Differential activation of the death receptor pathway in human target cells induced by cytotoxic T lymphocytes showing different kinetics of killing

Jeltje F. de Vries, Peter A. von dem Borne, Simone A.P. van Luxemburg-Heijs, Mirjam H.M. Heemskerk, Roel Willemze, J.H. Frederik Falkenburg, Renèe M.Y. Barge

From the Department of Hematology, Leiden University Medical Center, Leiden, The Netherlands.

Funding: supported by a grant from the Dutch Cancer Society: NKB 99-2122.

Manuscript received January 25, 2007.
Accepted August 31, 2007.

Correspondence:
Jeltje F. de Vries, MSc, Department of Hematology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
E-mail: j.f.vries@lumc.nl

ABSTRACT

Background and Objectives

Cytotoxic T lymphocytes (CTL) may use two effector mechanisms to kill their target cells: perforin (PFN) and granzyme B (GrB)-dependent granule-mediated cell death and death receptor-mediated cell death. Controversy exists whether, in addition to PFN/GrB-mediated apoptosis, death receptor-induced apoptosis contributes to the elimination of human tumor cells by CTL.

Design and Methods

Since the two CTL-mediated effector mechanisms differ in time required to eliminate target cells, lysis of target cells was analyzed using CTL clones with slow and rapid kinetics of killing derived from a patient with chronic myeloid leukemia. To determine the involvement of the death receptor pathway, a retroviral construct encoding the anti-apoptotic gene FLICE inhibitory protein (FLIP) was introduced into these target cells.

Results

A CTL clone capable of killing 50% of the target cells within 2 hours of incubation primarily acted by release of PFN and GrB. In contrast, two CTL clones showing slower target cell killing kinetics partially used the death receptor pathway (~30% inhibition by FLIP).

Interpretation and Conclusions

We demonstrated that the death receptor pathway contributes to T-cell-mediated cell death if not all target cells are destroyed by release of PFN and GrB.

Key words: apoptosis, cytotoxic T lymphocyte, retroviral vector, Fas, FLIP, death receptor pathway.

Haematologica 2007; 92:1671-1678. DOI: 10.3324/haematol.11308

©2007 Ferrata Storti Foundation

Cytotoxic T lymphocytes (CTL) mediate target cell death using two effector pathways: granule-mediated and death receptor-mediated killing.[1,2] Both pathways are activated by membrane T-cell receptors recognizing target antigen. In granule-mediated killing, T-cell receptor-triggering induces exocytosis of pre-formed cytotoxic granules, containing perforin (PFN) and a family of serine proteases termed granzymes,[3,4] of which granzyme B (GrB) is the most potent member. Intracellular delivery of GrB results in the initiation of a caspase cascade via proteolytic activation of caspase-3, directly[5] or through a mitochondrium-dependent pathway.[6] In death receptor-mediated killing, T-cell receptor-triggering induces surface membrane expression of apoptosis inducing ligands of the tumor necrosis factor (TNF) superfamily (Fas ligand (FasL), TNF-α, TRAIL), which cross-link death receptors expressed on the target cells.[1,7] Upon trimerization of the death receptor, Fas associated death domain (FADD) is recruited to the intracellular death domain, followed by engagement of pro-caspase 8, also called FADD-like interleukin-1-converting enzyme (FLICE).[8] In this complex, procaspase-8 is proteolytically cleaved, and the active caspase-8 that is formed either directly cleaves effector caspases resulting in apoptosis, or activates the mitochondrial pathway via cleavage of Bid, a member of the pro-apoptotic Bcl2 family.[9,10]

Granule-mediated cell death has been reported to play an important role in the elimination of virus-infected and tumorigenic cells.[11,12] In line with this, mice with defects in PFN expression develop spontaneous lymphoma and have a diminished ability to clear many viruses and tumor cells.[13] The kinetics of granule-mediated cell death is very rapid, with lytic concentrations of PFN inducing cell death virtually immediately (within 10 min). GrB combined with sublytic concentrations of PFN also results in rapid target cell death (in approximately 90 min), as recently demonstrated in a mouse fibroblast cell line.[14]

Death receptor-mediated cell death has primarily been described to be involved in eliminating autoreactive T cells and down-grading immune responses after infection,[15-18] as clearly demonstrated in various murine studies showing that mice genetically deficient in Fas (*lpr*) or Fas ligand (*gld*) develop lymphoproliferative disorders.[19,20] In other murine studies, Fas/FasL-mediated killing has also been reported to play a role in elimination of virus-infected cells.[21-23] The kinetics of death receptor-induced apoptosis is slow compared to that of PFN-GrB-mediated cell death,[24,25] which was underlined by a study by Shresta *et al.*, who showed in a murine model that apoptosis induced by $GrB^{-/-}$ CTL was delayed for 4 hours compared to lysis by $GrB^{+/+}$ CTL.[26] In the human setting it is largely unknown whether, besides secretion of PFN and GrB, cytotoxic T cells also use the death receptor pathway to kill virus-infected and tumor cells. In the current study, we investigated the role of the death receptor pathway in CTL-mediated cell death of human target cells. Because of the differences in kinetics of cell death between granule-induced and death receptor-induced cell death, we used not only rapidly lysing CTL but also T-cell clones displaying slow kinetics of killing, which were isolated from a patient with chronic myeloid leukemia (CML) at the time of clinical response to donor lymphocyte infusion. We investigated the involvement of the death receptor pathway in CTL-mediated lysis by introducing into EBV-transformed B cells (EBV-LCL target cells) a retroviral construct encoding the anti-apoptotic gene FLICE inhibitory protein (FLIP), which is an enzymatically inactive homolog to caspase 8 that interacts with FADD, preventing pro-caspase 8 from binding to the death domain of the death receptors.[27,28]

Design and Methods

Cells and culture conditions

HY-A1 is a $CD8^+$ HLA-A1-restricted anti-HY T-cell clone isolated from a patient with bone marrow rejection, recognizing a peptide derived from the DFFRY protein.[29] B57-2 and C6-2 are two minor histocompatibility regimens (mHag)-specific $CD8^+$ leukemia-reactive T-cell clones isolated from a patient (JTO) with CML at the time of clinical response to donor lymphocyte infusion.[30] These clones are HLA-B57- and Cw-6-restricted, respectively, and recognize EBV-LCL derived from the patient. The T-cell clones were cultured in IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin, 5% pooled human serum, 5% fetal bovine serum, and 100 IU/mL interleukin-2 (Chiron, Amsterdam, The Netherlands) and stimulated every 2 weeks with a mixture of irradiated allogeneic peripheral blood mononuclear cells, 800 ng/mL phytohemagglutinin (PHA, Murex Biotech Limited, Dartford, UK), and 120 IU/mL interleukin-2.

EBV-LCL were cultured in IMDM supplemented with 10% fetal bovine serum, 3 mM L-glutamine, 50 μg/mL streptomycin and 50 U/mL penicillin (all from Cambrex Bio Science, Verviers, Belgium).

CFSE cytotoxicity assay

Cytotoxicity was measured using carboxyfluorescein diacetate succinimidyl ester (CFSE)-based cytotoxicity assays as described by Jedema *et al.*[31] Target cells were labeled with 5 μM CFSE (Molecular Probes Europe, Leiden, The Netherlands), and incubated overnight in a humidified atmosphere of 5% CO_2 at 37 °C. For the cytotoxicity assay, 5,000 target cells/well (50 μL) were plated in 96-well microtiter plates (all in triplicate), and 5,000 effector T cells were added in a volume of 100 μL/well. After 2, 5 and 24 hours of co-culture, FACS analysis was performed to determine numbers of viable target cells. To exclude dead cells from the analy-

sis, propidium iodide (PI) (1 μg/mL; Sigma-Aldrich, St Louis, MO, USA) was added. To allow quantitative analysis of the viable cells, the wells were harvested, and transferred to FACS tubes containing 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). For each sample 3,000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (PI) $CFSE^+$ target cells. The percentage of specific cell death was defined as:

[(mean absolute number of viable CFSE+ target cells in control medium - absolute number of viable CFSE+ target cells^{experimental})/(mean absolute number of viable CFSE+ target cells in control medium)] x 100.

Activation of the death-receptor pathway

Death-receptor-mediated apoptosis was induced with Fas agonistic antibodies (10 to 1000 ng/mL) that cause cross-linking of the Fas receptor (Fas antibody, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), or with recombinant human TRAIL (rhs*Killer*TRAIL™) (Alexis Corp., Lausanne, Switzerland).

Generation of retroviral constructs and transduction of EBV-LCL cells

The complete coding region of human FLIP-long (U97074) with a FLAG tag in front of the start codon was amplified from plasmid pCR3.V64 (kindly provided by Dr. J.P. Medema, Leiden University Medical Center, Leiden, The Netherlands) by PCR using the forward primer 5'-tatagaagatctaccatggattacaaagacgatgac-3' and the reverse primer 5'-tataccgctcgagttatgtgtaggagag-3'. FLAG-FLIP-encoding PCR products were cloned into the Moloney murine leukemia virus-based retrovirus vector LZRS (G. Nolan, Stanford University, Palo Alto, CA, USA) containing truncated nerve growth factor receptor (ΔNGF-R) as the marker gene.[32] A retroviral pLZRS vector encoding ΔNGF-R alone was used as a control vector (mock) in the experiments. Generation of retroviral supernatant and retroviral transduction of EBV-LCL were performed as previously described.[33] Transduced cells were purified by FACS® sorting based on marker gene expression using a FACSVantage (Becton Dickinson, Mountain View, CA, USA).

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS PAGE) and western blot analysis

Cell lysates of 2×10^6 cells were obtained by freeze-thawing the cells in 100 μL NP40-lysis buffer (50 mM Tris-HCl, pH 7.6, 5 mM DTT, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and western blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described.[34] Primary antibody incubations were performed for 2 hours in 1% Ecl-blocking reagent. Horseradish peroxidase-conjugated antibodies specific for the FLAG epitope tag (Sigma) were used to detect the introduced FLIP (1:1,000).

Statistical analysis

Statistical analysis was performed using a two-paired Student's t test to calculate whether lysis in wild-type EBV-JTO cells differed significantly from that observed in EBV-JTO cells stably expressing FLIP. Differences were considered statistically significant when p values were ≤ 0.05.

Intracellular perforin and granzyme B analysis

Twenty thousand T cells were stimulated for 2 hours with 20,000 specific (EBV-JTO) or non-specific (EBV-JY) target cells. To discriminate between effector and target cells, T cells were stained with CD8 specific antibodies (BD, San José, CA, USA). Cells were fixed and permeabilized using Fix Buffer I and Perm/Wash Buffer I (both from BD), respectively, according to the manufacturer's protocol. The amounts of intracellular PFN and GrB present in the T cells were determined by flow cytometry using PFN (1:20) and GrB (1:20)-specific antibodies (BD). IgG antibodies were used to correct for background staining. The differences in PFN and GrB staining (ΔMFI) after specific and non-specific stimulation of the T cells reflect the amounts of PFN and GrB released by the different T-cell clones within 2 hours of specific stimulation with EBV-JTO.

Results

mHag-restricted CTL clones show different killing kinetics towards the same EBV-LCL

We examined three different CTL clones for their capacity to kill EBV-LCL from patient JTO (EBV-JTO). Percentages of specific lysis of EBV-JTO cells were determined after 2, 5 and 24 hours of co-culture with the T-cell clones using the CFSE-based cytotoxicity assay. CTL clone HY-A1 showed very fast kinetics of killing, resulting in 50% of target cell lysis after 2 hours of incubation and maximal lysis (90%) after 24 hours (Figure 1). CTL clone B57-2 showed 15% of lysis after 2 hours, almost 40% after 5 hours, and 70% after 24 hours of incubation, which we classified as intermediate killing kinetics. CTL clone C6-2 caused no EBV-JTO-specific cell death after 2 and 5 hours, but 50% lysis after overnight incubation, which we regarded as slow killing kinetics.

Kinetics of Fas-mediated cell death of EBV-JTO cells

To study the kinetics of death-receptor-mediated apoptosis in EBV-JTO cells, we performed cytotoxicity experiments in time using various concentrations of Fas antibody or TRAIL to activate the death receptor pathway. The EBV-JTO cells did not respond to various con-

centrations of TRAIL (*data not shown*) suggesting that the target cells do not express the receptor for TRAIL. As shown in Figure 2, in the first 2 hours of exposure to Fas antibody, no lysis was observed. After 5 hours of exposure, high concentrations of Fas antibody (≥500 ng/mL) caused 20-30% apoptosis of EBV-JTO cells, whereas hardly any (0-10%) target cells died in response to low concentrations of the antibody (≤100 ng/mL). Exposure to Fas antibody for 24 hours resulted in 60% apoptosis at high concentrations, and also significant cell death (~40%) at low concentrations (100 ng/mL) (Figure 2). These data show a correlation between the concentration of Fas antibody and the rate of Fas-induced apoptosis.

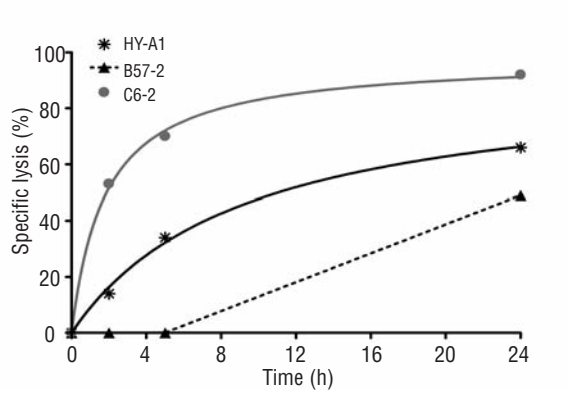

Figure 1. Kinetics of minor histocompatibility antigen-restricted CTL-mediated killing of EBV-JTO cells. EBV-LCL cells from patient JTO (EBV-JTO) were exposed for 2, 5, and 24 hours to the HLA-A1-restricted CTL clone HY-A1, the B57-restricted CTL clone B57-2 and the Cw6-restricted CTL clone C6-2, in an effector:target cell ratio of 1:1. Percentages of specific lysis were determined using CFSE-based cytotoxicity assays, and the mean percentages of three (HY-A1), five (B57-2) and seven (C6-2) independent experiments are indicated in the figure.

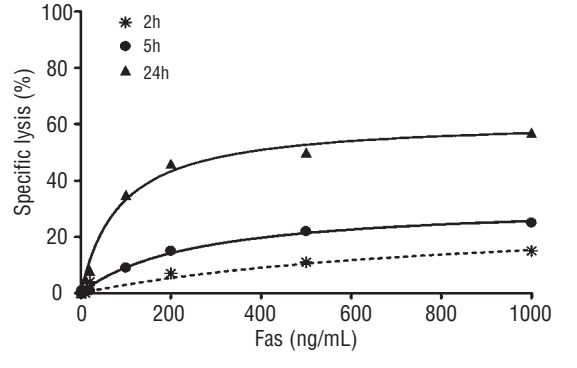

Figure 2. Kinetics of Fas antibody-induced cell death of EBV-JTO cells. Mean percentages of lysis, as determined by CFSE-based or ^{51}Cr-release cytotoxicity assays, obtained in four independent experiments are shown. Percentages of lysis after 2, 5 or 24 hours exposure to various concentrations of Fas antibody (Fas) are represented by dashed black lines, solid black lines, or solid gray lines, respectively.

Elevated FLIP expression efficiently inhibits the death receptor pathway

To study the role of the death-receptor pathway in CTL-mediated apoptosis, we introduced a retroviral construct into EBV-JTO target cells encoding the anti-apoptotic gene FLICE inhibitory protein (FLIP), which specifically blocks death-receptor-induced apoptosis. As a control, target cells were also transduced with an empty vector (mock). Transduced EBV-JTO cells were FACS sorted on the basis of NGFR expression, which resulted in >90% pure populations. Protein expression of the transduced cell lines was determined to verify proper translation of the introduced FLIP-encoding construct, as depicted in Figure 3A.

To test the functionality of the FLIP construct, wild-type, mock- and FLIP-transduced EBV-JTO cell lines were exposed for 5 or 24 hours to Fas antibody, and cell death was determined using the CFSE-based cytotoxicity assay (Figure 3B). Enhanced FLIP expression resulted in approximately 80% inhibition of lysis induced with Fas antibody, both after 5 and 24 hours of exposure, compared to wild type and mock-transduced EBV-JTO cells, illustrating effective inhibition of the death receptor pathway by FLIP.

Involvement of the death-receptor pathway in CTL-mediated killing of EBV-JTO cells

To determine the importance of the death receptor pathway in the execution mechanisms of the different T-cell clones, we tested wild-type, mock and FLIP-

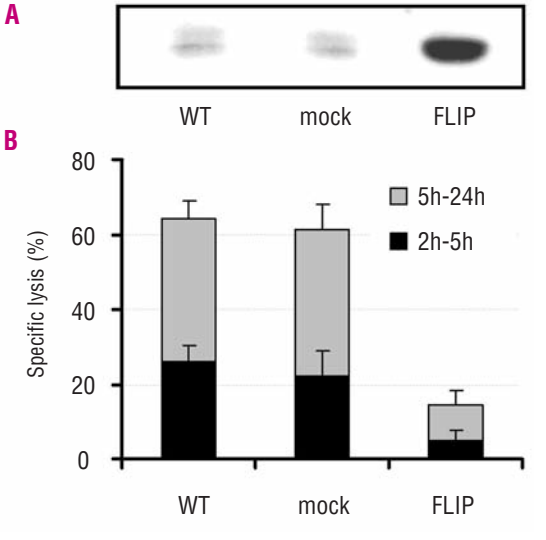

Figure 3. Functionality of EBV-JTO cells transduced with the anti-apoptotic gene FLIP. A. FLIP protein expression of wild-type (WT), mock-, and FLIP transduced EBV-JTO cells, as determined by western blot analysis. B. Effective inhibition of Fas-induced apoptosis in EBV-JTO cells stably expressing FLIP. WT, mock-, and FLIP-transduced EBV-JTO cells were exposed to Fas antibody (500 ng/mL), and cytotoxicity was determined after 5 and 24 hours of exposure using a CFSE-based assay. Mean percentages of lysis (±SD) of three independent experiments are shown.

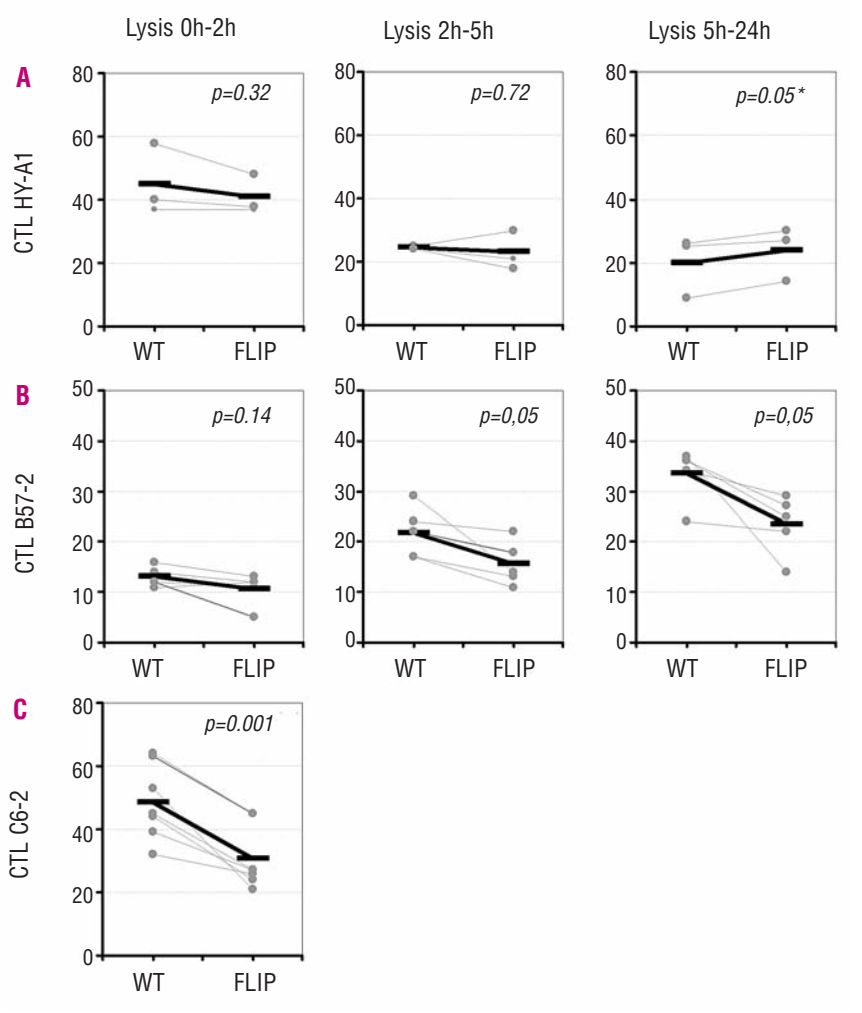

Figure 4. The effect of enhanced FLIP expression on CTL-mediated target cell death. Both wild-type (WT) and EBV-JTO cells stably expressing FLIP were exposed to CTL clones HY-A1 (A), B57-2 (B) and C6-2 (C), in an effector:target cell ratio of 1:1. Percentages of specific lysis were determined after 2, 5 and 24 hours of exposure using CFSE-based cytotoxicity assays. In the figures, lysis is compared between WT and FLIP-expressing cell lines, and is shown for three time-intervals: lysis from 0 to 2 hours, lysis from 2 to 5 hours, and lysis from 5 to 24 hours of exposure. Concerning clone C6-2, lysis in the intervals 0-2 hours and 2-5 hours was negligible and is not shown in the figure. Single experiments are represented in the figure by gray lines (n=3 for HY-A1, n=5 for B57-2 and n=7 for C6-2). Mean percentages of lysis of these independent experiments are indicated by a bold black line. Statistical analysis was performed, and p-values are shown in the figures. Lysis in EBV-JTO-FLIP was significantly lower than lysis in EBV-JTO WT if *p*-values are ≤0.05. *means that lysis was significantly higher in the FLIP-expressing cells than in the WT cells.

expressing EBV-JTO cells for their sensitivity to CTL clones HY-A1, B57-2 and C6-2 (described in Figure 1). Specific lysis was determined after 2, 5 and 24 hours of incubation with the CTL clones. Lysis of the wild-type and mock-transduced EBV-JTO cells was identical (*data not shown*). In our experiments we, therefore, compared the lysis of FLIP-transduced EBV-JTO cells with the lysis of wild type-EBV-JTO cells (Figure 4). Expression of FLIP did not significantly affect the lysis of the EBV-LCL by HY-A1, as shown in Figure 4A. Moreover, the majority of the target cells were killed within 2 hours of exposure, a period in which Fas-mediated apoptosis hardly takes place (Figure 2), suggesting that HY-A1-induced cell death of EBV-JTO is mainly mediated directly by PFN or PFN/GrB. As illustrated in Figure 4B, FLIP did not inhibit B57-2-induced lysis of the EBV-LCL in the first 2 hours of incubation, which correlates with the absence of Fas-induced cell death in this time interval (Figure 2). In the time intervals in which Fas-induced apoptosis theoretically plays a role (based on the results of Figure 2), an inhibitory effect of FLIP was observed. Significantly lower percentages of lysis of the FLIP-expressing EBV-JTO compared to the wild-type EBV-JTO cell line (mean inhibition of 27±19%, p=0.05) were observed between 2 and 5 hours of exposure to CTL clone B57-2. Similar results were found in the time interval from 5 to 24 hours. These data show that the death receptor pathway plays a role in the execution mechanisms used by the CTL clone B57-2. Clone C6-2 was not capable of lysing EBV-JTO cells in the first 5 hours of incubation (Figure 1). In the time interval from 5 to 24 hours, 50% target cell death was observed, which was even higher than that with clone B57-2 in the same time interval (35%). To investigate whether this lysis was caused by activation of the death receptor pathway in the target cell, we tested the effect of FLIP on C6-2-induced cell death after 24 hours of co-culture. As depicted in Figure 4C, inhibition of the death receptor pathway by FLIP resulted in significantly decreased cell death (p=0.001) compared to that of wild-type EBV-JTO cells (mean % inhibition=36±14).

Involvement of PFN/GrB release in CTL-mediated killing of EBV-JTO target cells

To investigate whether CTL HY-A1, which showed very fast kinetics of killing, did indeed rapidly secrete high levels of PFN and/or GrB, staining for intracellular PFN and GrB was performed. The amounts of PFN and

GrB present in the three CTL clones after 2 hours stimulation with an irrelevant target cell (JY) compared to the specific target JTO were determined. We supposed that upon recognition of a specific target cell, the CTL release PFN and GrB, resulting in lower amounts of intracellular PFN and GrB. The differences in PFN and GrB staining (ΔMFI) after specific and non-specific stimulation of the T cells were calculated and are shown in Figure 5A and B, respectively. These ΔMFI values reflect the amounts of PFN and GrB released by the different T-cell clones within 2 hours of specific stimulation with EBV-JTO.

In line with our assumption, we found that intracellular levels of both PFN and GrB in HY-A1 cytotoxic T cells were lower after 2 hours of stimulation with EBV-JTO cells than after to co-culture with EBV-JY cells, suggesting that HY-A1 released both PFN and GrB in this time-interval. Compared to the other two CTL clones HY-A1 secreted the highest amount of PFN, which may explain why JTO cells are killed within 2 hours by HY-A1 but not or barely by B57-2 or C6-2. All three clones secreted remarkable amounts of GrB within 2 hours of co-culture with EBV-JTO, but without PFN this apparently does not lead to apoptosis of the target cell.

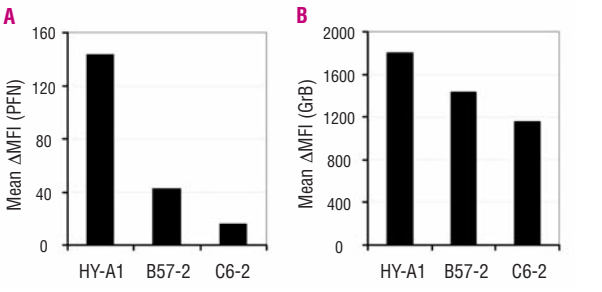

Figure 5. CTL-induced PFN and GrB release. CTL clones HY-A1, B57-2, and C6-2 were stimulated for 2 hours with the specific target EBV-JTO or with an irrelevant target EBV-JY. Intracellular levels of PFN and GrB were determined in the various CD8⁺ T cells by flow cytometry (n=2). MFI values were corrected for background staining using control IgG antibodies. The differences in PFN (A) and GrB (B) staining (ΔMFI) after specific and non-specific stimulation of the T cells were calculated, and are shown in the figures. These ΔMFI values reflect the amounts of PFN and GrB released by the different T-cell clones within 2 hours of specific stimulation with EBV-JTO.

Discussion

In this study, we investigated the role of the death receptor pathway in CTL-mediated death of human target cells. To determine the contribution of this slow execution mechanism, we introduced the anti-apoptotic protein FLIP, a specific inhibitor of the death receptor pathway. We used CTL clones isolated from a patient with CML that showed slow (C6-2, Figure 1), intermediate (B57-2) and fast (HY-A1) kinetics of killing EBV-LCL target cells. Concerning the rapidly lysing CTL clone HY-A1 no involvement of the death receptor pathway could be demonstrated (Figure 4A). Since 50% of target cells were already killed within 2 hours of exposure, a time-interval in which HY-A1 was shown to release PFN and GrB, whereas control experiments indicated that Fas-induced apoptosis did not occur during this time-interval (Figure 2), we argued that this CTL clone mainly acted through secretion of PFN and GrB. In contrast, the EBV-LCL target cells were at least partially killed via the death receptor pathway upon exposure to the other two T-cell clones (B57-2 and C6-2), suggesting that this pathway is important in the execution mechanism of a T cell if not all target cells have been destroyed by release of PFN/GrB.

Although cytotoxic T cells can mediate target cell death via different effector mechanisms, it is still questionable whether CTL clones preferentially use secretion of PFN and GrB or also use death ligands to kill human tumor cells.[11,12,15-18] Since the kinetics of death receptor-induced apoptosis is slow compared to that of PFN-GrB-mediated cell death, we analyzed CTL-mediated target cell death in different time intervals (0-2h, 2-5h, and 5-24h). We could not use the standard ^{51}Chromium (Cr) release assay,[35] because this assay is hampered by spontaneous release of ^{51}Cr, making it impossible to analyze cell death in EBV-LCL for longer than 4-10 hours of incubation. We, therefore, employed a CFSE-based cytotoxicity assay which can be used for longer periods of time. Results obtained with the CFSE assay have been demonstrated to correlate well with those obtained with the conventional ^{51}Cr release assay in 4-h incubations.[31]

To study the contribution of the death receptor pathway in T-cell-mediated target cell death, we introduced the anti-apoptotic gene FLIP into the target cell via retroviral transduction, and demonstrated that enhanced expression of FLIP caused almost complete inhibition of Fas-induced apoptosis (Figure 3B). The inhibitory effect of FLIP was variable when target cell death was induced with the different CTL clones. No inhibition was achieved when EBV-JTO cells were exposed to the very rapidly killing CTL HY-A1. Already 40-50% of target cells were killed within 2 hours of incubation, which coincided with PFN and GrB release by HY-A1 (Figure 5). Although besides PFN also high amounts of GrB were released by CTL HY-A1 after 2 hours of incubation with EBV-JTO, we hypothesize that these target cells may be killed via a PFN-dependent mechanism of cell death, since PFN has been shown to induce direct lysis of the target cell.[36,37] Although most of these studies were performed *in vitro* using non-physiological concentrations, Simon *et al*. illustrated in a murine study, in which CTL that lacked both GrA and GrB were used, the *in vivo* importance of direct PFN-induced lysis.[38] We observed that the death receptor pathway plays at least a partial role in the effector

mechanism of the CTL that killed its EBV-LCL target cells after 5 hours of exposure (B57-2) and of the CTL that were cytotoxic only after 24 hours of incubation (C6-2). This suggests that the death receptor pathway is important at a later time point after T cell-target cell interaction if not all cells have been destroyed by immediate release of PFN/GrB.

Two of the three T-cell clones used in this study (B57-2 and C6-2) were isolated from a patient (JTO) during the clinical response after a second donor lymphocyte infusion.[30] Analysis of the reactivity of the different CTL clones against different subsets of bone marrow cells from the patient revealed that B57-2 lysed only monocytic cells of the patient, whereas C6-2 was capable of lysing both monocytic and mature myeloid cells[30] (*data not shown*). The authors showed that resting B and T cells and immature CD34-positive cells representing bone marrow progenitor cells were not or only marginally recognized by these T-cell clones. The T-cell clone HY-A1 recognized all target cell populations. Since both B57-2 and C6-2 CTL clones were capable of rapidly killing monocytes of patient JTO[30] (*data not shown*), we postulate that the differential kinetics of killing EBV-LCL of the same patient by these two CTL clones may be caused by differences in the strength of interaction between effector and target cells. We hypothesize that Cw6-restricted CTL clones recognize minor antigens highly expressed on monocytes, but poorly expressed on EBV-LCL from the patient resulting in a low avidity interaction between the T-cell receptor and MHC/peptide complex. In the case of a low avidity interaction between the T-cell receptor and MHC/peptide complex, the CTL probably releases only low amounts of PFN and GrB, as illustrated for C6-2 in Figure 5, but may still activate the death receptor pathway, causing slow elimination of the target cell. The strength of triggering of the Fas-receptor on the target cell may determine the kinetics of the apoptosis induction, as was also illustrated by the correlation between the concentration of Fas antibody used to induce target cell death, and the rate of Fas-mediated apoptosis (see Figure 2).

In conclusion, in this study we demonstrate that not only PFN/GrB release but also the death receptor pathway plays a role in the execution mechanism of cytotoxic T cells derived from a patient with chronic myeloid leukemia.

Authors' Contributions

JFdV contributed to the design of the study, performed most of the experiments, analyzed and interpreted the data, and drafted the manuscript. PAvdB contributed to the design of the study and revised the manuscript. SAPvLH provided us with essential experimental tools and contributed to the design of the study. MHMH, JHFF, RW and RMYB contributed to the conception of the study and the interpretation of the data. Furthermore they critically revised the manuscript and their intellectual input was indispensable.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Henkart PA. Lymphocyte-mediated cytotoxicity: two pathways and multiple effector molecules. Immunity 1994;1:343-46.
2. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature 1994; 370:650-2.
3. Bleackley RC, Lobe CG, Duggan B, Ehrman N, Fregeau C, Meier M. The isolation and characterization of a family of serine protease genes Expressed in activated cytotoxic lymphocytes-T. Immunol Rev 1988; 103:5-19.
4. Tschopp J, Nabholz M. Perforin-mediated target-ccell lysis by cytolytic lymphocyte-T. Ann Rev Immunol 1990;8:279-302.
5. Yang XH, Stennicke HR, Wang BK, Green DR, Janicke RU, Srinivasan A, et al. Granzyme B mimics apical caspases - description of a unified pathway for trans-activation of executioner caspase-3 and -7. J Biol Chem 1998;273:34278-83.
6. MacDonald G, Shi LF, Velde CV, Lieberman J, Greenberg AH. Mitochondria-dependent and -independent regulation of granzyme B-induced apoptosis. J Exp Med 1999; 189:131-43.
7. Hanabuchi S, Koyanagi M, Kawasaki A, Shinohara N, Matsuzawa A, Nishimura Y, et al. Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. Proc Natl Acad Sci USA 1994; 91:4930-4.
8. Chen GQ, Goeddel DV. TNF-R1 signaling: a beautiful pathway. Science 2002;296:1634-5.
9. Fulda S, Meyer E, Friesen C, Susin SA, Kroemer G, Debatin KM. Cell type specific involvement of death receptor and mitochondrial pathways in drug-induced apoptosis. Oncogene 2001;20:1063-75.
10. Nagata S. Fas ligand-induced apoptosis. Ann Rev Genet 1999; 33:29-55.
11. Berke G. The Ctls kiss of death. Cell 1995;81:9-12.
12. Kagi D, Hengartner H. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. Curr Opin Immunol 1996;8:472-7.
13. vandenBroek MF, Kagi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK, et al. Decreased tumor surveillance in perforin-deficient mice. J Exp Med 1996; 184:1781-90.
14. Waterhouse NJ, Sutton VR, Sedelies KA, Ciccone A, Jenkins M, Turner SJ, et al. Cytotoxic T lymphocyte-induced killing in the absence of granzymes A and B is unique and distinct from both apoptosis and perforin-dependent lysis. J Cell Biol 2006;173:133-44.
15. Ju ST, Panka DJ, Cui HL, Ettinger R, Elkhatib M, Sherr DH, et al. Fas(Cd95) Fasl interactions required for programmed cell-death after T-cell activation. Nature 1995; 373:444-8.
16. Lynch DH, Ramsdell F, Alderson MR. Fas and Fasl in the homeostatic regulation of immune-responses. Immunol Today 1995;16:569-74.
17. Nagata S, Golstein P. The Fas death factor. Science 1995;267:1449-56.
18. Russell JH. Activation-Induced Death of mature T-cells in the regulation of immune-responses. Curr Opin Immunol 1995;7:382-8.
19. Rieuxlaucat F, Ledeist F, Hivroz C, Roberts IAG, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science 1995;268:1347-9.
20. Watanabefukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992;356:314-7.
21. Smyth MJ, Sedgwick JD. Delayed kinetics of tumor necrosis factor-

mediated bystander lysis by peptide-specific CD8(+) cytotoxic T lymphocytes. Eur J Immunol 1998; 28:4162-9.
22. Topham DJ, Tripp RA, Doherty PC. CD8(+) T cells clear influenza virus by perforin or Fas-dependent processes. J Immunol 1997;159: 5197-200.
23. Zajac AJ, Quinn DG, Cohen PL, Frelinger JA. Fas-dependent CD4(+) cytotoxic T-cell-mediated pathogenesis during virus infection. Proc Natl Acad Sci USA 1996;93:14730-5.
24. Maecker HT, Hedjbeli S, Alzona M, Le PT. Comparison of apoptosis signaling through T cell receptor, Fas, and calcium ionophore. Exp Cell Res 1996;222:95-102.
25. Jedema I, Barge RM, Willemze R, Falkenburg JH. High susceptibility of human leukemic cells to Fas-induced apoptosis is restricted to G1 phase of the cell cycle and can be increased by interferon treatment. Leukemia 2003;17:576-84.
26. Shresta S, Macivor DM, Heusel JW, Russell JH, Ley TJ. 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;92:5679-83.
27. Irmler M, Thome M, Hahne M, Schneider P, Hofmann B, Steiner V, et al. Inhibition of death receptor signals by cellular FLIP. Nature 1997; 388:190-5.
28. Thome M, Schneider P, Hofmann K, Fickenscher H, Meinl E, Neipel F, et al. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. Nature 1997; 386:517-21.
29. Vogt MHJ, de Paus RA, Voogt PJ, Willemze R, Falkenburg JHF. DFFRY codes for a new human male-specific minor transplantation antigen involved in bone marrow graft rejection. Blood 2000;95:1100-5.
30. Von dem Borne PA, Luxemburg-Heijs SAP, Heemskerk MHM, Jedema I, Mulder A, Willemze R, et al. Molecular persistence of chronic myeloid leukemia caused by donor T cells specific for lineage-restricted maturation antigens not recognizing immature progenitor-cells. Leukemia 2006;20:1040-6.
31. Jedema I, van der Werff NM, Barge RM, Willemze R, Falkenburg JH. New CFSE-based assay to determine susceptibility to lysis by cytotoxic T cells of leukemic precursor cells within a heterogeneous target cell population. Blood 2004; 103: 2677-82.
32. Kinsella TM, Nolan GP. Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. Human Gene Therapy 1996;7:1405-13.
33. Heemskerk MHM, de Paus RA, Lurvink EGA, Koning F, Mulder A, Willemze R, et al. Dual HLA class I and class II restricted recognition of alloreactive T lymphocytes mediated by a single T cell receptor complex. Proc Natl Acad Sci USA 2001; 98:6806-11.
34. Veuger MJT, Honders MW, Willemze R, Barge RMY. Deoxycytidine kinase expression and activity in patients with resistant versus sensitive acute myeloid leukemia. Eur J Haematol 2002;69:171-8.
35. Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the Fas/Fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. Blood 2000; 95:2352-5.
36. Liu CC, Persechini PM, Young JDE. Perforin and lymphocyte-mediated cytolysis. Immunol Rev 1995; 146: 145-75.
37. Rochel N, Cowan JA. Negative cooperativity exhibited by the lytic amino-terminal domain of human perforin: implications for perforin-mediated cell lysis. Chem Biol 1996; 3:31-6.
38. Simon MM, Hausmann M, Tran T, Ebnet K, Tschopp J, ThaHla R, Mullbacher A. In vitro- and ex vivo-derived cytolytic leukocytes from granzyme A x B double knockout mice are defective in granule-mediated apoptosis but not lysis of target cells. J Exp Med 1997;186:1781-6.