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MHC class I expression protects rat colon carcinoma cells from hepatic natural killer cell-mediated apoptosis and cytolysis, by blocking the perforin/granzyme pathway

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Published: 20 November 2002

Received: 28 August 2002

Comparative Hepatology 2002, 1:2

Accepted: 20 November 2002

This article is available from: <http://www.comparative-hepatology.com/content/1/1/2>

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Abstract

Background: Hepatic natural killer (NK) cells, the most cytotoxic cells of the natural occurring NK cells, are located in the liver sinusoids and are thus in a strategic position to kill arriving metastasising tumour cells, like colon carcinoma cells. It is known that major histocompatibility complex (MHC) class I on tumour cells negatively regulates NK cell-mediated cytolysis, but this is found using blood- or spleen-derived NK cells. Therefore, using isolated rat hepatic NK cells and the syngeneic colon carcinoma cell line CC531s, we investigated whether this protective role of MHC class I is also operative in hepatic NK cells, and addressed the mechanism of MHC class I protection.

Results: When MHC class I on CC531s cells was masked by preincubation with monoclonal antibody OX18, hepatic NK cell-mediated cytolysis (⁵¹Cr release) as well as apoptosis (DNA fragmentation, nucleus condensation and fragmentation) increased. When hepatic NK cells were preincubated with the granzyme inhibitor 3,4-dichloroisocoumarin, or when extracellular Ca²⁺ was chelated by ethylene glycol-bis(β-aminoethyl ether)-N, N-tetraacetic acid, the enhanced cytolysis and apoptosis were completely inhibited. The involvement of the perforin/granzyme pathway was confirmed by showing that the enhanced cytolysis was caspase-independent.

Conclusions: MHC class I expression protects CC531s colon carcinoma cells from hepatic NK cell-mediated apoptosis and cytolysis, by blocking the perforin/granzyme pathway.

Background

Natural killer (NK) cells are large granular lymphocytes that have the ability to kill cells without prior sensitisation and therefore play an important role in host defence [1]. NK cell-mediated target cell killing is mainly implemented by two pathways, namely the perforin/granzyme path-

way and the Fas ligand (FasL) pathway [2–5]. In the latter pathway, FasL on effector cells binds Fas present on the target cells which results in oligomerization of Fas and activation of caspase 8. Perforin and granzymes, of which granzyme B is the most potent one, reside in the granules of NK cells and are released by exocytosis after conjuga-

tion between the effector and target cell [4,5]. Inside the cytoplasm of the target cell, granzyme B activates caspase 3 directly [6] or indirectly, via a mitochondrion-dependent pathway [7]. Caspases play an essential role in the execution of apoptosis [6].

NK cells display two types of surface receptors: (i) activation receptors, such as the CD161 molecule that recognises structures on target cells and triggers NK cells to kill; (ii) inhibitory receptors, such as Ly-49 molecules, that recognise target cell MHC class I molecules and inhibit killing by NK cells [8,9]. When MHC class I molecules are absent or expressed in reduced amounts, the NK cells proceed with their attack [10]. The mechanism of MHC class I protection is not fully understood. MHC class I molecules do not block target cell recognition by NK cells [11]. A recent study shows that H-2D^d MHC class I molecules on target cells partially inhibit granzyme A release from mouse Ly-49A⁺ NK cells [12]. However, it is unclear whether such partial inhibition of granzyme A release is sufficient to protect target cells. Moreover, the assay used in the past to detect cytotoxicity by cytolysis is the release of ⁵¹Cr from loaded target cells. A recent study questioned the relevance of the ⁵¹Cr release assay compared to what occurs *in vivo*, whereas the DNA fragmentation assay to measure apoptosis coincided with *in vivo* results [13]. Therefore, it is needed to explore whether the protective role of MHC class I is also operative in apoptosis induced by NK cells.

Compared with NK cells from spleen and peripheral blood, hepatic NK cells, also called pit cells [14], are much more cytotoxic [15,16]. Strategically located in the liver sinusoids, they constitute a first line of cellular defence against invading cancer cells, like colon carcinoma cells [15,17–20]. In this study, using freshly isolated hepatic NK cells and CC531s, a syngeneic Fas ligand-resistant colon carcinoma cell line [21], we (i) demonstrated that MHC class I protects colon carcinoma cells from hepatic NK cell-mediated killing; and (ii) showed the involvement of the perforin/granzyme pathway in the mechanism of MHC class I protection.

Results and Discussion

Protection of target cells from NK cell lysis by expression of MHC class I molecules has been demonstrated in different experimental systems in human [11], mouse [12] and rat [10,22]. In rat, several MHC class I genes have been identified, *i.e.*, RT1.A, RT1.C and RT1.E [23]. It has been shown that transfection of RT1.A and RT1.C protects target cells from lysis by NK cells [10]. However, other data indicate that RT1.A molecules inhibit NK cells, whereas RT1.C region molecules activate natural killing [24,25]. Masking of RT1.A, RT1.C, or both alleles on target cells with allele-specific mAbs, has no effect on lysis by NK cells [26]. In view of these facts, mAb OX18 was chosen to

investigate the mechanism of MHC class I protection of CC531s target cells from hepatic NK cell-mediated killing. It has been found that (i) mAb OX18 binds total rat MHC class I [27], (ii) masking of MHC class I molecules on target cells by mAb OX18 or F(ab')₂ fragments of OX18 enhances the syngeneic target cell cytolysis by rat NK cells [22], and (iii) the enhanced NK cell-mediated target lysis by mAb OX18 is not caused by antibody dependent cellular cytotoxicity (ADCC) [22].

The expression of MHC class I molecules on CC531s cells was examined by flow cytometry. In agreement with a previous study [22], CC531s cells expressed MHC class I molecules (data not shown). The mAb CC52, used as a negative control during functional assays, was shown to bind to CC531s cells, as has also been shown previously [28] (data not shown). When CC531s cells were preincubated with mAb OX18 against MHC class I molecules, the hepatic NK cell-mediated cytolysis against CC531s cells was increased in comparison with the lysis of untreated or control mAb treated tumour cells (Fig. 1A). Similarly, the preincubation of CC531s cells with mAb OX18 increased fragmented DNA (apoptosis) in CC531s cells when coincubated with hepatic NK cells (Fig. 1B). CC531s cells showed the typical morphological characteristics of apoptosis such as nuclear fragmentation, chromatin condensation (Fig. 2A), blebbing, and rounding up (Fig. 2B), when they were coincubated with hepatic NK cells. When CC531s cells were pretreated with mAb OX18, the number of apoptotic CC531s cells increased (Figs. 2C,2D,2G). It has been reported that anti-MHC class I antibody alone can induce apoptosis in cancer cells [29]. In order to address the question whether the enhanced apoptosis and cytolysis in CC531s cells was induced by the binding of mAb OX18, CC531s cells were incubated with mAb OX18 alone. After 3, 24 or 48 hours of incubation, no apoptosis or cytolysis in CC531s cells was observed (data not shown). This is the first time it is shown that, besides cytolysis, MHC class I molecules protect cancer cells from apoptosis induced by NK cells. This is relevant because it has been suggested that *in vitro* assays quantifying effector cell-mediated apoptosis, but not cytolysis, are in accordance with *in vivo* results [13].

To address the mechanism of protection of MHC class I on CC531s cells, we used several approaches to assess the granule exocytosis pathway: granzyme inhibition by DCI, Ca²⁺ chelation by EGTA and caspase inhibition by Z-VAD-FMK.

When hepatic NK cells were preincubated with DCI, a granzyme inhibitor in intact cells [30], hepatic NK cell-mediated CC531s cytolysis was largely inhibited (Fig. 3A) and apoptosis was completely inhibited in the OX18-

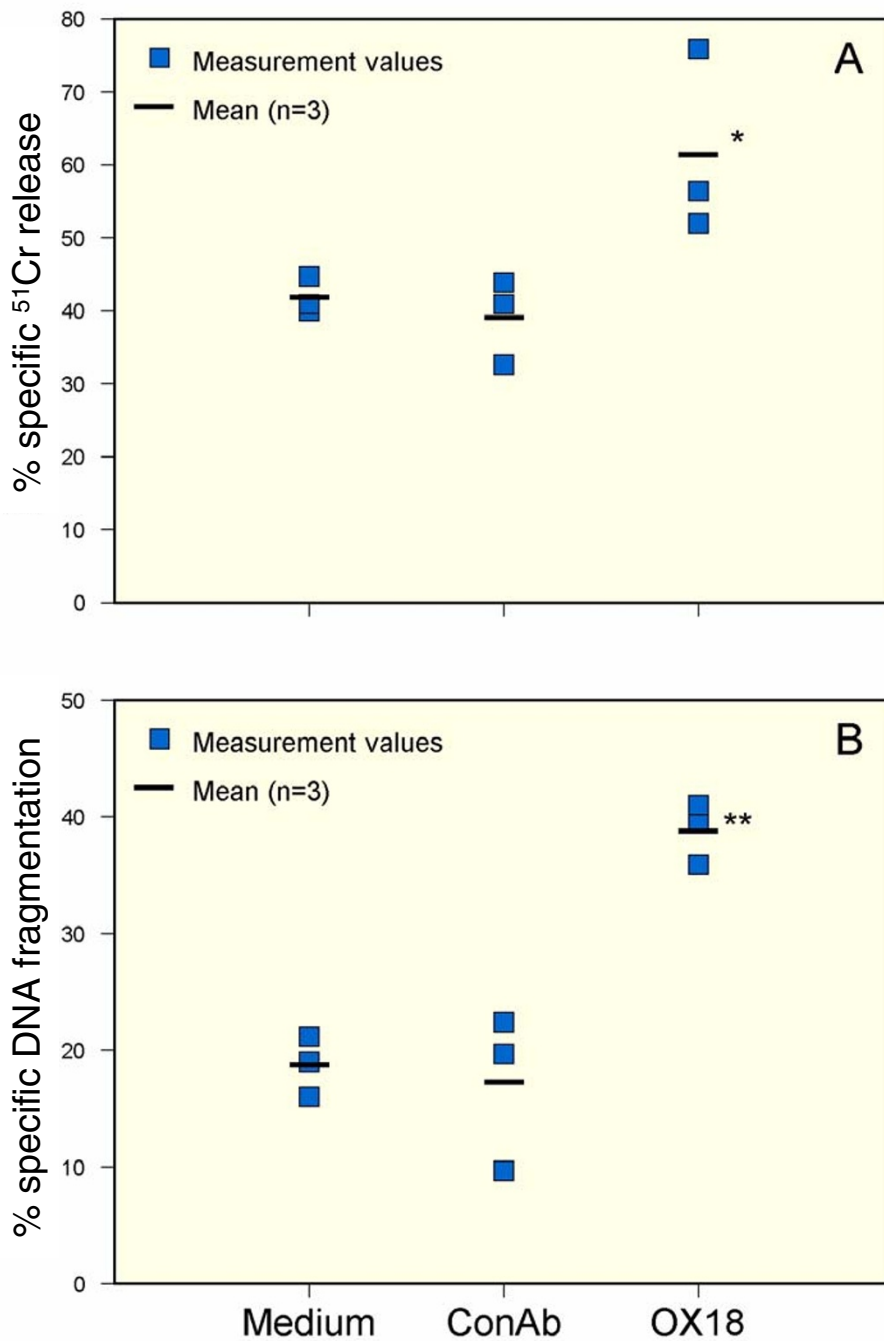


Figure 1
Effect of anti-MHC I mAb OX18 on hepatic NK cell-mediated CC531s cell cytolysis (A, ⁵¹Cr release) and apoptosis (B, DNA fragmentation). (A), ⁵¹Cr-labeled CC531s cells were incubated at an E:T ratio of 10:1 with freshly isolated hepatic NK cells for 18 hours. Cytolysis was measured in a ⁵¹Cr-release assay. (B), [³H]-TdR labeled CC531s cells were incubated at an E:T ratio of 10:1 with hepatic NK cells for 3 hours. Apoptosis was measured in a quantitative DNA fragmentation assay. ConAb, control antibody. *p < 0.05, **p < 0.01 vs. the treatment with medium only (LSD test).

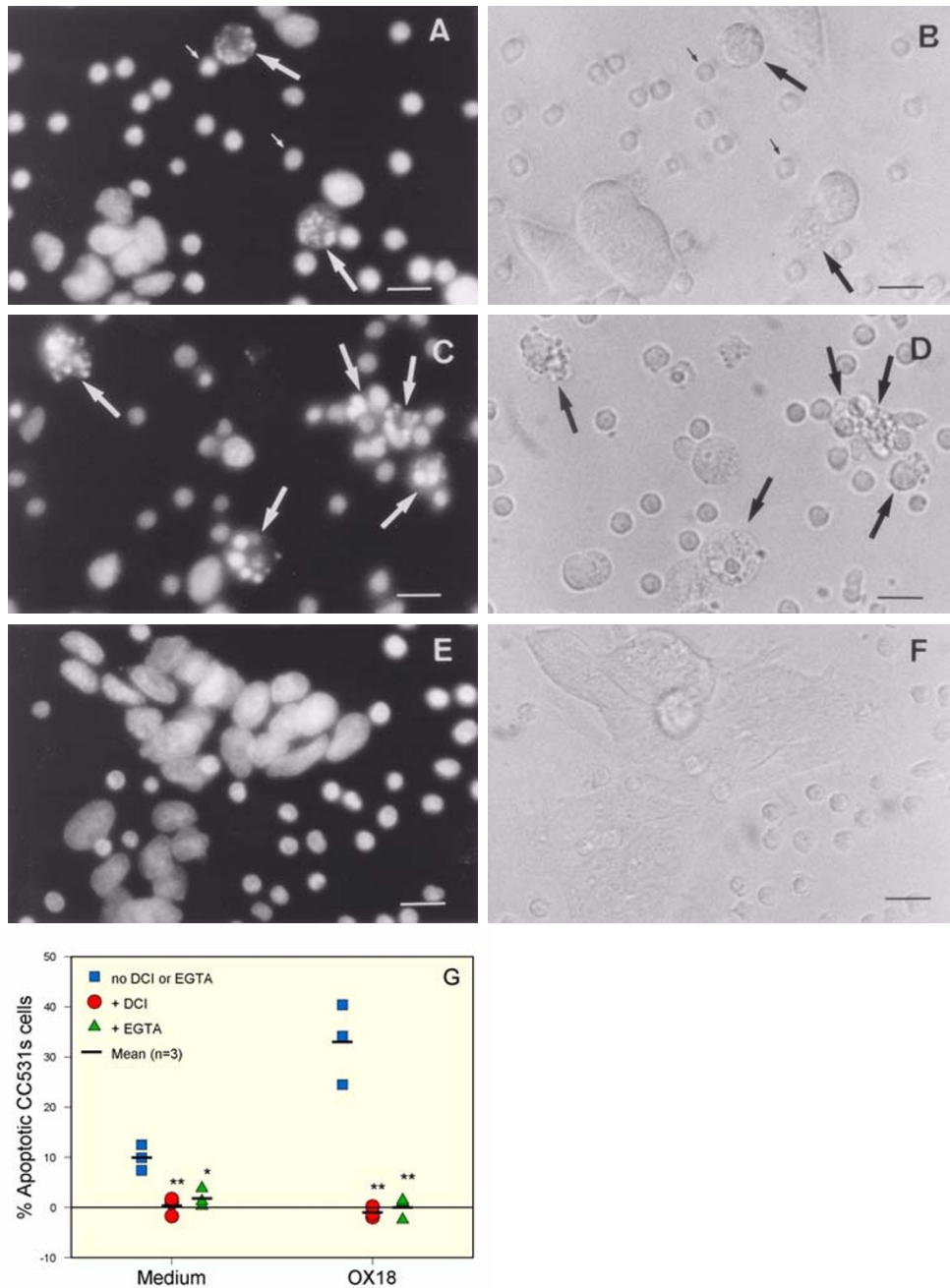


Figure 2
Hepatic NK cell-induced apoptosis in CC531s cells as observed by fluorescence microscopy (A, C, E) and light microscopy (B, D, F). CC531s cells were coincubated with hepatic NK cells at an E:T ratio of 10:1 for 3 hours. Cells were stained with Hoechst 33342/propidium iodide. The thick arrows indicate apoptotic CC531s cells with fragmented nuclei. The small cells are hepatic NK cells (thin arrows indicate examples). (A, B), Coincubation of CC531s cells with hepatic NK cells in medium. (C, D), CC531s cells were pretreated with anti-MHC I mAb OX18. (E, F), CC531s cells were pretreated with anti-MHC I mAb OX18 and hepatic NK cells were pretreated with DCI. The number of apoptotic CC531s cells dramatically decreased. Bar = 10 μ m. (G), The percentage of apoptotic CC531s cells was determined in preparations by counting at least 300 cells per sample. EGTA was present during the coincubation. *p < 0.05, **p < 0.01 vs. the corresponding control (LSD test).

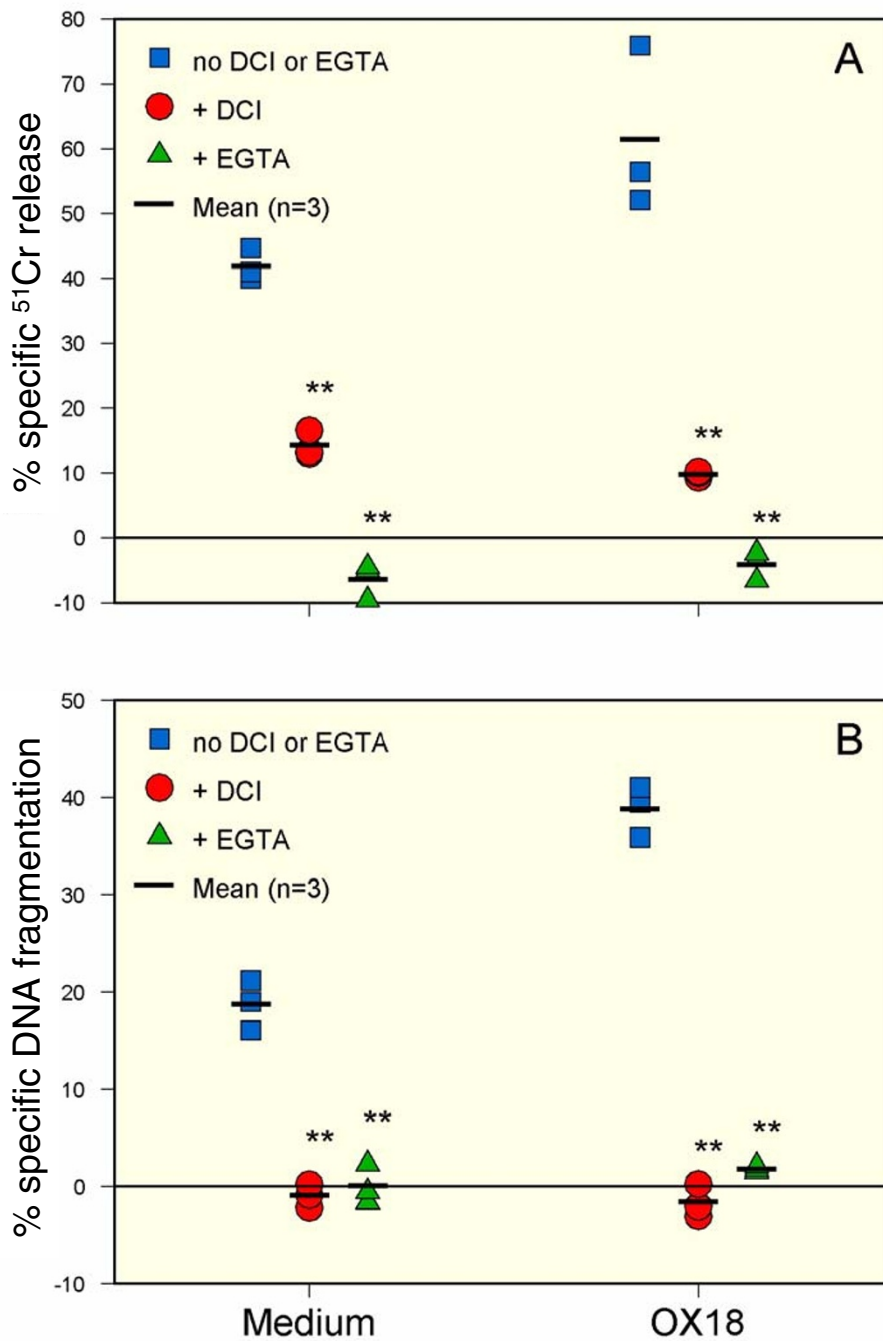


Figure 3
Effect of DCI and EGTA on anti-MHC I mAb OX18 enhanced cytotoxicity (A, ⁵¹Cr release) and apoptosis (B, DNA fragmentation) of CC531s cells by hepatic NK cells. CC531s cells were pretreated with mAb OX18 and hepatic NK cells were pretreated with DCI. EGTA was present during the coincubation. (A), Cytotoxicity was determined by a 18 hour ⁵¹Cr-release assay. (B), Apoptosis was determined by a 3 hour quantitative DNA fragmentation assay. The E:T ratio was 10:1. **p < 0.01 vs. the corresponding control (LSD test).

treated and untreated CC531s cells (Fig. 3B and Figs. 2E,2F and 2G).

The perforin/granzyme pathway is Ca^{2+} -dependent, involving extracellular Ca^{2+} at three distinct steps: (i) granule exocytosis; (ii) binding of secreted perforin to the membrane of target cells; and (iii) perforin polymerisation [5]. When extracellular Ca^{2+} was chelated by EGTA during the co-incubation, the anti-MHC class I mAb-enhanced cytolysis and apoptosis of CC531s cells by hepatic NK cells was completely inhibited (Figs. 2, 3).

The results obtained by granzyme inhibition and Ca^{2+} chelation strongly suggest the involvement of the perforin/granzyme pathway in the anti-MHC I mAb OX18 enhanced apoptosis and cytolysis. In order to verify these results, we made use, in a separate series of experiments, of the pan-caspase inhibitor Z-VAD-FMK. It has been shown that in several apoptotic pathways, including the perforin/granzyme pathway and the FasL pathway, DNA fragmentation is caspase dependent. On the other hand, cytolysis is also caspase dependent in the FasL pathway, but caspase independent in the perforin/granzyme pathway [31–33]. As a consequence, this caspase independent cytolysis induction can be used to characterise the perforin/granzyme pathway. Indeed, when Z-VAD-FMK was present during the coincubation of CC531s cells with hepatic NK cells, the OX18 enhanced apoptosis was completely inhibited, while cytolysis was not inhibited at all (Fig. 4). Fragmentation of the nucleus and condensation of the chromatin were also inhibited (data not shown).

Conclusions

MHC class I expression protects colon carcinoma cells from apoptosis and cytolysis induced by hepatic NK cells, by blocking the perforin/granzyme pathway. This mechanism of immune escape could possibly contribute to the incomplete killing by hepatic NK cells of arriving colon carcinoma cells in the liver sinusoids, resulting in the formation of liver metastases.

Materials and Methods

Isolation and purification of hepatic NK cells

Hepatic NK cells were isolated and purified from 3- to 5-month old male Wag/Rij rats (RT1^u, a Wistar-derived inbred strain, Harlan, The Netherlands) according to the method described before [34]. The purity of the isolated hepatic NK cells was at least 90%, as evaluated by light microscopy using May-Giemsa staining cytopins and by flow cytometric analysis using mAb 3.2.3, which recognises CD161A molecules on the surface of rat NK cells [35]. The viability of the recovered cells was more than 95%, as determined by trypan blue exclusion. The procedures used in this study were approved by the local ethical committee (license no. LA1230212).

Tumour cell line

CC531s, a dimethylhydrazine-induced colon carcinoma of Wag/Rij rats [36], was maintained in culture medium RPMI-1640 (Gibco, Life Technologies, Gent, Belgium), supplemented with 10% fetal calf serum (Eurobiochem, Bierges, Belgium), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and glutamin (0.2 mM) (Gibco, Life Technologies, Gent, Belgium).

Reagents and antibodies

3,4-dichloroisocoumarin (DCI) and ethylene glycol-bis(β -aminoethyl ether)-N, N-tetraacetic acid (EGTA), were purchased from ICN (Asse-Relegem, Belgium). The monoclonal antibody (mAb) OX18 (anti-rat MHC class I, IgG₁) [27] was purchased from ECACC (Porton Down, Salisbury, UK). MAb CC52 (IgG1) [28] was developed in the Department of Surgery and Pathology, Leiden University Medical Center, The Netherlands [37]. Z-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK) was obtained from Bachem (Bubendorf, Switzerland).

Flow cytometry

The expression of MHC class I molecules on the CC531s cells was determined by one-colour flow cytometric analysis, as described previously [38]. Briefly, 0.5×10^6 cells were incubated (30 minutes, 4°C) with the primary antibody OX18. Cells were then washed three times with cold phosphate-buffered saline (PBS), containing 1% bovine serum albumin and 0.02% sodium azide. Subsequently, cells were incubated with FITC-conjugated antimouse IgG₁ (Gilbertsville, PA). After incubation and washing, cells were fixed with 2% paraformaldehyde in PBS and analysed (FACSsort; Becton Dickinson, Mountain View, CA, USA). Isotype-matched irrelevant antibody was used as a negative control.

Quantitative DNA fragmentation assay

DNA fragmentation in the CC531s cells was determined as described previously [20]. In short, [methyl-³H]thymidine ([³H]-TdR) labeled CC531s cells were preincubated with the mAb OX18 (final concentration was 10 $\mu\text{g}/\text{ml}$) for 15 minutes, at room temperature, and freshly isolated hepatic NK cells were preincubated with 50 μM DCI for 30 minutes. It was shown that mAb CC52 binds to CC531s cells [28] and is used in this study as a negative control in the functional assays. The cells were then washed twice. EGTA (final concentration was 5 mM) and Z-VAD-FMK (final concentration 80 μM) were present during coincubation. The hepatic NK cells (10^5 cells in 100 μl) and CC531s cells (10^4 cells in 100 μl) were placed in triplicate in 1.5 ml microcentrifuge tubes. After 3 hours coincubation and centrifugation, the incubation medium was removed from the tubes. Subsequently, the pelleted cells were lysed. The lysates were ultracentrifuged (10,000 g for 15 minutes at 4°C) to separate fragmented DNA from in-

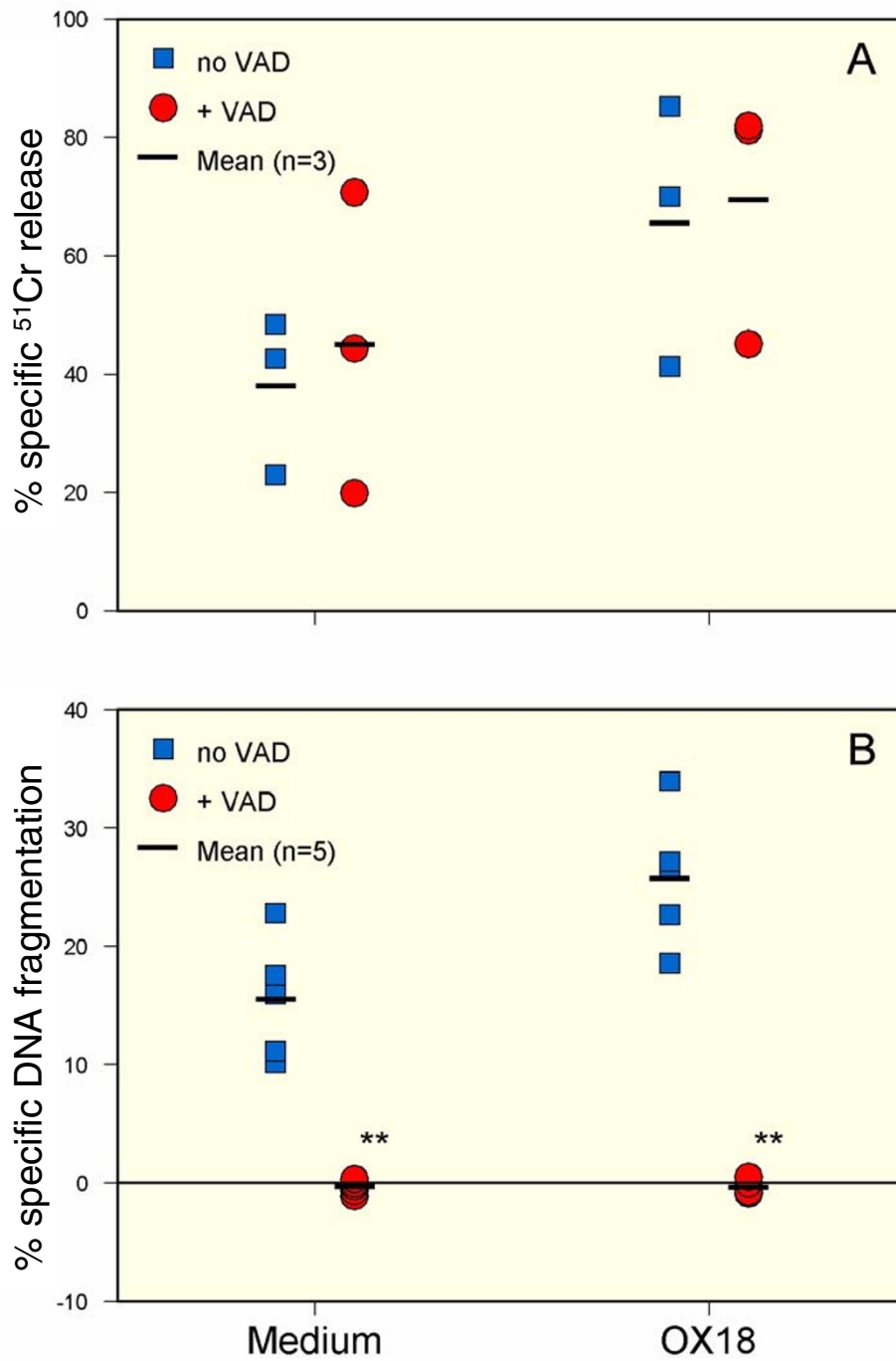


Figure 4
Effect of the pan caspase inhibitor Z-VAD-FMK on anti-MHC I mAb OX18 enhanced cytolysis (A, ⁵¹Cr release) and apoptosis (B, DNA fragmentation). Z-VAD-FMK was present during coinubation. CC531s cells were incubated at an E:T ratio of 10:1 with hepatic NK cells for 18 h (⁵¹Cr release) or 3 h (DNA fragmentation). **p < 0.01 vs. the corresponding control (LSD test).

tact DNA. Radioactivity (cpm) in the incubation medium, in the 10,000 g supernatant and in the 10,000 pellet was determined in a β counter (Beckman, Fullerton, CA, USA). The percentage of fragmented DNA was calculated using the following formula:

$$\% \text{ specific DNA fragmentation} = \frac{\text{cpm}_{\text{fr, exp}} - \text{cpm}_{\text{fr, spont}}}{\text{cpm}_{\text{total}} - \text{cpm}_{\text{fr, spont}}} \times 100\%$$

in which: cpm_{fr} = the radioactivity in the incubation medium plus the cpm in the 10,000 g supernatant; $\text{cpm}_{\text{total}}$ = cpm_{fr} + radioactivity in the 10,000 g pellet; exp = experimental (target cells with effector cells); spont = spontaneous (target cells and medium only).

Hoechst 33342/propidium iodide staining

CC531s cells and freshly isolated hepatic NK cells were treated as mentioned above. CC531s cells (10^4 cells in 100 μl), were coincubated with hepatic NK cells (10^5 cells in 100 μl) in a flat-bottom 96-multiwell plate, for 3 hours at 37°C. After coincubation, nuclei of the cells were stained with Hoechst 33342 and propidium iodide, as described previously [20]. Preparations were studied with a Leica DM IRB/E inverted fluorescence microscope (Leica, Heidelberg, Germany) with ultraviolet excitation, at 340 to 380 nm. The images were recorded and the number of apoptotic CC531s cells was determined by the characteristic morphological changes of the apoptotic nucleus, *i.e.*, condensation of chromatin and nuclear fragmentation. CC531s cells, not coincubated with hepatic NK cells, were used as a control. The fragmented nuclei were counted in at least 300 cells in each preparation and the percentage of apoptotic CC531s cells was calculated using the following formula:

$$\% \text{ specific fragmented nuclei} = \left[\left(\frac{\text{fragmented nuclei}}{\text{total nuclei}} \right)_{\text{EXP}} - \left(\frac{\text{fragmented nuclei}}{\text{total nuclei}} \right)_{\text{CON}} \right] \times 100\%$$

^{51}Cr -release assay

Cytolysis was measured in a 18 hour ^{51}Cr -release assay using 96-multiwell plates, as described previously [15]. Briefly, ^{51}Cr -labeled CC531s cells and hepatic NK cells were treated with the mAb or DCI or EGTA or Z-VAD-FMK, as mentioned above. CC531s cells at a concentration of 10^4 cells/well were coincubated with hepatic NK cells at an effector-to-target (E:T) ratio of 10:1 in a final volume of 200 μl . After 18 hours coincubation, the supernatant of each well was aspirated and radioactivity was determined in a γ counter. The cpm usually ranged from 1200 cpm (spontaneous release) to 5000 cpm (maximal release). Results were expressed as percentage of specific lysis according to the formula:

$$\% \text{ specific cytolysis} = \frac{\text{cpm}_{\text{exp}} - \text{cpm}_{\text{spont}}}{\text{cpm}_{\text{max}} - \text{cpm}_{\text{spont}}} \times 100\%$$

in which: exp = experimental (target cells with effector cells); spont = spontaneous (target cells and medium only); max = maximal, obtained after the addition of SDS (1% final concentration). All data are triplicate determinations.

Statistical analysis

Statistical analysis was performed by one-way ANOVA ($n = 3$ in each group, unless otherwise indicated) with post-hoc multiple comparison analysis made by LSD (the least-significant difference) test, using SPSS statistical package (SPSS Inc., Chicago, IL, USA). Variances were assumed to be homogeneous. Statistically significant difference between two groups was considered at the level of $p < 0.05$.

List of abbreviations used

ADCC, antibody dependent cellular cytotoxicity; cpm, counts per minute; DCI, 3,4-dichloroisocoumarin; EGTA, ethylene glycol-bis(β -aminoethyl ether)-N, N-tetraacetic acid; FasL, Fas ligand; mAb, monoclonal antibody; MHC, major histocompatibility complex; NK, natural killer; Z-VAD-FMK, Z-Val-Ala-Asp(OMe)-fluoromethylketone.

Authors' contributions

D.L. and D.V. designed and carried out the experiments. D.L. drafted the manuscript and D.V. contributed significantly to the text of the manuscript. P.J.K.K. provided the mAbs OX18 and CC52, and contributed to the text of the manuscript. E.W. co-ordinated the study and contributed to the text of the manuscript.

Acknowledgements

Supported by grants G038000 and G000599 from the Fund for Scientific Research – Flanders and grants OZR230 and OZR492 from the Research Council of the Free University of Brussels.

We thank Carine Seynaeve and Marijke Baekeland for their technical help, Karen Crits for her technical assistance in flow cytometry, Chris Derom for her photographic support and Ronald de Zanger for his assistance in statistics.

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