On the cell biology of pit cells, the liver-specific NK cells

Luo DZ, Vermijlen D, Ahishali B, Triantis V, Plakoutsi G, Braet F, Vanderkerken K and Wisse E

Subject headings pit cells; hepatic natural killer cells; large granular lymphocytes

INTRODUCTION

Natural killer (NK) cells are functionally defined by their ability to kill certain tumor cells and virus-infected cells without prior sensitization[1]. NK cells comprise about 10% to 15% of lymphocytes in the peripheral blood and most of these cells in human and rat have the morphology of large granular lymphocytes (LGL)[2]. However, recent studies have demonstrated that small agranular lymphocytes, lacking CD3 expression, have cytolytic activity comparable to NK cells[3]. These variations may be related to the stage of NK cell differentiation or heterogeneity[4]. Moreover, some cytotoxic T lymphocytes (CTL) also display LGL differentiation or heterogeneity[4]. Besides NK cells in peripheral blood, NK cells are also found in tissue compartments, such as the spleen, lung, intestine, lymph nodes, bone marrow and liver[4]. NK cells in the liver, also called pit cells[5], constitute a unique, resident NK population in the liver sinusoids. Their immunophenotypical, morphological and functional characteristics are different from blood NK cells[6]. Presently, several names, such as pit cells, hepatic NK cells and LGL are used to describe pit cells, referring to different aspects of their morphology or function[6]. We prefer to use the first-given name of pit cells for these cells in the liver, because it is not related to the ever varying levels of function and morphology or a person (like Kupffer cells)[7]. In addition, liver-associated lymphocytes (LAL), in some instances, are used to describe the total population of lymphoid cells in the liver[7,8]. However, LAL contain about 30% T lymphocytes, 3% B lymphocytes besides 43% pit cells in a human liver washout[9]. Rat liver washouts contain 43% T lymphocytes, 16% of B lymphocytes, 3.3% of monocytes and 26% pit cells[10].

The present review will discuss the biological relevance of pit cells with emphasis on rat liver.

IDENTIFICATION, STRUCTURE AND TISSUE DISTRIBUTION OF PIT CELLS

Pit cells were firstly described in 1976 by Wisse et al[5]. The name pit cell was introduced because of the characteristic cytoplasmic granules, which in Dutch language are called pit, resembling the pits in a grape[5]. The hypothesis that pit cells might possess NK activity was suggested by Kaneda et al[11], because of their morphologic resemblance to LGL. The deve lopment of a method for the isolation and purification of pit cells from rat liver and the evidence of pit cells possessing spontaneous cytotoxicity against NK sensitive YAC-1 lymphoma cells confirmed these cells to be hepatic NK cells[12,13].

Pit cells exist in the liver sinusoids and often adhere to endothelial cells (Ec), although they incidentally contact Kupffer cells (Kc) (Figure 1). They face the blood directly. Pseudopodia of pit cells can penetrate the fenestrae of the Ec, and enter the space of Disse and can directly contact the microvilli of hepatocytes[5,14]. Their appearance in the space of Disse is not a common feature[15]. By morphological investigation, the frequency of pit cells in liver tissue is about an average of 1 pit cell per 10 Kupffer cells. The number of pit cells, in untreated rats, is therefore estimated to be (1.4-2) ×10⁶ cells per gram liver weight[12]. By immunohistoc hemistry, using mAb 3.2.3 against NKR-P1A (a specific marker of NK cells), the
number of pit cells in frozen sections of rat liver is about 13.7 per mm²[16]. After intravenous injection of biological response modifiers (BRM), the number of pit cells increases 4-fold to 6-fold in rat liver treated with zymosan[17] and 43-fold with interleukin-2 (IL-2)[18]. The surplus of pit cells is considered to originate from local proliferation and from the bone marrow[17,18]. Pit cells were found to be more numerous in the perportal than in the pericentral region of the liver lobule[11,16].

Pit cells have essentially the same morphology as NK cells from blood and other organs, i.e. LGL (Figure 1). LGL morphology is characterized by a relatively large size, the presence of granules in the cytoplasm, a pronounced asymmetry of the cell and an indented or kidney shaped nucleus of high density[10]. Pit cells in the rat are about 7 µm in diameter and vary in shape, while possessing well developed pseudopodia. They show a pronounced polarity with an eccentric nucleus and most organelles lying at one side of the nucleus.

The most conspicuous organelles are the electron-dense granules. These granules have several characteristics. They are azurophilic, therefore, Giemsa staining of a cell smear or cytoospin preparation reveals the presence of the granules in pit cells with light microscopic examination. As measured by electron microscopy, the granules differ in size between different pit cell subpopulations (LD and HD pit cells, see next page), but within one cell type the granules are very homogeneous with respect to size, shape and electron density[19]. The granules are membrane bound and range in size between 0.2 µm in LD pit cells and 0.5 µm in LAK cells. These granules contain a number of lysosomal enzymes, such as acid phosphatase[12,20]. Although perforin and granzymes, which have been isolated from NK cell granules[21,22], are not yet been identified in pit cell granules, it is believed by analogy that these molecules are present in the granules of pit cells.

Rod-cored vesicles are small inclusions, ranging in diameter from 0.17 µm to 0.2 µm, and are exclusively found in LGL[11]. They contain a straight rod structure which is 30 nm-50 nm in length, that bridges the entire diameter of the vesicle[11,20]. Rod-cored vesicles derive from and distribute preferentially around the Golgi apparatus. Possibly rod-cored vesicles may also contain cytotoxic factors functioning in natural cytotoxicity[15].

Pit cells also exist in human and mouse liver, but the identification of pit cel ls is in human and mouse liver is more difficult than in rat because they contain a lower number and smaller size of the typical dense granules and very few rod-cored vesicles[9,23,24]. On the other hand, 5% to 25% of human pit cells contain ‘parallel tubular arrays’ (PTA), that were also reported in human blood NK cells and considered as a characteristic of these cells[8,23].

SURFACE PHENOTYPE OF PIT CELLS

Extensive phenotypic analysis has shown that no unique NK cell marker has been identified yet, but expression of a set of differentiation antigens in the absence of antigen-specific receptors of T and B lymphocytes serves to identify NK cells. NKR-P1 was first identified in the rat[25] and has now been shown to be expressed also by mouse and human NK cells[26,27]. NKR-P1 is a type II membrane glycoprotein of the C-type lectin superfamily[28]. The N KR-P1 genes are located on mouse chromosome 6[29], human chromosome 12 p 12-p13[27], and rat chromosome 4 in a region designated as the ‘NK gene complex’ (NKC)[28]. NKR-P1 antigen is present on 94% of rat LGL and serves as a triggering structure on these cells[25]. NKR-P1 is considered to be a useful marker for NK cell identification[25]. However, a subset of T lymphocytes and polymorphonuclear leukocytes also express NKR-P1[25,27]. CD56 and CD16 are expressed, either alone or in combination, on the majority of human NK cells and are most extensively used as human NK cell ‘markers’ for clinical and basic research purposes[22]. Other surface antigens expressed on NK cells are: CD2, CD8, CD11a-c, CD18, CD45, CD54, CD56, CD58 and CD69[2,4,30,31].

Most surface antigens found on rat pit cells are similar to that found on spleen or peripheral blood NK cells (Table 1)[13,16,32]. All LGLs from a rat liver washout, in preparations for light and electron microscopy, were found to express NKR-P1[16] (Figure 2) as recognized by using the monoclonal antibody (mAb) 3.2.3[25]. CD11a is present on 90% of rat pit cells, which is different from rat peripheral blood NK cells (54%)[32]. Approximately 90% of rat pit cells express CD18, 35% express CD54 and 80% express CD2[32]. Asialo-GM1, which is expressed by all rat blood NK cells[19], is present on 36% of LD pit cells and 70% of HD pit cells[13]. CD8, a marker of NK cells and cytotoxic T lymphocytes[2], is present on rat pit cells[13]. However, the composition of CD8 in NK cells and T lymphocyte is different. Most CD8+ NK cells express CD8α/CD8α homodimers rather than the CD8α/CD8α heterodimers prevalent on cytotoxic T cells[3]. In addition, rat pit cells do not express T cell receptor and CD5 antigen (a pan T cell marker)[13,15].

LAL from human liver contain about 35% CD56+ cells, in which three subsets are found: 1) CD3+/CD16, 2) CD3+/CD16, 3) CD3/CD16[40]. Moreover, all the CD56+ LAL express CD11a and CD18, and partly express CD2, CD11b, CD11c, CD54 and CD58[8,34,35].
Table 1  Characteristics of LD, HD pit cells and peripheral blood NK cells

| Item                        | LD pit cell | HD pit cell | Blood NK cell |
|-----------------------------|-------------|-------------|---------------|
| Morphology*                 |             |             |               |
| Size(µm²)                   | 27.7        | 24.5        | 24.8          |
| Rod-cored vesicles per cell | 1.0         | 0.8         | 0.5           |
| Microvilli per cell         | 5.2         | 5.9         | 7.1           |
| Size of granules (µm²)      | Smaller(0.09) | Intermediate(0.1) | Larger(0.14) |
| Number of granules per cell | Higher(50)  | Intermediate(20) | Lower(10)    |
| Surface antigens+           |             |             |               |
| CD2                         | 80          | 80          | 80            |
| CD8                         | 100         | 100         | 40            |
| CD11a                       | 90          | 90          | 90            |
| CD18                        | 90          | 90          | 90            |
| CD54                        | 35          | 35          | 35            |
| Asialo-GM1                  | 36          | 70          | 100           |
| NKR-P1                      | 95          | 95          | 94            |
| Functional features         |             |             |               |
| NK activity                 | High        | Intermediate | Low           |
| P915 cell killing           | Yes         | No          | No            |

*Data from reference 19.
+Approximate % of cells that express antigen; data summarized from references 2, 13, 16, 19, 25, and 32.

Figure 1  Transmission electron micrograph of a pit cell in a rat hepatic sinusoidal lumen (L). The pit cell shows polarity with an eccentric nucleus. The cytoplasm is abundant and contains characteristic electron-dense granules and other organelles lying mainly on one side of the nucleus. The cell contacts an endothelial cell (E) and a portion of a Kupffer cell (K) with a positive peroxidase reaction product in the rough endoplasmic reticulum. Bar = 1 µm. (from Hepatology,1988;8:46-52, with permission)

Figure 2  Immuno-transmission electron micrograph showing a 3.2.3 positive LGL (pit cell) (arrowhead) and a 3.2.3 negative agranular cell. The 3.2.3+ pit cell shows characteristic electron-dense granules in the cytoplasm and immunoperoxidase reaction product on the surface. Bar = 1 µm.(from Hepatology, 1995;21:1690-1694, with permission)

ISOLATION AND PURIFICATION OF PIT CELLS

Our isolation method for rat pit cells is based on a washout technique[12] followed by purification, based on the magnetic negative-selection of cells using mAbs against surface antigens and receptors found on T and B cells[6,36]. Since pit cells are apparently not heavily anchored in the liver sinusoids, the cells could be washed out by this non-enzymatic, high-pressure (50cm water) perfusion of the liver via the portal vein with phosphate-buffered saline supplemented with 0.1% EDTA[12]. The washout was collected from the vena cava. The erythrocytes, granulocytes and cell debris in the washout were removed by Ficoll-Paque gradient centrifugation. The mononuclear cells recovered from the interface of Ficoll-Paque gradient contained T cells, pit cells, B cells, monocytes, and a few Ec and Kc. Adherent monocytes and B cells in this population could be selectively removed in a nylon wool column[12]. Pit cells were further purified by magnetic cell sorting[36]. With this system, a highly purified population of pit cells was obtained by negative selection, i.e. by elimination of remaining monocytes, T and B cells using specific antibodies and immunomagnetic beads. By this method, we obtained pit cells with a purity of more than 90% and a viability of more than 95% (Figure 3). Moreover, this non-enzymatic method does not destroy cell surface molecules.

Alternatively, pit cells could be isolated by enzymatic methods[5]. However, this method is time consuming, labor-intensive and only provides pit cells with 30% purity and 90% viability[6,12].

HETEROGENEITY AND ORIGIN OF PIT CELLS

A considerable set of data indicates that rat pit cells constitute a heterogeneous us population. Based on the cell density, pit cells can be separated into a low density (LD) and high density (HD) fraction by 45% iso-osmotic Percoll gradient centrifugation[19]. These two cell populations have been shown to differ immunophenotypically, morphologically and functionally from each other and from m blood LGL (Table 1)[19,37]. LD pit cells (Figure 4A) contain more rod-cored vesicles and more, but smaller granules than blood NK cells (Figure 4B)[19,37]. The number of rod-cored vesicles and granule composition (number and size) of HD pit cells are intermediate between LD pit cells and blood NK cells[19,37]. Immunophenotypically, almost all blood NK cells are asialo-GM1 positive, and 70% of HD pit cells are strongly positive, whereas only 36 % of LD pit cells are weakly positive[19]. Furthermore, functional differences have been observed among these three populations. The LD pit cells are five to eight times more cytotoxic against YAC-1 cells and
colon carcinoma cells than blood NK cells\textsuperscript{37}. The HD pit cells have intermediate cytotoxic activity between LD pit cells and blood NK cells\textsuperscript{37}. In addition, LD pit cells are able to lyse LAK-sensitive P815 mastocytoma targets, which are resistant to normal blood NK cells and hepatic HD pit cells\textsuperscript{37}

Pit cells are considered to originate from blood NK cells\textsuperscript{38,39}. Several evidences support the concept that blood NK cells immigrate into the hepatic sinusoids to become HD pit cells, which further differentiate into LD pit cells. Importantly, the characteristics and functions of HD pit cells are intermediate between blood NK cells and LD pit cells\textsuperscript{19}. Kinetic experiments with sublethal total body irradiation (700cGy) showed that blood NK cells and HD pit cells were depleted in about one week after irradiation, whereas LD pit cells totally disappeared at two weeks after irradiation\textsuperscript{39}. Shielding of the liver gave similar results and splenectomy did not affect pit cell number\textsuperscript{39}. By using intravenous anti-asialo-GM1 antiserum injection, blood NK and HD pit cells totally disappeared within one week of treatment, whereas LD pit cells disappeared from the liver one week later\textsuperscript{39}. The direct evidence for LD pit cells originating from asialo-GM1 positive precursors (blood NK and HD pit cells) was given by the adoptive transfer of fluorescent-labeled HD pit cells into syngeneic rats\textsuperscript{39}. After three days, 5\% of labeled cells were recovered in the LD fraction and these cells displayed typical LD pit cell morphology\textsuperscript{39}. These observations also indicate that the life span of pit cells in the liver is about two weeks\textsuperscript{39,39}.

The mechanism behind the migration of blood NK cells to the liver sinusoids is not fully understood. Several adhesion molecules were found to be involved in the process\textsuperscript{32}. Rat blood NK and pit cells express LFA-1 (CD11a/CD18) and CD2 (LFA-2) adhesion molecules\textsuperscript{32}. Their ligands, CD54 (ICAM-1) and CD58 (LFA-3) were found to be present on liver Ec\textsuperscript{40}. After intravenous injection of antibodies against CD2, CD11a and CD18 into rats, the number of pit cells in the liver decreased significantly, indicating that the interactions of LFA-1/CD54 and CD2/CD58 are involved in the recruitment of pit cells in the liver\textsuperscript{32}.

Once marginated in the liver sinusoids, blood NK precursors further differentiate into HD pit cells, then into LD pit cells. The microenvironment of the liver sinusoid is believed to be responsible for this differentiation process\textsuperscript{41}. Vanderkerken et al found that Kc were selectively eliminated 3 days after intravenous injection of liposomes containing the cytotoxic drug dichloromethane diphosphonate\textsuperscript{41}. The number of HD pit cells declined 3 days after the injection. Conversely, the LD pit cell population showed no change in number after 3 days, but a decline of about 80\% was seen 7 days after the injection\textsuperscript{41}. These data indicate that pit cells constitute a Kupffer cell-dependent population and that Kc play an essential role in the differentiation of pit cells in the liver. However, it remains unclear what factor(s) secreted by Kc is responsible for this differentiation. On the other hand, other conditions present in the liver microenvironment, i.e. Ec and their secreted factors, may work synergically with Kc to contribute to pit cell differentiation, since coinoculation of HD pit cells with Kc failed to induce the full differentiation of HD into LD pit cells\textsuperscript{41}.

**FUNCTIONS OF PIT CELLS**

NK cells were initially defined as lymphoid cells capable of mediating spontaneous killing of target cells, including tumor and virus-infected cells\textsuperscript{1}. Such NK cytotoxic activity is mediated without prior sensitization and any obvious stimulation or activation\textsuperscript{1}. In addition to this natural spontaneous pathway of tumor killing, NK cells can mediate antibody-dependent cellular cytotoxicity (ADCC) by a mechanism involving CD16, an IgG Fe receptor\textsuperscript{42}. Most human and mouse NK cells express CD16\textsuperscript{2}. Rat NK cells contain genes with a high level of homology to human and murine Fe receptors\textsuperscript{43} and are able to display ADCC\textsuperscript{44}. Unfortunately, no antibodies against rat CD16 are available yet.

Although the cytotoxic function of NK cells is spontaneous, it can be significantly augmented by several cytokines\textsuperscript{2}. One of these, IL-2, has been shown to play a central role in the regulation of NK cells, including augmenting NK cell cytotoxicity, expanding NK cell antitumor spectrum and inducing NK cell proliferation\textsuperscript{10}.

NK cytotoxic (i.e. cytolytic) activity is usually determined by measuring the release of radiolabeled chromium from target cells after being exposed to effector cells\textsuperscript{2}. A new assay using flow cytometry to assess NK cell activity, in which various dyes are used to differentiate viable from dead target cells, has recently been described\textsuperscript{45}. Initial studies have shown that this method is quick, reliable, and correlates well with the standard \textsuperscript{51}Cr release assay\textsuperscript{45}.

Besides the cytotoxic function, NK cells can produce various cytokines\textsuperscript{46}, regulate the growth of hemopoietic tissues and bone marrow transplants\textsuperscript{47}, and participate in the resistance to microbial pathogens\textsuperscript{2}.
Figure 3 Light micrograph of an isolated and purified pit cell population in a May-Grünwald-Giemsa-stained cytospin. The cells contain cytoplasmic granules, which can be used to recognize and count the number of pit cells in freshly isolated liver-associated lymphocyte population. Bar = 5 \( \mu \text{m} \).

Figure 4 Transmission electron micrographs of a typical LD pit cell and a blood NK cell (B).
(A) The main morphological characteristic of LD pit cells, compared to HD cells and blood NK cells, is the presence of numerous small cytoplasmic granules.
(B) Note the few, but large granules in blood NK cell. Bar = 1 \( \mu \text{m} \). (from Hepatology, 1990;12:70-75, with permission)

Most investigations on pit cell functions focus on cytotoxic activity. Rat pit cells have high spontaneous cytotoxic activity against various tumor cell lines, such as YAC-1, P815, CC531s, DHD-K12, L929, 3LL, and 3LL-R[10]. Compared with blood NK cells, pit cells are four to eight times more cytotoxic against YAC-1 and CC531s cells, and are able to kill the NK-resistant but lymphokine activated killer (LAK)-sensitive P815 cells (Figure 5)[19,48]. This evidence seems to support the idea that pit cells become activated once they become liver residents. However, it is not understood yet what kind(s) of factor(s) is (are) responsible for the activation of pit cells in the liver, although it has been shown that pit cells are dependent on the presence of a healthy Kc population[41]. Furthermore, NK activity in the liver could be augmented by BRM, like Propionibacterium acnes or maleic anhydride divinyl ether[24]. Interestingly, an increase in function seems to coincide with a large increase in the number of LGL[17,24]. IL-2 treatment results in a dramatic accumulation of pit cells in the hepatic sinusoids in vivo[18], induces HD cell proliferation and augments liver HD pit cell cytotoxic activity in vitro[49]. In contrast, IL-2 treatment does not induce liver LD pit cell proliferation[49].

MOLECULAR MECHANISMS IN NK CELL-MEDIATED CYTOTOXICITY

It is believed that the cytotoxic function of NK cells is mediated by multiple pathways, and each pathway, in principle, encompasses a cascade of events, including recognition of target cells, binding of effector cells to target cells (conjugation), activation of effector cells, delivery of the lethal signal to target cells, and effector cell detachment and recycling[2,4,50]. Although the precise mechanisms of individual steps in this process have not been fully elucidated, significant progress has been made recently in identifying a number of molecules participating in NK cell-mediated cytotoxicity.

CONJUGATION

The prerequisite of NK cell killing is the binding of one or more effector cells to a target cell, that is, conjugation[6]. Several adhesion molecules on the NK cell, such as CD2, CD28 and LFA-1, and on the target cell, such as CD58, B7 and CD54, participate in this process, and some of them may also possess co-stimulatory or even triggering capacity in the cytotoxic cascade[28,50-52].

CD2 is an adhesion molecule of the immunoglobulin (Ig) superfamily expressed on T cells and NK cells[53]. Approximately 80% of rat pit
cells express CD2. Although CD2 is a well-known activation structure on T cells, mAbs against CD2, depending on experimental conditions, either induce or inhibit NK activity. Anti-CD2 mAb had no effect on the binding of pit cells to rat colon carcinoma cells (CC531s), on the cytotoxicity against CC531s. However, the anti-CD2 mAb enhanced the cytolytic function in rat pit cells against P815 target cells. The ligand of CD2 is another adhesion molecule, CD58 (LFA-3) that is widely expressed on various cell types. Transfection of CD58 into murine cell lines increased the lysis of these targets by some human CD2+ NK cell clones. However, expression of CD58 alone is insufficient to confer cells sensitive to NK cell-mediated lysis, indicating that CD2 may serve as a costimulatory receptor that augments, but not initiates, the primary activation of NK cells.

The interaction between β2 integrins (CD11a-c/CD18) and ICAMs (in tercellular adhesion molecules) has been found to be important in the binding of NK cells to their targets. β2 integrins are heterodimers containing a common β-chain (CD18) and one of three different β-chains (CD11a,CD11b,CD11c). β2 Integrins are expressed only in leukocytes, including NK and pit cells. Besides the effect on the binding to target cells, LFA-1 (CD11a/CD18) also participates in signal transduction in NK cells required for NK cell activation. Cross-linking of LFA-1 on NK cells with its antibody is known to induce a calcium influx, phosphoinositide turnover, tumor necrosis factor-α (TNF-α) production, and to inhibit the target cell killing by NK cells. LFA-1 was also found to be involved in pit cell-mediated cytotoxicity. The antibody against LFA-1 inhibits not only the binding of pit cells to target cells, but also the killing of target cells by pit cells. Taken together, this information suggests that LFA-1 on effector cells may have a dual function of binding to target cells and of triggering cytolysis.

Studies have shown that conjugation between NK cells and target cells is essential but not sufficient for NK activity. After conjugation, further recognition events mediated by triggering and inhibitory receptors on NK cells are required to trigger NK cell cytotoxic activity.

**NK Cell Receptors Involved in MHC Class I Recognition**

NK cell-mediated cytotoxicity was originally thought to be spontaneous and major histocompatibility complex (MHC) class I-unrestricted. However, increasing evidence indicates that NK cells preferentially kill cells lacking MHC class I. Expression of MHC class I on a number of target cells is correlated with target cell resistance to natural killing. Masking of MHC class I by an mAb, enhances pit cell-mediated cytotoxicity against CC531s cells, indicating that MHC class I on CC531s cells protects these cells from being killed by rat pit cells.

An explanation for these observations is, that the cytotoxic activity of NK cells is regulated by positive and negative signals from triggering and inhibitory membrane receptors. The final outcome, i.e. triggering of cytotoxic activity or inhibition of cytotoxicity, appears to depend on the balance between the positive and negative signals. An increasing number of triggering and inhibitory receptors have been described in recent years. Inhibitory receptors on NK cells recognize MHC class I, and these generally inhibit the lysis of MHC class I+ cells. Three receptor families, Ly49, CD94/NKG2 and Killer-cell inhibitory receptors (KIRs), are reported to be involved in the recognition of MHC class I molecules on target cells. The Ly49 family is the product of at least nine highly related genes. Ly49 receptors recognize a trimeric MHC class I complex composed of the H-2D or H-2K heavy chain, β2-microglobulin, and a bound peptide. However, the composition of the bound peptide does not appear to influence the interaction to a large extent. Most, but not all, Ly-49 receptors contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domains. Ly-49 receptors containing the ITIM sequence inhibit NK cell effector function, whereas Ly-49 lacking ITIM, such as Ly-49D and Ly-49H, can activate NK cell-mediated cytotoxicity when the receptor is ligated by ant i-Ly49D mAb.

KIRs, the inhibitory receptors recognizing MHC class I in human NK cells, are monomeric type I glycoproteins that contain Ig domains. They are encoded by genes located on human chromosome 19q13.4. Two subfamilies of KIRs can be identified by the number of Ig-like domains in the extracellular regions of the molecules. The KIR3D subfamily contains three Ig-like domains, whereas the KIR2D contains two Ig-like domains. A remar kable feature of both KIR2D and KIR3D is the heterogeneity in the length of the cytoplasmic domains. KIRs with long cytoplasmic...
domains, i.e. KIR2DL (p58) and KIR3DL (p70), contain two ITIM sequences that are responsible for the inhibitory function of these molecules[67,75]. KIRs containing short cytoplasmic domains, i.e. KIR2DS (p50) and KIR3DS, lack ITIM and potentially activate NK activity[76,77]. Both KIR2D and KIR3D molecules bind to HLA class I trimers, composed of a class I heavy chain, β2 microglobulin, and a bound peptide[28].

In addition to KIRs, human NK cells also express another type of receptor capable of recognizing MHC class I, namely CD94/ NKG2[78-80]. This receptor is a heterodimer and is composed of CD94 glycoprotein that is disulfide-bonded to either a NKG2A or a NKG2C subunit[58]. CD94 and NKG2 genes are pre sent on human chromosome 12p12.3 - p13.1 in the ‘NKC’[81]. Both CD94 and NKG2 molecules belong to the C-type lectin superfamily[81]. CD94 lacks a cytoplasmic domain, thus lacking intrinsic signal transduction capacity[81]. However, CD94 is required for the transport and membrane expression of the NKG2A or NKG2C glycoproteins[78,80]. Since NKG2A possesses an ITIM sequence in the cytoplasmic domain and NKG2C lacks an ITIM, the CD94/NKG2A complex acts as inhibitory receptor, whereas CD/NKG2C complex acts as a noninhibitory receptor for MHC class I on NK cells[28,68].

**Triggering NK cell receptors**

Several membrane molecules are described to serve as triggering receptors on NK cells, including CD16, NKR-P1, NK-TR1, 2B4, P38 and Lag3[28,51,69]. Only CD16 and NKR-P1 can be regarded as ‘established’ triggering receptors, while the role of the others is still undefined or controversial[81]. However, CD16 is responsible and necessary for ADCC and is not involved in natural killing activity[28].

NKR-P1, a marker of NK cells[25], is expressed by rat[25], mouse[82] and human NK cells[27], including pit cells[66]. There are three homologous NKR-P1 genes, NKR-P1A, NKR-P1B and NKR-P1C, in mice and rats[26,82,83], while only one human NKR-P1 gene has been found[27]. MAbs against mouse and rat NKR-P1 were found to trigger NK cell-mediated lysis of FcR+ target cells, termed re-directed ADCC[25]. This action also involves a rise in intracellular Ca2+ levels[84] and cytokine production[65]. Furthermore, mAbs to NKR-P1 stimulate phosphoinositide turnover[64], arachidonic acid generation[60] and granule exocytosis[25]. NKR-P1 on pit cells is involved in pit cell-mediated cytotoxicity against FcR+ P815 target, but not in FcR- CC531s tar get killing[66]. However, the function of NKR-P1 on human NK cells appears more complex. Treatment of human NK cells with anti-NKR-P1 mAb gives controversial results, such as activation, inhibition or no effect, depending on the NK cell population studied[27,87]. The conditions determining the outcome of the engagement of NKR-P1 in human NK cells are not known. When human NKR-P is compared with the corresponding rat and mouse proteins, it was found that all rodent NKR P1 has the CxCP motif that interacts with phosphorylated P56[28], whereas human NKR-P1 lacks this motif[28].

**THE TWO MAJOR PATHWAYS OF NK CELL-MEDIATED CYTOTOXICITY**

CTL and NK cells, including rat pit cells, kill target cells by one of two distinct mechanisms or both: necrosis and apoptosis[19,48,50,89]. Necrosis or cytolysis is characterized by swelling of the cell and organelles, and results in disruption and leakage of the cell membrane and in lysis[6]. Cell membrane damage is a key event in cytolysis and release of the cytoplasmic contents possibly leads to an inflammatory response in vivo[6]. The 51Cr-release assay is thought to reflect this type of damage[6].

Apoptosis or programmed cell death is morphologically recognizable by membrane blebbing, chromatin condensation, nuclear fragmentation, shrinking, condensation of cells and their organelles, and fragmentation of the cells into apoptotic bodies (Figure 6). The cellular remains are phagocytosed by neighboring cells or macrophages. When phagocytosing cells are absent, apoptotic bodies progress to secondary necrosis[6,90].

Recent studies have demonstrated that NK cell-mediated apoptosis can mainly be implemented by two pathways, i.e. the perforin/granzyme (granule exocytosis) pathway and the Fas/FasL (Fas ligand) pathway[91,92]. NK cell-mediated lysis is believed to be mainly based on granule exocytosis[91], whereas Fas-mediated necrosis has been recently reported when caspases are blocked[93].

The Fas pathway of apoptosis is mediated by the interaction of CD95 ligand (CD95 L, FasL) with the apoptosis-inducer CD95 (Fas/APO-1) molecule expressed on target cells[91,94,95]. CD95 is a member of the tumor necrosis factor (TN F) and nerve growth factor (NGF) receptor family[95,96]. CD95 is widely expressed on lymphoid and nonlymphoid tissues, and some tumor cells[95,96]. The expression of CD95 can be up-regulated by interferon γ (IFN-γ) in various cell lines[96,97]. The cytoplasmic tail of CD95 con tains a motif called ‘death domain’, that is essential for transmitting the apoptotic signal[98].
CD95L is a type II transmembrane protein of the TNF family[95]. CD95L is expressed by activated T cells, NK cells and pit cells[89,99,100]. The binding of CD95L to its receptor CD95 induces apoptosis of CD95-bearing cells[94]. It is demonstrated that CD95/CD95L plays an important role in the killing of virus-infected cells and tumor cells by CTL and NK cells[98]. Although CD95 is expressed on CC531s cells and CD95L is expressed on rat pit cells, pit cell-mediated CC531s apoptosis was found to be exclusively implemented by the perforin/granzyme exocytosis pathway[89].

The perforin/granzyme pathway is a Ca^{2+}-dependent pathway and is mediated by the pore-forming protein perforin and granzymes, especially granzyme B, both of which are stored in NK cell granules[92]. After the contact between effector and target cells, perforin and granzymes are released in a directed manner into the intercellular space between these cells. Perforin alone induces lysis without inducing apoptosis, i.e. fragmentation of target cell DNA. Granzymes play a critical role in the rapid induction of DNA fragmentation by CTL, NK cells and pit cells (Figure 7)[89,101]. Entrance of granzymes into target cells is postulated to occur through pores produced in the target cell membrane by perforin. Recent studies have shown that granzyme B is endocytosed by target cells independently of perforin, possibly through saturable high affinity cell surface binding sites. In the absence of perforin, granzyme B shows a cytoplasmic localization. When perforin is added, granzyme B relocalizes to a nuclear position, rapidly inducing apoptosis[102,103]. These data indicate that the cooperation of the two molecules is necessary to induce apoptosis including DNA fragmentation.

**Figure 5** Comparison of cytolysis between rat blood NK, HD and LD pit cells. The ratio of freshly isolated effector cells to target cells was 20:1. The cytolysis was measured in a 4 hour ^{51}Cr-release assay for YAC-1 and P815 cells and a 16 hour ^{51}Cr-release assay for CC531s cells. The data show that LD pit cells are more cytotoxic against YAC-1, P815 and CC531s than HD cells and blood NK cells. Values were means±SD of three to five independent experiments. (Hepatology, 1990;12:70-75, with permission)

**Figure 6** Transmission electron micrograph of an apoptotic CC531s cell (T) coincubated with pit cells (E) for 3 hours. The apoptotic CC531s cell (T) shows vacuolization (large arrowhead), blebbing of the cell surface (small arrowhead), chromatin condensation (thin arrow), and fragmentation of the nucleus (thick arrow). Bar:2µm. (Hepatology, 1999;29:51-56, with permission)

**Figure 7** The involvement of the perforin/granzyme pathway in pit cell-induced CC531s apoptosis. The ratio of freshly isolated pit cells to CC531s cells was 10:1. Apoptosis was measured in a 3 hour DNA fragmentation assay. EGTA is a Ca^{2+} chelator that blocks granule exocytosis and the action of perforin. DCI is a granzyme inhibitor. Z-DEVD-FMK is an inhibitor of caspase 3. These treatments completely inhibit pit cell-induced CC531s apoptosis. Values were mean±SD of three independent experiments. (Hepatology, 1999;29:51-56, with permission)

**SUMMARY**

There is a growing evidence that pit cells are highly active, liver-specific NK cells. Pit cells are located in the liver sinusoids and can be easily isolated and purified by liver sinusoidal lavage and a magnetic separation method. Furthermore, pit cells can be separated into a LD and HD fraction by 45% isosmotic Percoll gradient centrifugation. These two populations are shown to differ morphologically, phenotypically and functionally from each other and from blood NK cells. LD pit cells contain more rod-cored vesicles and more, but smaller granules than blood NK cells although both of them share LGL morphology. Phenotypically, LD cells have a higher expression of LFA-1 and a lower expression of asialo-GM1 molecules compared to blood or spleen NK cells. Functionally, pit cells are more cytotoxic against several tumor cell lines as...
compared to blood NK cells, and are able to kill-NK- resistant but LAK-sensitive P815 cells. These data indicate that pit cells are a kind of naturally activated NK cells and their cytotoxic function is comparable to IL-2 in vitro activated NK cells. The characteristics of HD cells are intermediate between LD pit cells and blood NK cells. Pit cells most probably originate from blood NK cells, although they show mitosis in the liver after certain stimuli. The recruitment of pit cells in the liver is mediated by adhesion molecules. A major challenge is to achieve a better understanding of the mechanisms of pit cell cytotoxicity and the cooperation between pit cells and other cells in the liver, i.e. Kc, Ec and LAL. Moreover, since pit cells are located in a strategic position in the hepatic sinusoids, they represent a first line of cellular defense against metastasizing colon cancer cells. The role of pit cells in a number of liver pathologies deserves more attention.

ACKNOWLEDGEMENTS We thank Carine Seynaeve and Marijke Baekeland for their excellent technical support and Chris Derom for her photographic support.

REFERENCES
1 Trinchieri G. Biology of natural killer cells. Adv Immunol, 1989; 47:187-376
2 Robertson MJ, Ritz J. Biology and clinical relevance of human natural killer cells. Blood, 1990;76:2421-2438
3 Inverardi L, Watson JC, Faud SA, Winkler Pickett RT, Ortaldo JR, Bach FH. CD3 negative “small agranular lymphocytes” are natural killer cells. J Immunol, 1991;146:4048-4052
4 Lotzova E. Definition and functions of natural killer cells. Nat Immun, 1993;12:169-176
5 Wisse E, van’t Noorden JM, van der Meulen J, Daems WTh. The pit cell: description of a new type of cell occurring in rat liver sinusoidal and portal blood. Cell Tissues,1976;173:423-435
6 Wisse E, Luo D, Vermijlen D, Kanellopoulou C, De Zanger R, Kc, Ec and LAL. Large granular lymphocytes or "pit cells" from rat liver: isolation, ultrastructural characterization and natural killer activity. Eur J Immunol, 1987;17:37-42
13 Bouwens L, Wisse E. Immuno-electron microscopic characterization of large granular lymphocytes (natural killer cells) from rat liver. Eur J Immunol, 1987;17:1423-1428
14 Wisse E, De Zanger RB, Charels K, Van Der Smitten P, McCuskey RS. The liver sieve: considerations concerning the structure and function of endothelial fenestratae, the sinusoidal wall and the space of Disse. Hepatology, 1985;5:683-692
15 Bouwens L, Wisse E. Pit cells in the liver. Liver,1992;12:3-9
16 Luo D, Vanderkerken K, Bouwens L, Kuppen PJK, Crebbe E, Wisse E. The number and distribution of hepatic natural killer cells (pit cells) in normal rat liver: an immunohistochemical study. Hepatology,1995;21:1690-1694
17 Bouwens L, Wisse E. Tissue localization and kinetics of pit cells or large granular lymphocytes in the liver of rats treated with biological response modifiers. Hepatology,1988;8:46-52
18 Bouwens L, Marinelli A, Kuppen PJK, Eggemont AMM, Van De Velde C, Wisse E. Electron microscopic observations on the accumulation of large granular lymphocytes (pit cells) and Kupffer cells in the liver of rats treated with continuous infusion of interleukin-2. Hepatology, 1990;12:1356-1370
19 Vanderkerken K, Bouwens L, Wisse E. Characterization of a phenotypically and functionally distinct subset of large granular lymphocytes (pit cells) in rat liver sinusoids. Hepatology,1990;12:70-75
20 Kaneda K. Liver-associated large granular lymphocytes: morphological and functional aspects. Arch Histol Cytol,1989;52:447-459
21 Liu CC, Perussia B, Cohn ZA, Young JDE. Identification and characterization of a pore-forming protein of human peripheral blood natural killer cell lines. J Exp Med,1986;164:2061-2076
22 Kamada MM, Michon J, Ritz J, Holdack J, Serafin WE, Austen KF, MacDermott RP, Stevens RL. Identification of carboxypeptidase and trypsin esterase activities that are complexed to proteoglycans in the secretory granules of human cloned natural killer cells. J Immunol,1989;142:699-615
23 Bouwens L, Brouwer A, Wisse E. Ultrastructure of human hepatic pit cells. In: Wisse E, Knoek DL, Decker K, eds. Cells of the hepatic sinusoid. The Kupffer Cell Foundation, PO Box 5815, 2280 HV Rijwijk, The Netherlands,1989;2:471-476
24 Wiltrout RH, Mathieson BJ, Talmadge JE, Reynolds CW, Zhang SR, Herberman RB, Ortaldo JR. Augmentation of organ-associated natural killer activity by biological response modifiers. Isolation and characterization of large granular lymphocytes from the liver. J Exp Med,1984;159:1431-1449
25 Chambers WH, Vujanovic NL, Deleo AB, Olszowy MW, Herberman RB, Hiserodt JC. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine activated killer cells. J Exp Med, 1989;169:1373-1389
26 Giorda R, Trucco M. Mouse NKR P1:a family of genes selectively coexpressed in adherent lymphokine activated killer cells. J Immunol, 1991;147:1701-1708
27 Lanier LL, Chang C, Philips JH, Human NKR-P1A: a disulfide linked homodimer of the C type lectin superfamily expressed by NK and T lymphocytes. J Immunol,1994;153:2417-2428
28 Lanier LL. NK cell receptors. Annu Rev Immunol,1998;16:359-393
29 Yokoyama WM, Ryan JC, Hunter JJ, Smith HRC, Stark M, Sea- man WE. cDNA cloning of mouse NKR-P1 and genetic linkage with Ly 49: identification of a natural killer cell gene complex on mouse chromosome 6. J Immunol,1991;147:3229-3236
30 Siliciano RF, Pratt JC, Schmidt RE, Ritz J, Reinherz EL. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. Nature, 1985; 317:428-430
31 Timonen T, Gahmberg CG, Patarroyo M. Participation of CD11a/CD18, CD12 and CD2 and RGD binding receptors in endogenous and interleukin-2-stimulated NK activity of CD3 mono- large granular lymphocytes. Int J Cancer,1990;46:1035-1040
32 Luo D, Vanderkerken K, Bouwens L, Kuppen PJK, Baekeland M, Seynaeve C, Wisse E. The role of adhesion molecules in the recruitment of hepatic natural killer cells (pit cells) in rat liver. Liver,1996;4:1475-1480
33 Baume DM, Caligiuri MA, Manley TJ, Daley JF, Ritz J. Differential expression of CD8α and CD8β associated with MHC-restricted or large granular lymphocytes. Cell Immunol, 1990;131:352-365
34 Garcia-Barcina M, Winnock M, Huet S, Dubuisson L, Neaud V, Bidaurrazaga I, Bernard P, Bedin C, Saric J, Bioulac-Sage P, Balabaud C. Expression of cell-adhesion molecules on liver-associated lymphocytes and peripheral blood lymphocytes in patients with benign or malignant liver diseases. In: Knook DL, Wisse E, eds. Cells of the hepatic sinusoid. The Kupffer Cell Foundation, Rijswijk.
antigen (HLA)-C-specific "activatory" or "inhibitory" natural killer cell receptors display highly homologous extracellular domains but differ in their transmembrane and intracytoplasmic portions. J Exp Med, 1996;183:643-650

Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C, Moretta L. Existence of both inhibitory (p58) and activatory (p50) receptor for HLA-C molecules in human natural killer cells. J Exp Med, 1995;182:875-884

Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH. Human NK cell receptors involved in MHC class I recognition are disulfide linked heterodimers of CD94 and NKG2 subunits. J Immunol, 1996;157:4741-4745

Carretero M, Cantoni C, Bellon T, Bottino C, Biassoni R, Rodriguez A, Perez-Villar JI, Moretta L, Moretta A, Lopez-Botet M. The CD94 and NKG2A C-type lectins covalently assemble to form a natural killer cell inhibitory receptor for HLA class I molecules. Eur J Immunol, 1997;27:563-575

Brooks AG, Posch PE, Scorzelli CJ, Borrego F, Coligan JE. NKG2A complexed with CD94 declines a novel inhibitory NK cell receptor. J Exp Med, 1997;185:795-800

Chang C, Rodriguez A, Carretero M, Lopez-Botet M, Phillips JH, Lanier LL. Molecular characterization of human CD94: a type II membrane glycoprotein related to the C-type lectin superfamily. Eur J Immunol, 1995;25:2433-2437

Ryan JC, Turck J, Niemi EC, Yokoyama WM, Seaman WE. Molecular cloning of the NK1.1 antigen, a member of the NKR-P1 family of natural killer cell activation molecules. J Immunol, 1992;149:1631-1635

Giorda R, Rudert WA, Vavassori C, Chambers WH, Hiserodt JC, Trucco M. NKR-P1, a signal transduction molecule on natural killer cells. Science, 1996;249:1298-1300

Ryan JC, Niemi EC, Goldfien RD, Hiserodt JC, Seaman WE. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. J Immunol, 1991;147:3244-3250

Arase H, Arase N, Saito T. Interferon production by natural killer (NK) cells and NK1-1+T cells upon NKR-P1 cross-linking. J Exp Med, 1996;183:2391-2396

Cifone MG, Roncuzzi CJ, Borrego F, Coligan JE. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. J Immunol, 1991;147:3244-3250

Arase H, Arase N, Saito T. Interferon production by natural killer (NK) cells and NK1-1+T cells upon NKR-P1 cross-linking. J Exp Med, 1996;183:2391-2396

Cifone MG, Roncuzzi CJ, Borrego F, Coligan JE. NKR-P1, an activating molecule on rat natural killer cells, stimulates phosphoinositide turnover and a rise in intracellular calcium. J Immunol, 1991;147:3244-3250

Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine activated killer cells. J Immunol, 1996;157:2909-2915

Lee BK, Spelman J, Zhao DY, Olsen KJ, Podack ER. Perforin, Fas ligand, and tumor necrosis factor are the major cytotoxic molecules used by lymphokine activated killer cells. J Immunol, 1996;157:2909-2915

Shresta S, Machov DM, Heusel JW, Russell JH, Ley T. Natural killer and lymphokine-activated killer cells require granzyme B for the rapid induction of apoptosis in susceptible target cells. Proc Natl Acad Sci USA, 1995;92:5679-5683

Jans DA, Jans P, Briggs LJ, Sutton V, Trapani JA. Nuclear transport of granzyme B (fragminin 2). Dependence on perforin for the rapid induction of apoptosis in susceptible target cells. Proc Natl Acad Sci USA, 1995;92:5679-5683

Proofread by Miao QH