share similarities with previously reported mucin-specific CTLs (12–15, 29, 45, 46), but they also display characteristics that also distinguish them as a unique T-cell population. Although the CD8+ CD56+ NKT cells phenotype described gives the impression of a mucin-specific CTL, CD56 was shown to be up-regulated. Thus, we do not believe that the expression of this NK marker is alone sufficient to distinguish between an NKT cell and a CTL. However, we believe that a more appropriate definition of an NKT cell should be based on criteria of effector function. Namely, although the CD56+ and CD56+ NKT cells and CTLs display mucin-specific cytolytic activity against non-HLA matched allogeneic target cells, the NKT cells described here displayed strong NK-like cytolytic activity, whereas the previously reported mucin-specific CD8+ CTLs were devoid of NK-like cytolytic activity. This difference alone suggests that the lymphocytes reported herein are distinct from previously reported mucin-specific CTLs. Other differences include the observations that the cytolytic activity of the CD8+ CTLs was inhibited by the W6/32 anti-HLA class I mAb, whereas the cytolytic activity of the NKT cells reported herein was not inhibited by the antibody. This suggests that, although the CD8+ CTLs are not restricted to a single HLA haplotype, TCR-HLA interactions play an important role in target recognition. By contrast, the cytolytic activity of the mucin-specific NKT cells was independent, not only of HLA class I molecules, but also the CD1 family of antigen-presenting molecules. The mechanism of target cell recognition by the mucin-specific NKT cells is not yet known, but may involve intercellular adhesion molecules, similar to the HLA-unrestricted, mucin-specific CD8+ CTLs (27). Work is in progress to determine the extent to which intercellular adhesion molecules play in the antigen-specific and NK-like cytolytic activities of NKT cells.

Adaptive immunotherapy using tumor-specific CD8+ CTLs provides an avenue for the eradication of tumor having minimal to no nonspecific damage of normal cells. However, the effectiveness of CTLs may be limited because many tumors, including mucin-expressing adenocarcinomas, evade immune detection through mechanisms that include antigen shedding and down-regulation of HLA class I molecules (5, 6). Because NK cells also infiltrate tumor and cause regression (8, 47, 48), we speculate that both the dual cytolytic activities of the effector/memory NKT cells described herein that allow the cells to sense their antigenic environment as a CTL, while also having the ability to react to stimuli of NK cells when loss of HLA molecules occur, may make these lymphocytes a more versatile cell type for the active immunity against cancer and future prevention against relapse.

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Ex Vivo Expansion of CD8+CD56+ and CD8+CD56− Natural Killer T Cells Specific for MUC1 Mucin

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