Cytotoxic T lymphocytes and natural killer cells display impaired cytotoxic functions and reduced activation in patients with alcoholic hepatitis

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The class of lymphocytes collectively known as cytotoxic effector cells forms a central part of our defense against infections, but excessive activation may be injurious to our tissues. Their role in alcoholic hepatitis (AH), in which both increased susceptibility to infections and hepatic injury are hallmarks, remains poorly understood (35). Thus, the frequency, functionality, and activation of cytotoxic effector cells have not been investigated in such patients.

The dominant cytotoxic effector cells comprise cytotoxic (CD8+ T lymphocytes (CTLs), natural killer (NK) cells, and natural killer T (NKT) cells) among which invariant NKT (iNKT) cells are a well-defined subgroup (11, 20, 34). These cells share the ability to directly kill infected cells (6). A classical NK cell killing mechanism is the release of cytotoxic granules, and this degranulation can be detected by the surface expression of the lysosomal membrane protein CD107a (2). Such functional activation requires the expression of and signals via stimulatory surface receptors from infected, stressed, or damaged target cells. A central and well-characterized stimulatory receptor is the natural killer group 2 D (NKG2D) (50). For NK cells, the level of CD56 expression is believed to assess maturation: immature CD56bright cells are regarded as precursors of mature CD56dim cells (9).

The production of the hepatoprotective, T-helper17-related cytokine IL-22, which holds anti-apoptotic and proliferative actions, is of particular interest in AH because of the extensive liver tissue destruction (5, 38, 42). The cytotoxic effector cells also produce the Th1-related cytokine interferon-γ (IFNγ) which, conversely, has proinflammatory and also cytotoxic effects by sensitizing target cells (15).

On this basis, the aim of this study was to describe and explore the status of the cytotoxic effector cell system in severe AH. We measured the frequencies of the CTLs and NK, NKT, and iNKT cells and characterized their functionality and state of activation.

MATERIALS AND METHODS

Patients. We included 24 consecutive patients at diagnosis with severe AH [Glasgow alcoholic hepatitis score (GAHS) >9] according to the following established diagnostic criteria: a history of heavy alcohol consumption until at least 3 wk before admission; acute jaundice (serum bilirubin >80 μmol/l); modified Maddrey’s discriminant function >32 (29); the absence of suspected infectious foci and the absence of liver tumors or other cancers. The disease severity was further scored according to the model of end-stage liver disease (MELD score) and Child-Pugh score (7, 18, 40). We excluded patients aged below 18 yr or above 75 yr, with gastrointestinal bleeding within the past 3 mo, or prior immune-modulating therapy. Blood samples were collected at the time of diagnosis and at day 14 and 30. All patients were treated with 400 mg pentoxifylline three times per day according to the national guidelines. No patient received corticosteroids at any time. Five patients (20%) developed blood culture-verified infections during the follow-up period (2 fungal and 3 bacterial). One of these died 2 days following diagnosis of AH. All patients were abstinent on recruitment and during the study period. Ten age (51.3 ± 9.2 yr)- and sex (4 females/6 males)-matched abstinent volunteers with no history of liver or other diseases were included as healthy controls. As a sick control group, we included 10 abstinent patients with stable biopsy-proven alcoholic cirrhosis. The study was approved by the local ethics committee (i.no. 20100281), and written informed consent was obtained from all participants. The study was registered at clinicaltrials.gov (NCT00992888).

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Table 1.  Patient characteristics

<table>
<thead>
<tr>
<th>Gender (F/M)</th>
<th>Alcoholic Cirrhosis</th>
<th>Alcoholic Hepatitis-Infected</th>
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</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>Day 14</td>
<td>Day 30</td>
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<tr>
<td>Age, yr</td>
<td>8/16</td>
<td>8/15</td>
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<tr>
<td>BMI</td>
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<td>53 ± 9</td>
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<tr>
<td>ALT, U/l</td>
<td>22.5 ± 5.3</td>
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<td>Sodium, mmol/l</td>
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<td>11.80 ± 9.00</td>
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</tr>
<tr>
<td>GAHS</td>
<td>54.8 ± 33.1</td>
<td>28.6 ± 55.2</td>
</tr>
</tbody>
</table>

Values are medians ± interquartile ranges. F, females; M, males; BMI, body mass index; ALT, alanine aminotransferase; INR, international normalized ratio; CRP, C-reactive protein; MELD, model of end-stage liver disease; GAHS, Glasgow alcoholic hepatitis score; MDF, modified Maddrey’s discriminant function.

Phenotypic characterization. Flow cytometry was carried out on peripheral blood mononuclear cells (PBMCs) isolated from EDTA-whole blood samples by Ficoll-Hypaque (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation and stored until analysis at −140°C. Samples originating from the same patient at different time points were analyzed together. At the time of analysis, the PBMCs were thawed in PBS containing 20% heat-inactivated pooled human AB-serum. To minimize nonspecific binding, 0.5 × 10⁶ cells in 100 µl washing buffer (PBS with 2% BSA and 0.9% azide) were initially blocked with 10 µl heat-inactivated mouse serum (Invitrogen, Carlsbad, CA) and then stained with optimized concentrations of fluorescent-conjugated monoclonal antibodies: anti-Vα24Jα18-FITC (clone 6B11), anti-CD56-PE (clone B159), anti-CD3-PerCP (clone SK7), anti-CD8-PE-Cy7 (clone RPT8), anti-NKGD2-APC (clone 1D11), anti-CD4-APC-Cy7 (clone RPA-T4), and the viability marker 7AAD (all from BD Bioscience, San Diego, CA). Following 20 min of incubation in the dark, the cells were washed in washing buffer and fixed in PBS with 1% formaldehyde. The samples were analyzed within 24 h of preparation using a FACs Canto-II instrument (BD Bioscience).

Based on a forward-side scatter plot, a large lymphocyte gate was set, and 200,000 events were recorded. CTLs were identified as CD3+ CD8+, NK cells as CD3− CD56+, NKT cells as CD3+ CD56+, and iNKT cells as CD3− CD56− Vα24Jα18+. The data were analyzed using FlowJo v10.1 (Tree Star, Ashland, OR). The expression levels of NKGD2 and CD56 are reported as the median fluorescence intensity. Fluorescence minus one and isotype controls were used throughout the flow cytometric experiments.

Degranulation assay and cytokine production. The thawed cells were kept at 37°C and 5% CO₂ overnight in culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated pooled human AB-serum). The human chronic myeloid leukemia cell line K562 was cultured under the same conditions. The cells were counted and set to a concentration of 2 × 10⁶/ml. For stimulation, a 100-µl sample of PBMC (2 × 10⁶/ml) was stimulated with 100 µl K562 cells (2 × 10⁶/ml) and centrifuged at 100 g for 1 min before being incubated for 2 h at 37°C and 5% CO₂. Nonstimulated samples were included as controls. Following incubation, the cultured cells were washed, and surface staining was performed as described above including anti-CD107a-FITC (BD Bioscience) as a degranulation marker (4). To assess the potential to produce cytokines, the remaining cells were stimulated with 50 µg/ml phorbol 12-myristate,13-acetate (PMA; Sigma-Aldrich), 1 µg/ml ionomycin (Sigma-Aldrich), and 10 µg/ml of the Golgi-blocking agent brefeldin A (Sigma-Aldrich)/2.5 ml cell suspension for 4 h at 37°C and 5% CO₂. The cells were harvested, and intracellular cytokine staining was performed using anti-CD4-FITC (clone RPA-T4), anti-CD3-PerCP, anti-CD8-APC (BD Bioscience), anti-IFNγ-PE (clone 4S.B3), anti-CD56-PE-Cy7 (eBioscience, San Diego, CA), anti-IL-22-PE (clone 142928) (R&D Systems, Minneapolis, MN), and Live-Dead-APC-Cy7 (Life Technologies, Carlsbad, CA). Statistics. We used the Kruskal-Wallis and the Wilcoxon rank-sum tests to assess differences among the groups. We used ANOVA for repeated measurements to examine the changes in cell and cytokine frequencies during follow-up. The measurements were normally distributed after a logarithmic transformation and were included as such in the analyses. Histograms and Q-Q plots were used to check normality, and Levene’s test was used to test the homogeneity of variance. Correlation analyses were performed using the Spearman rank-order correlation coefficient. The results are expressed as medians and interquartile ranges (25–75), and a two-tailed P value <0.05 was considered statistically significant.

RESULTS

Lymphopenia and a reduced frequency of CTLs. At the diagnosis of AH the total lymphocyte count was low (1.45 × 10⁹/l ± 0.70) in the reference interval (1.3–3.5 × 10⁹/l) and the frequency of lymphocytes among total leukocytes was decreased (41.6 ± 20.1 vs. 72.7 ± 10.6% in the healthy controls, P < 0.0001) (1). There was a substantially decreased frequency of CTLs (9.2 ± 11.6 vs. 22.2 ± 18.2% in the healthy controls; P < 0.001) but not so vs. the cirrhosis patients (10.2 ± 6.8; P = 0.5). With regard to NK cells we found a similar trend toward decreased frequencies (9.9 ± 8.4 vs. 14.0 ± 10.2% in healthy controls, P = 0.089) and vs. cirrhosis patients (15.6 ± 10.9%; P = 0.067). The frequency of NKT cells was not different among the patient groups. We similarly found no difference in frequencies of the iNKT cells. During the 30 days of follow-up we observed no dynamics.
in these cell frequencies (Fig. 1). Furthermore, we stratified the frequencies of CTL and NK, NKT, and iNKT cells in the AH patients after verified follow-up acquired infection and detected no differences (Fig. 1).

**Decreased degranulation capacity in the NK cell population.** The AH patients’ NK cell degranulation capacity was reduced (by 25%: 9.4 ± 3.7 vs. 14.1 ± 5.4% in the healthy controls, *P* = 0.02 and by 50%: 18.8 ± 17.3% in the cirrhosis patients, *P* < 0.05) (Fig. 2B). This capacity was not different in the patients who developed infections.

**Decreased expression of the activation receptor NKG2D but higher expression of CD56.** The expression of the activation receptor NKG2D was markedly decreased on both the CTLs and NK and NKT cells in patients with AH compared with

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**Fig. 1. Lymphocyte subset frequencies.** The cell frequencies are quantified by flow cytometry and expressed as percentages of the total peripheral blood mononuclear cells (PBMCs), the lymphocyte population, or the natural killer T (NKT) cell population. Values are presented as medians ± interquartile ranges. The cell frequencies are reported for healthy controls (HC) and alcoholic cirrhosis patients (AC) and on the day of diagnosis (d0), day 14 (d14), and day 30 (d30) for all alcoholic hepatitis (AH) patients and for the 5 AH patients who developed infections. NK, natural killer; iNKT, invariant NKT. *Significant differences between AH at diagnosis and healthy controls or cirrhosis patients. No difference was found among the study days in the group of AH patients and neither when comparing infected AH patients with noninfected AH patients at any of the study days.

**Fig. 2. NK cell degranulation capacity.** PBMCs were cocultured with K562 cells for 2 h. Following incubation, the expression of CD107a on NK cells was measured by flow cytometry. A: the flow cytometric gating strategy using a representative AH patient. NK cells are gated as CD3−CD56+ cells among the lymphocytes (a lymphogate was set before the first image) (top). Bottom, the percentage of CD107a+ NK cells is shown in a K562-stimulated sample (left) and in an unstimulated control sample (right). The gates are set based on fluorescence minus one (FMO) (data not shown).

**B:** the percentage of NK cells expressing CD107a is shown for AH patients, alcoholic cirrhosis patients, and healthy controls. The boxes show the medians and interquartile ranges (*P* < 0.05 and **P** = 0.02).
both control groups ($P < 0.05$, all) (Fig. 3). In contrast, the CD56 expression was markedly increased on the NK and NKT cell populations from the AH patients compared with both control groups ($P < 0.05$, all) (Fig. 4). The NKG2D expression was not different in the AH patients who developed infection.

**Increased production of IL-22 in CTLs and NK cells.** The frequency of IL-22-producing CTLs was doubled in AH compared with healthy controls ($P = 0.03$). The same was found for NK cells ($P = 0.005$) (Fig. 5). We found no difference in the frequency of IFN-$\gamma$-producing CTLs and NK cells among the groups (data not shown).

**NKG2D expression is related to disease severity.** We found that the expression of NKG2D on CTLs and NK and NKT cells was lower in a stepwise manner with an increasing Child-Pugh class ($-0.48$, $P = 0.01$; $-0.43$, $P = 0.02$; and $-0.48$, $P = 0.01$, respectively). We found a similar relationship between lower frequencies of NK cells and a higher MELD score ($-0.37$, $P < 0.05$). At study entry there was no relation between our findings and the patients’ GAHS. In terms of disease progression we found that the change over time in GAHS correlated with the change in CTLs ($-0.58$, $P = 0.04$) but with changes in none of the other measurements. We found parallel relative changes in NKG2D expression (day 30 to diagnosis) on NK cells and CTLs (0.76, $P = 0.002$) and on NKT cells (0.80, $P = 0.001$) and comparing the CTLs with NKT cells (0.52, $P = 0.07$). The parallel relative changes in NKG2D expression

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**Fig. 3. Natural killer group 2 D (NKG2D) expression.** The surface expression of NKG2D was assessed by flow cytometry. The median fluorescence intensity (MFI) of NKG2D on cytotoxic T lymphocytes (CTLs, CD3$^+$CD8$^+$) ($\ast P = 0.01$ and $\ast\ast P = 0.004$) (A), NK cells (CD3$^+$CD56$^+$) ($\ast P = 0.05$ and $\ast\ast P = 0.004$) (B), and NKT cells (CD3$^+$CD56$^+$) ($\ast P = 0.04$ and $\ast\ast P = 0.05$) (C) is shown.

**Fig. 4. CD56 expression on NK and NKT cells.** The expression of CD56 on NK (CD3$^+$CD56$^+$) and NKT (CD3$^+$CD56$^+$) cells was quantified by flow cytometry. MFI of CD56 in the three groups for NK cells ($\ast P = 0.001$ and $\ast\ast P = 0.0001$) and NKT cells ($\ast P = 0.02$ and $\ast\ast P = 0.006$) is shown.
progresses with the disease severity, or is a trait of the disease feature already of early stages of alcoholic liver damage and another question, namely whether immune dysfunction is a (25). Inclusion of a group of patients with less florid alcoholic withdrawal period demonstrate few changes and only ones that studies on immune changes following a 3-mo alcohol with-
alcohol consumers with recent alcohol withdrawal. However, our study, the healthy control group could have been heavy evaluate the effects of long-term alcohol exposure as such in demonstrating correlations with clinical measures. To fully limitations on the degree of detail with which the findings can be interpreted. Additionally, the large interindividual varia-
tional assays we employed are ex vivo and, therefore, exclu-
sively reflect the functional potential of the specific cell subsets analyzed, which may be a limitation. We studied unsorted NK cells, and post hoc calculations of the NK-to-K562 ratio showed the median ratio in AH patients to be approximately twofold higher than in control groups. This seems, however, not to be an important limitation because a recent study showed no difference in CD107a expression within a broad range of effector-to-target ratios (28). Finally, we studied peripheral blood cells because these are accessible in sufficient numbers and are believed to a certain extent to reflect the hepatic environment (45).

The relatively low number of patients we studied imposes limitations on the degree of detail with which the findings can be interpreted. Additionally, the large interindividual vari-
tions in our outcome variables weaken the probability of demonstrating correlations with clinical measures. To fully evaluate the effects of long-term alcohol exposure as such in our study, the healthy control group could have been heavy alcohol consumers with recent alcohol withdrawal. However, studies on immune changes following a 3-mo alcohol withdrawal period demonstrate few changes and only ones that would cause us to underestimatethe immune paralysis in AH (25). Inclusion of a group of patients with less florid alcoholic liver disease, e.g., alcoholic steatosis, might have shed light on another question, namely whether immune dysfunction is a feature already of early stages of alcoholic liver damage and progresses with the disease severity, or is a trait of the disease AH. We cannot answer this question from our patients. However, a recent animal study indicates immune dysfunction even in dietary fatty livers (48). Also, with the progression from alcoholic steatosis to AH both the overall mortality (5-yr mortality; 17 vs. 56%) and the fraction of deaths attributed to infections increase (0.4 vs. 15%), which seems to point mostly at the former possibility (8, 16, 37, 44).

The lymphopenia and decreased CTLs frequency have previously been described, but the reason and significance are unclear (43). The same may occur in decompensated cirrhosis and be ascribed to cell exhaustion and cell death due to chronic inflammation based on high expression of senescence markers on CTLs (30). A similar mechanism is likely in AH due to the florid inflammation. Lymphopenia has been found to be a risk factor for severe infections in systemic lupus erythematosus (32). CTLs and NK cells have long been known to be important for viral clearance, and there is now evidence for a role also in the clearing of bacterial and fungal infections (3, 10, 21, 24, 27). Reduced numbers of these cells may, thus, render patients susceptible to all types of infections, which we also see in this cohort where both bacterial and fungal infections arise.

The markedly impaired NK cell degranulation probably contributes further toward the immune incompetence. The defect appears to be disease specific, since it was neither present in our cirrhosis controls nor is reported in HBV and HCV patients (36). However, there are no previous AH results with which directly to compare our finding. One report on mild AH indicated increased killing efficiency by peripheral blood leukocytes, but both the patient and cell populations were different from ours (26). It is still a possibility that the degranulation defect we describe evolves with the severity of the disease.

The defective degranulation may be related to the decreased activation state with low NKG2D expression that we observed in the NK cells and also in the CTLs and NKT cells. Reduced NKG2D receptor expression may be caused by receptor down-regulation by large amounts of cell-damage fragments acting as ligands (22). This has been directly shown in transgenic mice, and such ligand excess is a feature of nonalcoholic steatohepatitis, a disease with liver cell damage in many ways resembling AH (17, 50).

Expectedly, the reductions in frequency, function, and activation of the cytotoxic effector cells together will lead to a reduced capacity to conquest invading microorganisms and be

Fig. 5. Cytokine production. The cells were stimulated in vitro with phorbol 13-myristate,13-acetate, ionomycin, and brefeldin A for 4 h, followed by intracellular staining for cytokines. The percentage of IL-22-producing CTLs (*P = 0.03) and NK cells (*P = 0.005) is displayed for the three groups.
part of the well-known severe immune incompetence in severe AH. In our cohort, five patients (20%) developed verified infections during the 30-day follow-up period. Within our data structure, these patients were not distinguishable with regard to the cell frequencies or the activation state, but it should be kept in mind that our study was not powered to detect such differences. Thus, we cannot determine whether the observed dysfunctions in cytotoxic effector cells are significant for the immune deficiency and the susceptibility to infections. Nonetheless, the association between decreased cell activation and high disease scores supports that the functionality of CTLs and NK and NKT cells may be important for the disease course. Also, the internal correlation between the changes in NKG2D expression on the different cell subsets supports that the findings, rather than being random, reflect a common underlying mechanism, namely the disease AH. Again, however, the large interindividually variations in our immune measures preclude identification of correlations with the disease development.

The NKG2D receptor is not only central for the NK cells' cytotoxicity but also mediates their proinflammatory cross talk with macrophages and is a potent costimulator of T cell receptor-mediated functions in CTLs (12, 13). Therefore, the downregulation of NKG2D that we demonstrated, although likely initially secondary to liver cell death, at the same time may counteract further liver cell damage by limiting proinflammatory and cytotoxic cell activities. This corresponds to the finding that NKG2D-blocking antibodies prevent the onset of hepatitis in HBV-infected mice (49).

On the other hand, low frequencies of NK cells and decreased expression of NKG2D may also accelerate fibrosis formation due to defective killing of activated stellate cells that express NKG2D ligands (31, 41). Indeed, for an AH patient, the cumulative 10-yr risk of developing cirrhosis is 24% (37). Additionally, we demonstrated an increased median expression of CD56 on both NK and NKT cells. This has not previously been described in AH, but the prevailing interpretation of CD56 could indicate a more regulatory and less cytotoxic profile of these cells in AH (39). This like the decreased NKG2D expression may be beneficial to prevent hepatocyte damage.

Our data thus argue against the hypothesis that CTLs and NK cells are primarily involved in liver cell injury in AH. Rather, we found increased frequencies of IL-22-producing cells, which may be considered beneficial in AH, since IL-22 may also support the deficient NK cell fibrosis control mechanism, namely the disease AH, since IL-22 is a functional marker for the identification of natural killer cell activity. J Immunol Methods 294: 15–22, 2004.


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