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<td><strong>Author(s)</strong></td>
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Complementary activation of peripheral natural killer cell immunity in nasopharyngeal carcinoma

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NK cells and αβ- and γδ-CTL play important roles in cellular immunity against tumors. We previously demonstrated that NPC patients have a quantitative and qualitative deficit in γδ-CTL and EBV-specific αβ-CTL when compared to normal subjects and NPC long-term survivors. In this study we report further observations of a complementary activation of peripheral NK cells in NPC patients. The NK cells in these patients, compared to those of healthy subjects and NPC survivors, were preferentially activated in response to the stimulation of myeloma cell line XG-7 and expanded in the presence of exogenous IL-2. The production of IFN-γ was lowest in the patient group, whereas IL-12, IL-15 and TNF-α were produced in higher levels in patients than in the donors and survivors. The cytolytic effect of the NK cells against NPC cells in the patient group was also higher than that of the donors and survivors. Furthermore, the patients at later stages of NPC had lower γδ-CTL activity but higher NK cytotoxicity towards NPC targets, with higher production of IL-12, IL-15 and TNF-α but lower production of IFN-γ than in patients at earlier stages. This might be part of a triggered compensatory re-activation of the innate immunity, believed to be mediated through various cytokines and chemokines when adaptive T cell immunity is breached. Together, these data suggest complementary roles of innate and adaptive immune response in tumor immunity where NK cells, γδ- and αβ-CTL compensate for the deficits of one another at different stages of tumor invasion. (Cancer Sci 2006; 97: 912–919)

NK cells, and αβ- and γδ-CTL are the dominant players in cellular immunity to tumors.1(1) The presence of cellular dysregulation manifested as abnormal expressions on the surface of tumor cells are potential targets for immune surveillance by these cytolytic lymphocytes.1(2) The effector function of αβ-CTL of the adaptive immune response is MHC restricted and hence provides more specific targeted, albeit delayed, cytotoxicity.1(3) NK cells and γδ-CTL are thought to contribute principally to innate immune surveillance due to their non-MHC restricted cytotoxicity, which will accommodate a more rapid response to cellular stress and disregulation signals that trigger these cells’ cytolytic functions.1(4–6) The escape mechanisms of tumor cells from these cytolytic lymphocytes depend on the expression of different cell surface molecules such as inhibitory ligands.1(7) The presence of these inhibitory ligand-mediated escape mechanisms is essential for the host to control excessive autoimmune reactions in otherwise healthy individuals.1(8) Different inhibitory ligands are recognized by receptors, such as NKG2A and KIR, distributed amongst these different cytolytic cells, thus reducing the chance of the tumor cells’ complete escape from them.1(9–11) The nature of these lymphocytes, taken together, thus provides a broad defense net at different stages of cellular immunity as well as broader coverage of cytotoxicity towards their respective targets.

The cytolytic effect of NK cells mainly occurs through the granulysin–perforin mechanism after activation of the appropriate receptors and in the absence of inhibitory ligands such as MHC class I molecules.1(12) Circulating NK cells recognize and kill cells which express abnormally downregulated levels of MHC class I molecules resulting from stress, infection or cellular disregulation.1(13) However, tumor cells expressing MHC class I molecules are also susceptible to NK cytotoxicity when ligands to the NKG2A receptor, such as MIC and RAE-1, are coexpressed1(10,12) providing an impediment to escape mechanisms depending on MHC class I alone.

The roles of NK cells and T cells in host immunity are extensively interdependent through the sharing of cytokines and chemokines in a complex web of stimulatory and inhibitory interactions which remains to be further explored.1(14–20) NK cells are generally believed to take part in early innate host defense through a non-specific response to inflammatory cytokines. They are distinguished from T cells in that they do not partake in adaptive immunity through expression of rearranged antigen receptors.1(21) NK cells do contribute to the development of the adaptive immune response,1(22) but their role diminishes as the more specific adaptive immune response begins to mature in the body’s defense. Kasaian et al.1(23) recently demonstrated that IL-21, a product of activated T cells, can reduce NK cells by antagonizing their survival, which provides one possible pathway for innate immunity to

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Abbreviations: CTL, cytotoxic T lymphocytes; EBV, Epstein–Barr virus; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; IFN, interferon; IL, interleukin; mAbs, monoclonal antibodies; MEC, methylcellulose; MHC, major histocompatibility complex; NPC, nasopharyngeal carcinoma; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; TCR, T-cell receptor; TNF, tumor necrosis factor.

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be taken over by adaptive immunity as the immune response progresses.

NPC is an EBV-associated cancer common in southern China. We have previously shown that NPC patients display quantitative and qualitative deficits in γδ-CTL and EBV-specific αβ-CTL compared to NPC long-term survivors and healthy donors. (24, 25) The deficits were restored in the survivors after successful treatment to remove the tumor. In this study, we further demonstrate that NPC patients exhibit preferential activation of NK cells with concomitant increase in cytotoxicity against NPC cells and different profiles of tumor-induced cytokine production.

Materials and methods
Study subjects
Study subjects included 15 active NPC patients, five long-term survivors who had been in disease-free remission for 5–13 years and five healthy donors. All study subjects were male ethnic Chinese whose ages coincided with the peak age incidence of the disease. Diagnosis of NPC patients was confirmed by characteristic pathology of poorly or undifferentiated carcinoma. The disease stage was defined according to Ho’s standard. (26) The 15 active NPC patients were divided into three groups according to their NPC stages, which were varied from T1N1 to T3N3 (Table 1).

Cytokines, cell lines and cell cultures
All recombinant cytokines were obtained from R&D Systems (USA). XG-7 is a human myeloma cell line that expresses both HLA-I (97% with intensity 511) and HLA-II (94% with intensity 458). (27) This cell line has been demonstrated to be able to effectively simulate growth of γδ T cells using Hsp70 on its surface. (28) CNE2 and 915 are NPC cell lines. They were generous gifts of Dr X. Zhang (Su Zhou Medical College, China) and Dr Y. Zeng (Chinese Academy of Preventive Medicine, Beijing, China), respectively. XG-7 was cultured in RPMI-1640 medium (Gibco-BRL, USA) supplemented with 10% FCS (Gibco-BRL) (10% RPMI), 0.2 mM L-glutamine and antibiotics (100 U/mL of penicillin, 100 µg/mL streptomycin, 20 µg/mL garamycin and 100 units of nystatin) and 1 U/mL rIL-6. The NPC cell lines were cultured in modified Eagle’s medium (Gibco-BRL) supplemented with 10% FCS (10% MEC), 0.2 mM L-glutamine and antibiotics. PBMC obtained from the study subjects were separated by Ficoll-Hypaque (Pharmacia Biotech, Sweden) according to standard procedure. CD40-activated B cell lines were established as described previously. (28)

Ex vivo expansion of non-HLA-restricted tumor-effector cells
PBMC were cultured with a γδ T cell stimulant, XG-7, for 10 days. The non-HLA-restricted tumor-effector cells were expanded ex vivo as described previously. (28, 29) Briefly, 1 × 10^7 of PBMC was mixed with 5 × 10^6 irradiated (7000 cGy) allogeneic XG-7 cells and incubated in 10% RPMI. Irradiated XG-7 cells (3 × 10^6) were added to the cultures on day 3, 6 and 9. Exogenous rIL-2 (100 U/mL) was added to the cultures on day 11 and fed every 2–3 days or as needed thereafter.

Table 1. NPC statuses of 25 study subjects, with disease stages defined according to Ho’s standard(26)

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<th>Number</th>
<th>Patients (groups)</th>
<th>Survivors</th>
<th>Donors</th>
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<tr>
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<td>II (P1)</td>
<td>III (P2)</td>
<td>IV (P3)</td>
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<tr>
<td>1</td>
<td>T2N1</td>
<td>T3N1</td>
<td>T2N3</td>
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<tr>
<td>2</td>
<td>T1N1</td>
<td>T2N2</td>
<td>T3N3</td>
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<td>3</td>
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Diagnosis of NPC patients was confirmed by characteristic pathology of poorly or undifferentiated carcinoma. NA: Not applicable.

Flow cytometry analysis using FACS
Approximately 1 × 10^6 cells were stained with FITC- or PE-labeled mAbs specific for CD3, CD34, CD56, Lineage, pan TCR-αβ, pan TCR-γδ and mouse immunoglobulin G1 (BD Biosciences, USA) for 30 min at 4°C. After three washes with phosphate-buffered saline, the stained cells were analyzed using a flow cytometer (BD Biosciences).

Purification of NK cells and γδ T cells
NK cells and γδ T cells were purified from 4-week expansion cultures using MACS CD56 and TCR-γδ MicroBeads (Miltenyi Biotec, Germany). Briefly, approximately 10^7 cells in 0.5 mL of phosphate-buffered saline containing 2 mM EDTA and 0.5% FCS were mixed with 20 µL of the CD56 or TCR-γδ beads and allowed to stand at 6–10°C for 20 min. The beads-bound NK cells or γδ T cells were magnetically separated. The procedure was repeated to ensure the purity of NK and γδ T cells (>95%).

Detection of cytokine secretion
The production of IFN-γ, TNF-α, IL-15 and IL-12 in XG-7 stimulated cell cultures was detected by ELISA using OptEIA kits (PharMingen, USA) according to the manufacturer’s instructions. Levels of these cytokines in supernatant obtained on day 2, 4, 6, 8 and 10 post-stimulation were quantified using at least six concentrations of standard cytokines provided by the kits.

Detection of CTL activity
The CTL activity of the purified NK and γδ T cells was determined in triplicate by a standard 4 h Calcein AM release assay (Molecular Probes, USA) in U-bottom 96-well microplates. (28, 30, 31) Briefly, triplicate cultures were seeded with a graded number of purified NK or γδ T cells and 5000 Calcein AM (Molecular Probes) labeled target cells at effector:target ratios from 30/1 to 0.3/1. The cytolysis of the targets was determined by measuring Calcein AM FI using a fluorometer. The maximum release was estimated by incubating target cells with 5% sodium dodecylsulfate (total lysis) and the spontaneous release estimated by incubating the targets in medium alone (target control). The percentage specific cytolysis was calculated as follows:

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Statistical analysis
The significance of differences between groups was analyzed by paired Student’s t-test.

Results

Preferential activation and expansion of NK cells from NPC patients in response to tumor cell stimulator

FACS analysis of freshly prepared PBMC revealed that the frequencies of CD3-/CD56+ NK cells were similar among NPC patients, survivors and healthy donors. However, the percentage of γδ T cells was slightly lower in the patients than survivors and donors, and CD34+/Lin cells, NK cell precursors, showed markedly increased frequencies in the patients than in the other groups (Fig. 1a). PBMC were ex vivo stimulated with XG-7 cells, a non-HLA restricted tumor stimulant, for 10 days and further cultured in the presence of exogenous IL-2 thereafter. The number of NK cells was increased rapidly in the cultures of the patient groups in response to the tumor cell stimulation, reaching $7.9 \pm 1.2 \times 10^6$ (mean ± standard deviation, the same below) on day 10 (Fig. 2a), which was approximately 12- and 24-fold higher than those in the cultures from survivors (0.66 ± 0.3 × 10^6) and donors (0.33 ± 0.3 × 10^6), respectively ($P < 0.05$). Notably, the frequency of NK cells from the patient groups was markedly higher after 4 weeks of culture, reaching 38.6% ± 21.5%, as compared to that of survivor (1.5% ± 1.1%) and donor (0.5% ± 0.2%) groups. However, no CD34+/Lin+ cells were detectable in these cultures (Fig. 1b). Interestingly, the number of γδ T cells in the cultures from the patient group was significantly less than that of donors and survivors after 10 days with tumor cell stimulation (Fig. 2b), suggesting impaired activation of γδ T cells from the patients. In the presence of exogenous IL-2, rapid γδ T cell growth was sustained for approximately 5 weeks in the cultures of PBMC from survivors and donors, reaching approximately $8.8 \times 10^9$ and $5.4 \times 10^9$, respectively. However, the number of γδ T cells in cultures from NPC patients was at least 20-fold lower than that from survivors and donors ($P < 0.01$) (data not shown). After 4 weeks of culture, NK cells from patients were increased to approximately $1.1 \times 10^9$, which was 6- and 10-fold more than those from survivors and donors. The results showed that NK cells from NPC patients display markedly enhanced growth after tumor cell stimulation, whereas their γδ T cell expansion was significantly reduced as compared with those from the donors and survivors.

Increased cytotoxicity of NK cells from patients to target tumor cells

The non-HLA restricted cytotoxicity of NK and γδ T cells to tumor targets was measured in total cells from 4-week cultures of study subjects (Fig. 3). The cytotoxicity of purified NK cells from the cultures of patient groups against two NPC cell lines, CNE2 and 915, was significantly higher than that of healthy donors (data not shown). Furthermore, NK cells from survivors showed less cytotoxicity against the tumor cells, after γδ T cell immunity was restored, than patients with active disease. Compared to survivors, however, the cytotoxicity of purified γδ T cells from patients and donors was markedly reduced. These total culture cells, purified NK and γδ T cells did not lyse autologous B cells (data not shown).

Complementary regulation of NK cell and γδ T cell immunity associated with the progression of NPC

Cytolytic activities of purified NK and γδ T cells derived from NPC patients at different disease stages were further compared (Fig. 4). Patients who had progressed to a later
Tumor-induced cytokine profiles varied in NPC patients with different disease status

Further analysis revealed that the production of cytokines induced by tumor cell stimulation was varied in patients with different disease status (Fig. 6). The cultures from patients who had less disease progression (group P1) showed higher production of IFN-γ but lower production of IL-12p40, IL-15 and TNF-α than those from patients at later stages (groups P2 and P3). The level of IFN-γ at day 10 of culture from group P1 was 115 ± 18 pg/mL, which was significantly higher than that of groups P2 and P3 (73 ± 9 pg/mL and 66 ± 13 pg/mL, P < 0.05). However, levels of IL-12, IL-15 and TNF-α in day 10 cultures from group P1 were 30 ± 5 pg/mL, 22 ± 6 pg/mL and 73 ± 11 pg/mL, respectively, which were much lower than those from groups P2 (39 ± 7 pg/mL, 37 ± 7 pg/mL and 82 ± 6 pg/mL) and P3 (39 ± 7 pg/mL, 28 ± 6 pg/mL and 84 ± 9 pg/mL). These results were consistent with those compared to survivors and donors (Fig. 5). The cytokine profile of patients with earlier disease status (group P1) was comparable to that of donors than those of patients at later disease stages (groups P2 and P3).

Discussion

In our previous studies on NPC patients, it was observed that both patients' EBV-specific αβ-CTLs and tumor cytolytic γδ-CTLs suffer from reductions in cell quantity as well as their respective cytolytic effects against tumor cells. Moreover, increased deficits of these cells were also observed during the progression of the tumor. In this study, we further investigated NK cells, another important antitumor immune cell type, and found that although both EBV-specific αβ-CTLs and tumor cytolytic γδ-CTLs appeared to have been breached, NK cells were indeed preferentially activated in these patients. Their cytotoxicity against NPC cells was also increased, accompanied by impaired antitumor function of γδ T cells, as compared to the NK cells from healthy donors. In contrast, when the tumor has been removed and successfully treated, as reflected in the survivors, the reverse is observed, where γδ- and αβ-CTLs appear to be the dominant antitumor immune cells with increased cytotoxicity towards tumor
cells. Our findings suggest a complementary activation of NK cell-mediated immunity in these NPC patients.

In general, the progression of immune response is sequentially activated from innate to adaptive immunity. NK and γδ T cells, which are broadly cytolytic, initially play dominant roles before target-specific αβ T cells take over. It is thus probable that the reverse is true when adaptive immunity is breached by tumors, especially in view of the intercommunication
between these cells through molecules such as cytokines and chemokines. Patients at earlier stage II of NPC (group P1) had higher $\gamma\delta$-CTL cytotoxicity towards NPC targets than patients at later stages III and IV (groups P2 and P3). Conversely, their respective NK cell cytotoxicity was lower than their later stage counterparts. These results suggest that $\gamma\delta$-CTLs were probably recruited first as part of the complementary immune response as the adaptive immunity, that is, specific $\alpha\beta$-CTLs, was first breached by the tumor cells. With the progression of the tumor disease, the immune response elicited by $\gamma\delta$ T cells began to show deficiency. Our current observation that enhanced immune response mediated by NK cells in patients might reflect some potential compensatory mechanisms. In patients at a more progressed disease stage (group P3), $\gamma\delta$ T immunity against the tumor targets was further defected, but not accompanied with continually increased NK cell cytolitic activity toward the tumors, which suggest that the remobilized innate immunity would be also exhausted by the tumors at the later stage of the disease.

In the present study, NK cells are expanded by co-cultures of PBMC from NPC patients with a tumor stimulator, XG-7. There is evidence that tumor-cytolytic NK cells can be expanded ex vivo by co-culture of PBMC obtained from adult and pediatric acute lymphoid leukemia patients with a feeder cell line RPMI-8866.\textsuperscript{(32)} It is still not clear why NK cells could be expanded in ex vivo cultures of PBMC from these patients. Interestingly, numbers of CD34$^+$/Lin$^-$ cells, a precursor of NK cells, were markedly higher in PBMC taken

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**Fig. 5.** Cytokine profiles in study subjects’ PBMC cultures stimulated with XG-7 cells. PBMC ($10 \times 10^7$ cells) from NPC patients, survivors and donors were stimulated with XG-7 and cultured for 10 days in the absence of exogenous IL-2. The culture supernatants were collected every 2 days for determination of IFN-$\gamma$ (A), IL-12p40 (B), IL-15 (C) and TNF-$\alpha$ (D) by ELISA.

**Fig. 6.** Cytokine productions in response to the stimulation of XG-7 cells in PBMC cultures from NPC patients with different disease statuses. Production of IFN-$\gamma$ (A), IL-12p40 (B), IL-15 (C) and TNF-$\alpha$ (D) in PBMC cultures from NPC patients with different disease statuses of II (P1), III (P2) and IV (P3) were tested by ELISA.
from patients than those from survivors and donors in our study (Fig. 1a). Markedly increased CD34+ cells were also observed in patients with acute myelogenous leukemia.\textsuperscript{13–36} Further study is required to elucidate if the successful \textit{ex vivo} expansion of NK cells in these patients is associated with the increase numbers of CD34+ cells in their blood circulation.

Although the mechanism leading to this resurgence of NPC cytotoxic NK cells is not clear, cytokines produced in the cultures upon stimulation of tumors seem to be involved in immune regulation. The reduced level of IFN-γ, an important cytokine for regulating immune response, was observed in cultures of patient groups (Fig. 3a). This is probably attributable to the decreased total numbers of effector cells in the cultures, as IFN-γ is produced by both T cells and NK cells. Increased levels of IL-12, IL-15 and TNF-α were detected in the patient group, compared to those of the survivors and donors (Fig. 5b–d). As IL-12 is an activator for both NK cells and T cells,\textsuperscript{6,15,17} the increased level of this cytokine might be a signal indicating T cell immunity failure, thus triggering the response of NK cell activation. On the other hand, IL-12 receptors on macrophages are primed by IFN-γ.\textsuperscript{49} The reduced level of IFN-γ in patient groups might also contribute indirectly to the increase of IL-12 in the cultures by reducing the amount of IL-12 binding receptor. IL-15 is known to be important for activation and growth of NK cells, and it is also able to improve cytolytic activity of NK cells.\textsuperscript{41,42} The rapid expansion of NK cells with higher cytotoxicity to tumor target cells in the cultures from patient groups might be partly attributed to the increased IL-15 production. The upregulation of TNF-α might be a result of NK cell proliferation and activation, as it is a product of activated NK cells. Furthermore, this cytokine can promote NK cell-mediated killing.\textsuperscript{43,44} Increased levels of TNF-α in cultures from patient groups might alternatively enhance the cytolytic ability of NK cells purified from these cultures. Distinct cytokine profiles were also observed in three groups of patients with different NPC status, in which patients with earlier stage disease showed a cytokine profile closer to that of healthy donors than those with later stage disease (Fig. 6). Notably, these cytokine profiles were accompanied with lower γδ T and higher NK cell activities in cultures from patient groups, but higher γδ T and lower NK cell activities in cultures from survivors and donors, suggesting that these cytokines might play roles in the complementary regulation between NK cells and T cells.

Although the mechanism of complementary regulation of innate and adaptive immunity remains to be elucidated, we have demonstrated that NK cells can be preferentially activated and expanded \textit{ex vivo}, when the immune responses mediated by γ<sub>δ</sub>- and γδ-CTLs are breached in NPC patients. Further work is underway to investigate the roles of other immune cells, such as antigen-presenting cells, NKT cells, and other cytokines, in regulating the immune system to achieve effective immunity against tumors and infections.

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