Increased numbers but functional defects of CD56+CD3+ cells in lung cancer

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Abstract

CD56+ T cells were studied in samples of peripheral blood from small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC) patients compared with healthy controls. Relative numbers of CD56+CD3+ cells were increased in NSCLC (P = 0.001) and SCLC (P = 0.002) compared with normal subjects but their ability to respond to activation by up-regulating CD25 or producing IFN-γ were both significantly impaired. Expression of the killer-immunoglobulin-like receptor CD158a was significantly lower on CD56+ cells in lung cancer, which may have implications for a reduction of direct or indirect anti-tumour responses.

Keywords: CD25; CD56; CD69; lung cancer: killer immunoglobulin-like receptors; IFN-γ; T cells

Introduction

CD56 is expressed by most human NK cells and a small subpopulation of T cells (1). This, or a similar population of CD56+CD3+ cells, has been variously described as Natural Killer T (NKT) cells, NKT-like cells or natural killer-like T cells. CD56+CD3+ cells can be expanded following cytokine treatment in vitro and have been named cytokine-induced killer (CIK) cells, which represent a major contributor to the MHC non-restricted cytotoxicity induced by IL-2 (2). However, the relationship of these cells to each other and to other unconventional T-cell subsets (3) is unclear. From relative percentages in peripheral blood, most are probably not classical invariant Vα24+ NK T (/NKT) cells (4) and CIK cells generally fail to express Vα24 (5). Their relationship to Type II or non-invariant NKT cells (6) is also uncertain.

Human CD56+CD3+ cells are scarce in cord blood but increased in adult peripheral blood (7). They are increased following hepatitis B (8) or malaria infection (9) but decreased in patients infected with hepatitis E (10). There is no consensus about changes in inflammatory diseases, with decreases reported in systemic lupus erythematosus (11) and haemodialysis patients with chronic inflammation (12) but increases in vitiligo (13), chronic obstructive pulmonary disease (14) and fatty liver disease, although these were predominantly Vα24+ /NKT cells (15).

CD56+CD3+ CIK cells generated after cytokine stimulation in vitro were effective at preventing tumour growth in a mouse model of human lymphoma (16). Numbers were decreased locally in higher grade ovarian (17) or metastatic colorectal cancer (18). NKT-like cell cytotoxicity was mediated by NKG2D, expression of which was reduced in ovarian and prostate cancer patients, leading to impaired lytic function (19). Following activation, enhanced levels of pro-inflammatory cytokines were produced by CIK cells (20, 21).

Killer immunoglobulin-like receptors (KIRs) of the CD158 family are used by NK cells to recognize virus-infected or malignant cells. Those with long cytoplasmic regions are inhibitory, while those with short cytoplasmic regions are activating receptors. KIR2DL1/2DS1 are specific for HLA-C2 ligands and KIR2DL2/3/DS2 are specific for HLA-C1 ligands, while KIR3DL1 recognizes HLA-Bw4 ligands (22). The inhibitory forms of these three KIRs are designated CD158a, CD158b and CD158e, respectively, although the available mAbs also recognize the activating forms of the receptor. Individuals homozygous for the A haplotype express almost...
exclusively inhibitory receptors, while the B haplotype additionally encodes several activating receptors (22). KIRs are also expressed by a minority of T cells in tumour patients (23, 24) but their expression has not been extensively studied in relation to CD56 expression on T cells. CIK cells derived from CD56–CD3+ precursors lack inhibitory forms of CD158 as well as other activating NK receptors (25).

The aims of the present experiments were to quantify CD56+CD3+ cells in lung cancer patients compared with normal healthy subjects and to investigate their expression of markers normally associated with conventional T cells or NK cells.

Methods

Patients and controls
Blood samples were obtained following informed consent from patients with lung cancer attending St Helens & Knowsley NHS Trust, Liverpool Cardiothoracic Centre NHS Trust and Aintree Hospital NHS Trust, Merseyside, UK. These comprised 67 patients with NSCLC and 30 with SCLC. Sixty-nine age-matched normal healthy volunteers from Merseyside and of the same ethnic background (predominantly Caucasian) were used as controls. Ethical approval for the study was obtained from Cheshire and Liverpool NHS Research Ethics Committees.

Reagents

mAbs used in the study were: anti-human CD3-Pe-Cy5; mouse IgG1-PE isotype control (both Dako, Ely, UK); anti-human CD56-FITC; anti-human CD158b-PE (anti-KIR2DL2/DL3; also recognizes CD158h, KIR2DS1); anti-human CD158e-PE (anti-KIR3DL1) and anti-human CD158a-PE (anti-KIR2DL1; also recognizes CD158h, KIR2DS1); anti-human CD158e-PE (anti-KIR3DL1) and anti-human IFN-γ-PE (Beckman Coulter, High Wycombe, UK).

Cell preparation and phenotypic analysis

PBMC were isolated from anticoagulated blood by density gradient centrifugation on an equal volume of Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) by centrifugation at 3000 g for 20 min at room temperature. PBMC were harvested from the interface, washed twice with phosphate-buffered isotonic saline (PBS) and centrifuged at 400 g for 10 min.

Surface phenotyping was carried out using three-colour immunofluorescence staining. PBMC (5 × 10⁵) were stained with mouse anti-human mAbs conjugated with FITC, PE or PE-Cy5 for 30 min in the dark at 4°C. Cells were washed twice with cold PBS, re-suspended in 0.5 ml of PBS and analysed using an EPICS XL-MCL flow cytometer (Coulter, Luton, UK), gated for lymphocytes. Negative controls used appropriate mouse IgG1 isotype controls and fluorescence thresholds were set so that <1% of lymphocytes showed non-specific antibody binding. Results were expressed as % of total gated lymphocytes.

Detection of NK cell activation markers

PBMC were washed twice with PBS, re-suspended in RPMI 1640 + 10% heat-inactivated foetal bovine serum + antibiotics (culture medium) and adjusted to 1 × 10⁶ cells ml⁻¹. To study CD25 expression, PBMC (200 μl) were stimulated with K562 cells for 24 h at a ratio of 1.1 (25 × 10⁶:25 × 10⁵ effector:target cells) in a 96-well U-bottomed plate at 37°C. Expression of CD69 was studied on cells stimulated with 20 μg ml⁻¹ PHA (Sigma, Poole, UK) for 15 h at 37°C. Cells were then washed in PBS and stained with 2 μl mouse anti-human CD56-FITC, mouse anti-human CD3-PE-Cy5 and mouse anti-human CD25-PE or mouse anti-human CD69-PE for 30 min in the dark at 4°C, washed twice with PBS and re-suspended in 500 μl of PBS before analysis by flow cytometry.

Intracellular staining

For measurement of intracellular IFN-γ, PBMC suspended in culture medium at 10⁶ ml⁻¹ were stimulated for 24 h with 10 ng ml⁻¹ IL-12 and 100 ng ml⁻¹ IL-18 (Peprotec, London, UK). Two hours before analysis, brefeldin A was added at 10 μg ml⁻¹ and cells were washed and labelled with anti-CD3-PE-Cy5 and anti-CD56-FITC as above. Cells were then fixed with 1% PFA for 20 min and washed twice with saponin buffer (BD Pharmingen), re-suspended in 100 μl saponin buffer and incubated with anti-human IFN-γ-PE antibody for 40 min at 4°C. After a final wash in saponin buffer, cells were analysed on the flow cytometer, re-gating for lymphocytes. Intracellular staining for perforin A was carried out similarly on fresh cells using anti-human perforin A-PE antibody but without cytokine stimulation or brefeldin A treatment. Appropriate isotype control reagents were used in all experiments.

Statistical analysis

Differences between healthy subjects and lung cancer patients (NSCLC and SCLC) were evaluated using the Mann–Whitney U-test or using an unpaired t-test. Graphic representation was performed using GraphPad Prism software.

Results

CD56+CD3+ cell populations in lung cancer patients

The mean proportion of CD56+CD3+ cells in NSCLC and SCLC patients was significantly higher than in healthy controls. The proportions of CD56+CD3+ cells in NSCLC patients, SCLC patients and controls were 2.91, 2.97 and 1.6%, respectively (P = 0.001 and 0.002; Fig. 1(a)). Several NSCLC patients had >20% CD56+CD3+ cells comprising two distinct populations with different levels of CD3 expression. The two-colour dot plot of a representative NSCLC patient is shown in Fig. 1(b). These distinct populations of CD64+CD3+ cells were both tested for expression of CD25, a marker of Regulatory and activated T cells, but no differences in staining were found between these two populations in all patients analysed (data not shown).

KIR expression by CD56+CD3+ cells in lung cancer

KIR expression was studied in CD56+CD3+ cells by three-colour flow cytometry. Cells gated on the basis of positivity for CD56 and CD3 were then analysed for CD158 staining, as shown in Fig. 1(c). CD56+CD3+ cells from SCLC patients contained a lower percentage of CD158a+ cells than healthy controls (0.84 versus 2.3%, P = 0.005; Fig. 2(a)) despite the
variability within groups. However, overall percentages of CD158b+ cells were not significantly lower in SCLC patients compared with controls (Fig. 2b). Although there were no significant differences in proportions of CD56+CD3+CD158e+ cells between lung cancer patients and controls (Fig. 3a), median levels of CD158e in NSCLC were significantly higher than in controls (21.6 versus 12.4 arbitrary units, P = 0.004; Fig. 3b). SCLC patients with the KIR BB haplotype had significantly lower median levels of CD158b on CD56+CD3+ cells than controls (P = 0.03; Fig. 3c). However, NSCLC patients with the KIR haplotype AA had significantly higher median levels of CD158e per cell than healthy controls (P = 0.01; Fig. 3d).

CD56+CD3+KIR+ cells in relation to ligand expression in lung cancer

Neither the per cent CD56+CD3+ cells expressing CD158a or b nor the mean fluorescence intensity (MFI) for these KIR differed between NSCLC and SCLC patients and normal subjects for combinations of CD158a or CD158b with their corresponding ligand (C2 or C1, respectively; Figs 4 and data not shown). In NSCLC patients who lacked the CD158a ligand (C2), the frequency of CD158a+ cells was lower than in controls (P = 0.01 and 0.002, respectively; Fig. 4c). Conversely, the MFI of CD158b in the absence of its ligand HLA-C1 was significantly increased in NSCLC patients compared with controls (P = 0.004; data not shown). SCLC patients had a lower percentage of CD56+CD3+CD158a+ cells and a lower MFI in the absence of its corresponding ligand C2 compared with controls (P = 0.002 and 0.01, respectively; Figs 4c and d). The frequency of CD56+CD3+CD158e+ cells in the presence of its
corresponding ligand (Bw4) was similar in all groups (data not shown). However, a significantly higher MFI for CD158e was observed in the presence of its ligand in NSCLC and SCLC patients compared with controls (MFI 21.8 versus 14.9 versus 10.9 arbitrary units; \( P = 0.006 \) and \( P = 0.03 \); data not shown). There were no significant differences in the percentage CD158e+ cells and the MFI in the absence of its corresponding ligand (Bw4) between the controls and lung cancer patients (data not shown).

**KIR expression according to lung cancer stage**

Stage IV NSCLC patients had a lower percentage of CD56+CD3+ cells compared with stage I patients (3.8 versus 9.0%, \( P = 0.013 \); Table 1). A significantly higher percentage of CD56+CD3+CD158a+ cells was seen in stage II compared with stage I patients (5.9 versus 0.9%, \( P = 0.04 \); Table 1). The MFI of CD158a for CD56+CD3+ cells expressing CD158a was higher in stage III compared with stage I (\( P = 0.036 \); data not shown).

**Activation markers and functional molecules in CD56+CD3+ cells in lung cancer**

NSCLC patients showed a significantly lower percentage of CD56+CD3+ cells expressing CD25 following stimulation with K562 cells overnight compared with healthy controls (\( P = 0.049 \); Fig. 5a). Unstimulated cells from all groups showed very low levels of CD25+ cells (data not shown). The percentage of CD56+CD3+ cells expressing CD69 was lower, but not significantly, in cells from NSCLC patients stimulated with PHA compared with healthy subjects (Fig. 5b). When PBMC were stimulated with IL-12 and IL-18 overnight, both the percentage of IFN-\( \gamma \)+CD56+CD3+ cells and mean IFN-\( \gamma \) levels were significantly lower in NSCLC patients than controls (Fig. 5c and d; \( P = 0.003 \) and \( P = 0.02 \), respectively). However, perforin was found in a similar percentage of CD56+CD3+ cells in control and NSCLC patients (data not shown).

**Discussion**

CD56+CD3+ cells activated in vitro have enhanced cytotoxicity against tumour cells (2, 3, 16, 26). As proportions of CD56+CD3+ cells in both NSCLC and SCLC patients were significantly increased, particularly at the early stages of lung cancer, there is the potential for enhanced cytotoxic function against autologous tumour cells. However, phenotypic and functional analyses suggest that these cells were functionally compromised in lung cancer. CD25 induction was significantly impaired in CD56+CD3+ cells from lung cancer patients. As IL-2 may be the most important inducer of CD56+CD3+ cell expansion (27), reduced CD25 expression would limit their further proliferation or activation in response to IL-2. This would substantially impair induction of CIK cells capable of targeting autologous tumour cells in vivo.

A second potentially important function of CD56+CD3+ cells is their ability to produce IFN-\( \gamma \) (28). Following T\(_h\)1
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Cytokine stimulation, fewer CD56+CD3+ cells from lung cancer patients were found to produce IFN-γ and mean levels per cell were also reduced. This reduction in pro-inflammatory cytokine-producing ability would potentially limit the capacity of CD56+CD3+ cells to enhance effector functions of other T cell types, NK cells or macrophages in lung cancer, thereby potentially reducing Th1 responses to autologous tumour. However, this may be an indirect effect, the response of another cell type being deficient in lung cancer, leading to impaired induction of IFN-γ in CD56+CD3+ cells. However, sample sizes were small and though statistically significant a larger sample would need to be tested to confirm this result.

Previous infection with the herpes viruses cytomegalovirus or Epstein-Barr virus may have influenced levels or function of CD56+CD3+ cells but information on viral status was unavailable for most patients. It is considered unlikely, however, that viral status would differ between patients and controls.

CIK cells generated in vitro have been reported to lack inhibitory KIR expression (25) although no differences in KIR expression were noted between CD56+CD3+ cells in hepatocellular carcinoma patients and controls (28). Here, the proportion of CD56+CD3+ cells expressing CD158a was significantly lower in SCLC patients compared with controls. However, the proportions of CD158a+ cells and the median levels of CD158a increased with advancing cancer stage, which would potentially reduce cytotoxicity, assuming that

Fig. 4. CD158a expression by CD56+CD3+ cells in lung cancer patients and healthy controls in relation to HLA-C ligand: (a and b) Per cent CD56+CD3+CD158a+ cells and MFI for CD158a in the presence of HLA-C2 ligand; (c and d) Per cent CD56+CD3+CD158a+ cells and MFI for CD158a in the absence of HLA-C2 ligand. In (c), controls > NSCLC (P = 0.01), controls > SCLC (P = 0.002). In (d), controls > NSCLC (P = 0.01).

Table 1. Per cent of CD56+CD3+ cells positive for the three KIRs CD158a, b and e in NSCLC patients with tumours at different stages

<table>
<thead>
<tr>
<th>% Total cell population</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD56+CD3+ cells</td>
<td>9 ± 7.3</td>
<td>6.1 ± 6.4</td>
<td>5.7 ± 6.4</td>
<td>3.8 ± 6.2a</td>
</tr>
<tr>
<td>% CD56+CD3+ cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56+CD3+CD158a+ cells</td>
<td>0.9 ± 1.3b</td>
<td>5.9 ± 5.4</td>
<td>6.4 ± 8.2</td>
<td>2.4 ± 2.2</td>
</tr>
<tr>
<td>CD56+CD3+CD158b+ cells</td>
<td>11.6 ± 10.5</td>
<td>9.7 ± 9.8</td>
<td>10.4 ± 9.2</td>
<td>7.4 ± 9.1</td>
</tr>
<tr>
<td>CD56+CD3+CD158e+ cells</td>
<td>12.2 ± 16.8</td>
<td>15.3 ± 21</td>
<td>8.3 ± 9.3</td>
<td>6.7 ± 9.8</td>
</tr>
<tr>
<td>No. of patients</td>
<td>11</td>
<td>5</td>
<td>31</td>
<td>16</td>
</tr>
</tbody>
</table>

aStage IV < stage I (P = 0.013, Mann–Whitney test).
bStage I < stage II (P = 0.04, Mann–Whitney test); all other differences not significant.
Inhibitory KIR expression by CD56+CD3+ cells is functionally comparable to that by CD3− NK cells. A similar reduction in %CD158a+CD56+CD3+ cells was noted in hepatocellular carcinoma (29). KIRs expressed by T cells may modulate cytotoxic and cytokine-producing functions (30). Decreased inhibitory KIR expression by CD56+CD3+ cells would reduce inhibition of cytotoxicity, although reduced natural cytotoxicity receptor expression (25) would negate any potential enhancement of cytotoxicity. However, median levels of CD158e on CD56+CD3+ cells in NSCLC and SCLC were significantly increased, potentially strengthening inhibitory function. The relevance of the increased %KIR+ cells and median KIR densities in CD56+CD3+ cells in the absence of the cognate ligand in lung cancer patients is unclear as these cells would not be "licensed" to recognize loss of the ligand if KIR repertoire development in CD56+CD3+ cells resembles that of NK cells.

The significantly decreased levels of CD158b in SCLC patients with haplotype BB, containing several activating KIR genes, including KIR2DS2, may result in lower levels of activation of CD56+CD3+ cells in the presence of an HLA-C1 ligand on tumour cells, particularly if CD56+CD3+ cells lack activating NCRs (25). Also, the highly significantly increased density of CD158e on CD56+CD3+ cells from NSCLC patients with KIR haplotype AA would be expected to enhance the inhibition of cytotoxic function as KIR3DS1, also recognized by anti-CD158e, is absent from the A haplotype and the antibody must be detecting only the inhibitory KIR3DL1 in AA haplotype patients.

Relative changes in numbers and phenotype of CD56+CD3+ cells according to tumour progression in lung cancer patients were consistent with decreased effector function with increasing stage of disease. In a previous study, decreased numbers of CD56+CD3+CD16+ cells were associated with disease progression in chronic lymphocytic leukaemia patients (31). CIK cells generated from normal subjects in vitro were highly cytotoxic towards allogeneic lung cancer cell lines (32), emphasizing their potential importance in tumour resistance. However, it is possible that peripheral blood CD56+CD3+ cells are not representative of those present at the tumour site. Most patients in the present study did not undergo tumour biopsies so it was not possible to test this. Taken together, these findings suggest that aspects of CD56+CD3+ T-cell function are defective in lung cancer but that this generally does not extend to KIR expression. This would potentially impair anti-tumour responses. A possible mechanism might be induction of increased regulatory T-cell activity via increased thymic stromal lymphopoietin, which has recently been reported in the lung cancer microenvironment (33). This, or another mechanism, might result in decreased IFN-γ production by CD56+CD3+ cells. We have previously found KIR expression to be enhanced in NK cells in lung cancer (34), which would potentially inhibit NK cell cytolytic function. The function of purified CD56+CD3+ cells in lung cancer and delineation of their relationship to Type I and Type II NKT cells warrants further study.
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References


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