Review

Perforin-mediated Lysis of Tumor Cells by *Mycobacterium Bovis Bacillus Calmette-Guérin*-activated Killer Cells[1]

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Abstract

Immunotherapy with *Bacillus Calmette-Guérin* (BCG) is clinically established in the treatment of superficial bladder cancer. In our attempt to clarify the underlying immunological mechanism, we could previously show that stimulation of PBMC with BCG leads to the generation of cytotoxic BCG-activated killer (BAK) cells. Among others, these BAK cells as well as lymphokine-activated killer (LAK) cells have been suggested as possible effector cells during BCG therapy. To understand BCG-induced activation of effector lymphocytes more precisely, we investigated the lytic pathways of human BAK cells and compared BAK cell cytotoxicity with LAK cell cytotoxicity.

Perforin and Fas ligand (FasL) are the major cytolytic molecules of cytotoxic lymphocytes. Our results demonstrate that BAK and LAK cells showed an increased expression of perforin and FasL as compared with unstimulated controls. Killing of T-24 bladder tumor as well as Jurkat cells by BAK and LAK cells was predominantly mediated via perforin as demonstrated by a drastically reduced lysis in the presence of concanamycin A and EGTA/MgCl$_2$, respectively. In contrast, lysis (radioactive release assay) and membrane disintegration (Annexin V binding) of both targets by BAK and LAK cells could not be blocked with an inhibitory anti-FasL monoclonal antibody (NOK-1). Nevertheless, T-24 and Jurkat were susceptible to killing by recombinant soluble FasL and by Chinese hamster ovary cells expressing membrane-bound FasL.

We conclude that cellular mediators of BCG effector mechanisms, such as BAK and LAK cells, kill their targets via perforin and independent of the FasL pathway. Because we also found increased numbers of perforin-expressing lymphocytes in patients after BCG therapy, our findings have potential clinical relevance because BCG therapy would not be impaired by FasL resistance of target cells, which recently has been described for some tumors.

Introduction

Intravesical BCG[3] therapy is highly effective in the treatment of superficial bladder cancer (1–3). Although initially the mode of action of this therapy was only poorly understood, our recent knowledge about the immunological mechanisms involved has increased considerably (reviewed in Ref. 4).

In particular, it has been shown that lymphocytes (5) and various cytokines (4, 6–8) are released into the patient's urine after BCG instillation. Immunohistochemical analysis revealed an infiltration of mononuclear cells into the bladder wall (9, 10). Furthermore, *in vivo* therapy studies with bladder tumor-bearing mice suggest the requirement for both CD4$^+$ and CD8$^+$ T cells in BCG-mediated antitumor activity (11, 12). It has become clear that cell-mediated immunity plays a key role (4), and LAK and BAK cells have been proposed as possible effector cells (13–15). However, until now the cytotoxic pathways of these effector cell populations have not been identified.

Perforin and FasL have recently been shown to be the key effector molecules in cell-mediated cytotoxicity (16, 17). In the murine system CTLs lyse their targets via perforin or Fas-FasL interactions (18–21), whereas at least for tumor cell killing, NK cells predominantly use the perforin-dependent pathway (22). Interestingly, alternative lysis via FasL has been reported for human NK cells *in vitro* (23–26).

In our attempt to elucidate the immunological mechanisms responsible for the BCG-induced antitumor effect, we have previously described BAK cells, which were induced by stimulation of human PBMCs with BCG (14). These BAK cells were shown to effectively lyse bladder tumor cells in a MHC-unrestricted manner and differ from LAK cells in thus far as they require CD4$^+$ T cells and monocytes as well as IL-2, IFN-γ, and IL-12 during the stimulation period (14, 27).[4] Whereas LAK cells usually contain a mixture of different cytotoxic lymphocyte subsets, BAK cell activity could be attributed to a small subpopulation of activated lymphocytes, which belong to the CD3$^-$/CD8$^+$/CD56$^+$ NK cell phenotype (28). In

addition, BAK cells but not LAK cells show a reduced lytic activity against normal as compared with malignant urothelial cells (29).

In this study, we examined the lytic pathways of BAK cells and compared killing by BAK cells with LAK cell-mediated lysis. Furthermore, we present preliminary results about the expression of perforin in bladder specimens of patients undergoing BCG therapy.

Materials and Methods

Cell Culture. The bladder tumor cell lines T-24 and J-82 as well as Jurkat were cultured at 37°C and 5% CO_2 in RPMI 1640 (Biochrom, Berlin, Germany) containing 10% FCS, 1% L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. T-24 cells have been described to be TNF-resistant (30–32). CHO-K1 cells were cultured in Ham's F12 containing 10% FCS, 1% L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. In some experiments, T-24 and J-82 were stimulated with 1000 units/ml IFN-γ and 400 units/ml TNF-α (both reagents kindly provided by Dr. Ernst, Research Center Borstel) for 48 h.

Generation of CHOFasL. CHO-K1 cells were transfected with plasmid pBOSHFLD4 (Ref. 33; kindly provided by S. Nagata) using FUGENE 6 (Boehringer Mannheim, Germany) according to the manufacturer's recommendations. pBOSH-FLD4 encodes for a noncleavable mutant of human FasL. After 48 h, cells were harvested and checked for expression of FasL by flow cytometry using anti-FasL mAb NOK-1 (PharMingen, Hamburg, Germany). CHOFasL cell cultures contained about 15–25% of cells expressing FasL (data not shown), and they were not further purified or selected but directly used in subsequent cytotoxicity assays.

Isolation and Stimulation of PBMCs. PBMCs from heparinized blood of healthy human donors were obtained by Ficoll-Paque (Pharmacia, Freiburg, Germany) centrifugation. Cells were adjusted to a concentration of 2×10^6/ml in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 5% human serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Reconstituted lyophilizate of BCG (Connaught substrain, 4×10^4 colony-forming units/ml, Immucyst, kindly provided by Cytochemia, Ihringen, Germany) was added, and the cells were cultured for 7 days in six-well microtiter plates at 37°C and 5% CO_2 to generate BAK cells. LAK cells were generated in parallel with human native IL-2 (200 units/ml; kindly provided by Dr. H. Mohr, Blood Transfusion Service of Lower Saxony, Springe, Germany). Unstimulated cultured PBMCs served as controls.

sFasL ELISA. PBMCs were stimulated with BCG or IL-2 for 7 days. Unstimulated cells served as controls. At day 7, cells were counted and the culture supernatant was harvested. BCG- and IL-2-stimulated cells had a higher cell density than controls, and their culture supernatant was diluted according to the cell density obtained from unstimulated control cells. One hundred µl of each culture supernatant were transferred to a 96-well microtiter plate. The samples were processed using a sFasL sandwich ELISA (Coulter Immunotech, Krefeld, Germany) according to the manufacturer's recommendations.

Cytotoxicity Assay. Cytotoxicity was determined in a L-[^{3}H]methionine release assay as previously described (14). Specific lysis was calculated according to the formula: specific lysis (%) = $100 \times (Exp - Spo)/(Max - Spo)$ where Exp is the experimental release, Spo is the spontaneous release, and Max is the maximum release. All assays were performed in triplicates.

When cell-mediated cytotoxicity was measured in the presence of inhibitory antibodies, we used a standard ^{51}Cr-release assay because this assay can be performed as a long-term 18-h as well as a short-term 4-h test. For the chromium release assay, target cells were labeled with Na_2^{51}CrO$_4$ for 1.5 h at 37°C, washed, and resuspended in 5×10^4 cells/ml. Effector cells, recombinant FasL (Alexis, Grünberg, Germany), or CHOFasL cells were added to a total of 100 µl of target cells and coincubated between 4 and 18 h as indicated in the figure legends. All assays were performed in triplicate, and the specific lysis was determined as described for the methionine release assay.

To inhibit FasL- and perforin-dependent lysis, respectively, anti-FasL mAb NOK-1 (PharMingen, Hamburg, Germany) or CMA (Sigma, Munich, Germany) was added to the culture. To assure effective inhibition, treatment of effector cells with NOK-1 was started 30 min and with CMA 120 min before target cells were added. To inhibit Ca^{2+}-dependent cytotoxicity, EGTA/MgCl$_2$ was added during the coincubation of effector and target cells.

Flow Cytometry. T-24 or J-82 were incubated at 10^6 cells/100 µl with mouse antihuman Fas (clone ZB4) or an isotype control for 30 min at 4°C in the presence of 3% human AB serum. Cells were washed in PBS and subsequently incubated with a FITC-labeled goat-antimouse IgG (Dianova, Hamburg, Germany). After washing with PBS, the cells were analyzed on a FACS StarPlus (Becton Dickinson, Heidelberg, Germany).

For perforin staining, 10^6 BAK and LAK cells were fixed with 3% *p*-formaldehyde and permeabilized by 0.1% saponin and 5% human serum in PBS for 10 min at 4°C. Cells were then stained with 0.5 µg of mouse antihuman perforin (PharMingen, Hamburg, Germany) or an isotype control antibody. After washing in PBS, 1% FCS, and 0.1% saponin, cells were incubated with FITC-labeled goat-antimouse IgG (Dianova, Hamburg, Germany). Analysis was performed on a FACS Calibur (Becton Dickinson, Heidelberg, Germany), and data were processed using WinMDi.

Annexin V-binding Assay. Jurkat cells were harvested, and Annexin V-positive cells were removed by magnetic cell separation using a dead cell removal kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's recommendations. Annexin V-negative Jurkat cells were labeled for 15 min with 5 µM CFSE and washed twice in PBS. Stimulated effector cells (BAK and LAK) at an effector target ratio of 2:1 or recombinant FasL (100 ng/ml) were added. To inhibit FasL-mediated killing, effector cells and sFasL were preincubated with 4 µg/ml NOK-1 or an isotype control antibody for 1 h. After 12 h of coincubation in the presence of 2.5 mM EGTA/MgCl$_2$ and 2 µg/ml NOK-1/isotype control, the cells were harvested. Annexin V-PE (PharMingen, Hamburg, Germany) staining was performed according to the manufacturer's recommendations.

Immunoenzymatic Staining of Bladder Specimens. Cold cup biopsies of bladder urothelium were taken from patients before initiation of BCG therapy or 24 h after the sixth instillation. Cryostat frozen sections were fixed in acetone for 30 min, followed by fixation in chloroform for 30 min. Incubation with mouse antihuman perforin (PharMingen, Hamburg, Germany) was performed for 30 min, and immunostaining was undertaken according to the alkaline phosphatase antialkaline phosphatase method with New Fuchsin development (34). Finally, slides were counterstained with hematoxylin and mounted. Immunostainings were controlled by implementing the secondary reagents alone to confirm specificity or enzyme development alone to rule out endogenous enzyme activities.

Studies involving patient material were performed according to the Declaration of Helsinki and were approved by the Institutional Review Board.

Results

Stimulation of PBMCs with BCG or IL-2 Leads to Increased Expression of Perforin and FasL. Perforin and FasL have been described as the major cytotoxic principles in cell-mediated cytotoxicity (16, 18, 21). We determined the expression of FasL and perforin by unstimulated PBMCs and by PBMCs stimulated with BCG or IL-2. As depicted in Fig. 1*A*, unstimulated cells express low levels of FasL. Expression is induced in BAK and LAK cells. Perforin expression was determined by flow cytometric analyses of PBMCs of three different donors. In unstimulated PBMCs, roughly 10% of the cells stained positive for perforin. After stimulation with BCG, the number of positive cells increased to 15–30%. IL-2 stimulation lead to 40–75% of perforin-positive PBMCs. One representative experiment of three is shown in Fig. 1*B*. Cytotoxicity of unstimulated cells against T-24 bladder tumor targets was low (12% specific lysis), whereas BAK and LAK cells showed a strong induction in cytotoxicity (61% and 94%, respectively; data not shown). Taken together, BAK and LAK cells displayed an increased expression of the cytolytic molecules perforin and FasL and an increased cytolytic activity as compared with unstimulated PBMCs.

Expression of Fas on Bladder Carcinoma Cells. We next investigated the expression of the FasL receptor Fas on the cell surface of T-24 and J82 bladder tumor cells. J-82 showed only very little expression of Fas. In contrast, on T-24, a strong signal was detected by flow cytometry. Fas expression was slightly up-regulated on both cell lines after stimulation with IFN-γ and TNF-α for 48 h (Fig. 2).

Lysis of T-24 by BAK Cells and LAK Cells Is Largely Mediated by Perforin. Because BAK and LAK cells both expressed perforin and FasL and showed an induced cytolytic activity, we analyzed the relative contribution of these two pathways to the cellular cytotoxicity. In this context, CMA has been described as a specific inhibitor of perforin-based lytic activity. It specifically inhibits vacuolar type H^+-ATPases and the function of lytic granules, but at the same time, Fas-based cytotoxicity is not impaired (35, 36). When we coincubated BAK cells and T-24 in the presence of CMA, the specific lysis was almost completely inhibited (Fig. 3*B*). The cytotoxicity of LAK cells was also strongly reduced in the presence of CMA
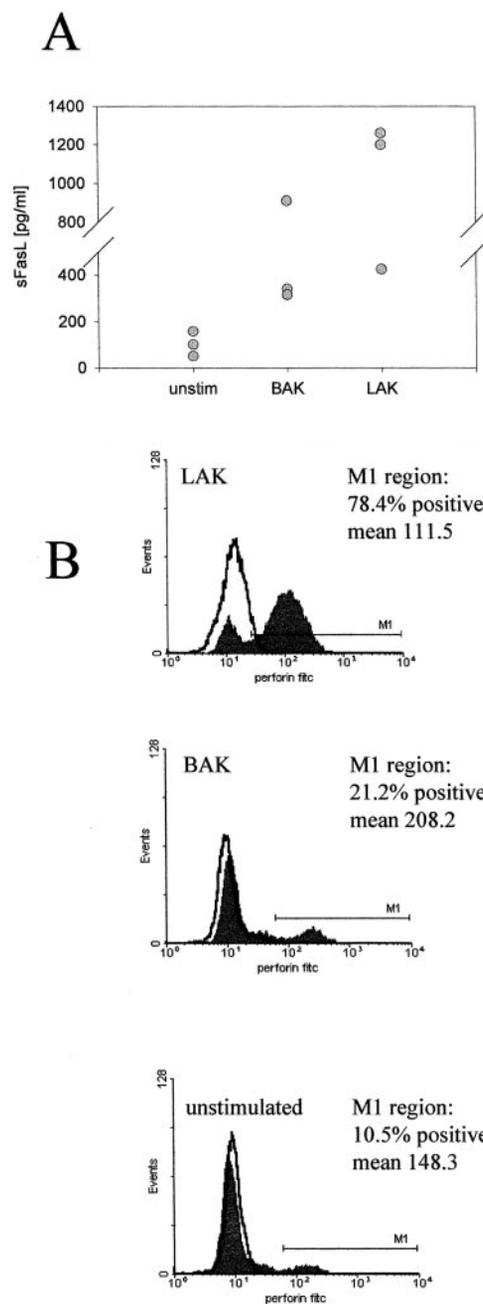

Fig. 1 Induced expression of perforin and sFasL by BAK and LAK cells. PBMCs were stimulated with BCG or IL-2 to generate BAK and LAK cells. *A,* after 7 days, culture supernatant was harvested and the amount of secreted sFasL was determined by a sandwich ELISA. The results of three independent experiments are shown. *B,* PBMCs were fixed, permeabilized, and stained with an antiperforin mAb (*dark histogram*) or an isotype control (*white histogram*) followed by FITC-conjugated goat antimouse IgG. The percentage of positive cells and the mean of positive cells are indicated.

(Fig. 3*A*). Each of these experiments was performed with four different donors, and BAK cell cytotoxicity was always reduced to a specific lysis of ≤10%. Reduction of LAK cell cytotoxicity varied in the range of 30–75% inhibition of the original specific
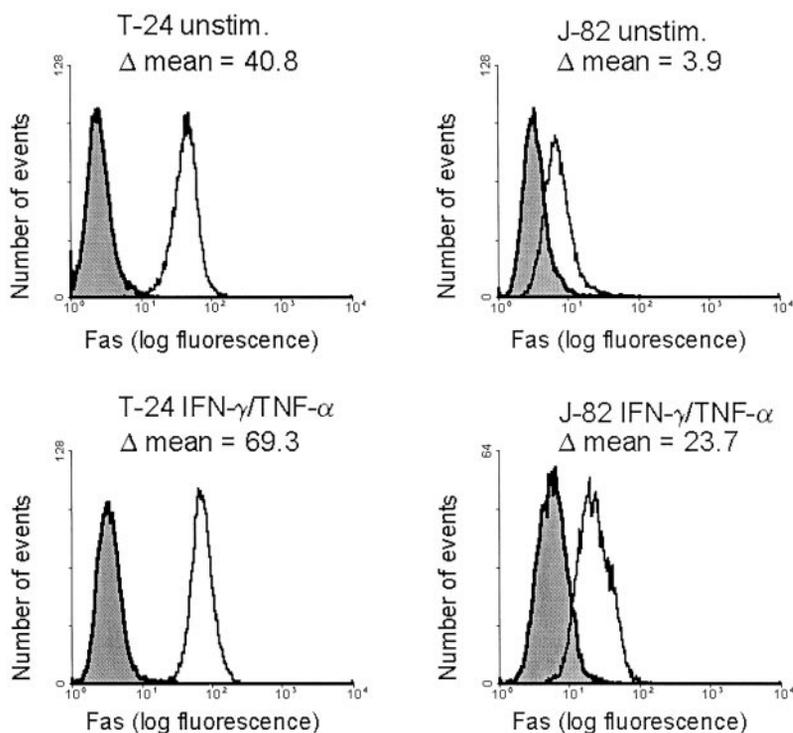
Fig. 2 Expression of Fas by malignant urothelial cells. T-24 and J-82 were cultured with or without 400 units/ml TNF-α and 1000 units/ml IFN-γ for 48 h. Tumor cells were then incubated with mouse anti-Fas antibody ZB-4 (*white histogram*) or an isotype control (*dark histogram*) and subsequently developed with FITC-conjugated goat antimouse IgG. Cell surface expression was determined by flow cytometry with a FACS Calibur (Becton Dickinson). The difference of the mean fluorescence intensity between isotype control and anti-Fas-antibody is shown for each staining. Note the slight increase of Δ mean after cytokine stimulation.

lysis. These experiments suggest that perforin is the predominant pathway used by BAK cells in the killing of T-24 and a major pathway of LAK cells.

To further substantiate these data, cytotoxicity assays were performed in the presence of the calcium-chelator EGTA. Although perforin-mediated killing is Ca^{2+}-dependent, the interaction of FasL and Fas is the only Ca^{2+}-independent killing mechanism (19, 37), and recently it became clear that it is the membrane-bound FasL that is operative in the absence of Ca^{2+} (38, 39). Unstimulated PBMCs showed no cytotoxicity against the bladder tumor cells, whereas BAK and LAK cells effectively killed their targets. However, in the presence of EGTA, the cytotoxicity of both BAK and LAK cells was almost completely inhibited, and only a residual activity was detectable (Fig. 3, *C* and *D*; Fig. 4, *E* and *F*). This confirms the important role for Ca^{2+}-dependent perforin in the killing by BAK and LAK cells and suggests that Ca^{2+}-independent lysis by FasL is of minor importance during the killing process.

Neither BAK nor LAK Cells Kill via FasL. To directly assess the role of FasL-mediated killing, we analyzed target cell lysis in the presence of the inhibitory anti-FasL-antibody NOK-1. This antibody has been described to inhibit not only sFasL (38) but also FasL-based killing in cell-mediated lysis (40, 41). First, we tested whether T-24 cells were susceptible to FasL-based killing. As a control, we used low Fas-expressing J-82 and FasL-susceptible Jurkat cells. T-24 cells were lysed by sFasL (Fig. 5*A*) and CHOFasL (Fig. 5*B*), and killing was comparable with the killing of Jurkat. This killing could be inhibited by anti-FasL mAb NOK-1. Low Fas-expressing J82 cells were neither lysed by sFasL (Fig. 5*A*) nor by CHOFasL (Fig. 5*B*). These data show that T-24 and Jurkat but not J-82 cells are

susceptible to FasL-mediated lysis and that NOK-1 specifically inhibits killing via sFasL and membrane-bound FasL. However, killing of T-24 and Jurkat by BAK and LAK cells was not inhibited by NOK-1 (Fig. 4, *A–D*), suggesting that FasL is not involved in the killing process. To exclude the possibility that the effector cells switch to an alternative lytic pathway (*e.g.,* perforin) in a situation where FasL-mediated lysis is inhibited by NOK-1, we also added NOK-1 in the presence of 5 mM EGTA. In this experimental setup, which excludes a switch from FasL- to perforin-mediated killing, no reduction of specific lysis by anti-FasL mAb NOK-1 was found (Fig. 4, *E–H*). With J-82 target cells, we obtained similar results as obtained with T-24. J-82 cells were not killed by unstimulated PBMCs but readily lysed by BAK and LAK cells, and killing of J-82 could not be inhibited by addition of NOK-1 (data not shown).

As shown in Fig. 2, Fas expression of bladder tumor cell lines could be slightly up-regulated after stimulation with IFN-γ and TNF-α. However, stimulation with these cytokines for up to 48 h did not increase susceptibility to killing by sFasL. In addition, lysis of cytokine-stimulated T-24 and J-82 by BAK and LAK cells could not be inhibited by mAb NOK-1 (data not shown).

To further confirm the exclusion of FasL-mediated killing by BAK and LAK cells, we looked into the induction of cell membrane alterations induced by the effector cells. Beginning at the early stages of apoptosis, phosphatidylserine is translocated from the inner part of the plasma membrane to the outer layer. Annexin V is a phospholipid-binding protein with high affinity to phosphatidylserine and can be used to detect loss of cell membrane integrity (42) of apoptotic and necrotic cells. We determined Annexin V binding of fluorescently labeled Jurkat
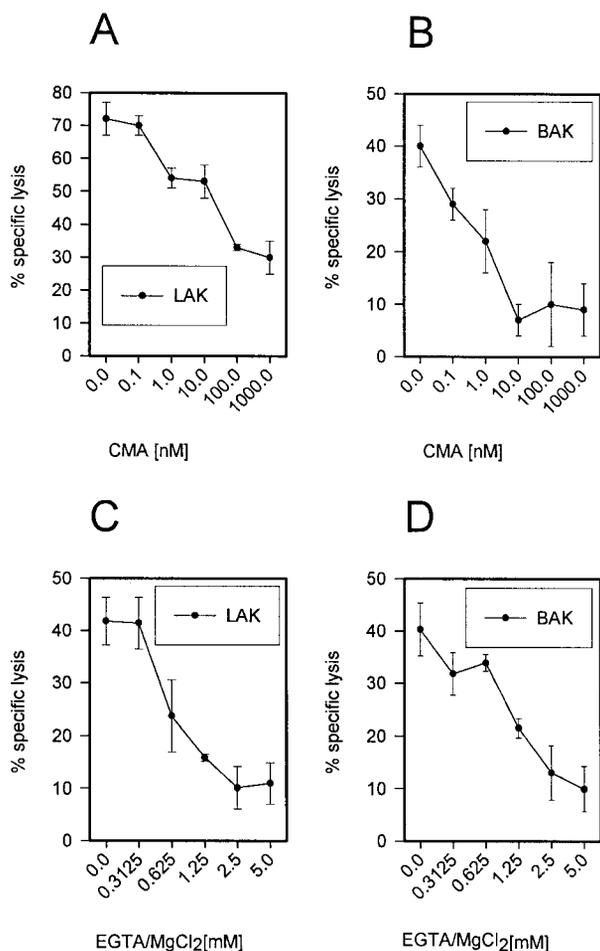
Fig. 3 Cytolytic activity of BAK and LAK cells against T-24 is Ca^{2+}-dependent and perforin-dependent. PBMCs were stimulated with IL-2 (*A* and *C*) or BCG (*B* and *D*) for 7 days. Cytolytic activity against T-24 bladder carcinoma cells was tested in a 20-h methionine release assay in the presence of increasing amounts of CMA (*A* and *B*) and EGTA (*C* and *D*), respectively. The E:T ratio was 40:1, experiments were done in triplicate, and results are shown as mean ± SD. Specific lysis of unstimulated cells was <10%.

after coincubation with recombinant FasL (positive control) and unlabeled effector cells, respectively. After coincubation of Jurkat with 100 ng/ml recombinant FasL for 12 h, essentially all target cells stained positive for Annexin V (Fig. 6*A*). By the addition of anti-FasL NOK-1, the amount of dead cells was strongly reduced (Fig. 6*B*). A marked cytotoxicity (66.7% and 78.8% Annexin V-positive cells, respectively) could be observed after coincubation of CFSE-labeled Jurkat with unlabeled BAK or LAK cells (Fig. 6, *C* and *E*). However, in the presence of inhibitory anti-FasL mAb, the number of compromised target cells remained unchanged (Fig. 6, *D* and *F*). Finally, this notion was further confirmed by our observation that an inhibitory antibody to Fas (clone ZB4) also had no impact on the killing of Jurkat by both effector cell populations (data not shown). Therefore, several lines of evidence suggest that human PBMCs stimulated with BCG or IL-2 for 7 days do not exert

cytotoxicity via FasL irrespective of the presence of Fas on the target cell surface.

Perforin Is Expressed in the Human Urothelium after BCG Immunotherapy. After we had found perforin to be the major cytolytic mechanism of BCG-stimulated human PBMCs *in vitro*, we analyzed the distribution of perforin-positive lymphocytes in the bladder wall of patients. Therefore, perforin expression before and after BCG immunotherapy was assessed by immunohistochemistry. In normal, untreated bladder specimens, only very few perforin-positive cells were detected (Fig. 7*A*). These cells were located in the suburothelial stroma but not in the upper urothelial layers. After BCG immunotherapy, a higher number of perforin-positive cells was present, and these cells were primarily located in the urothelium (Fig. 7*B*). These findings indicate that perforin is expressed in human bladder tissue and therefore BCG-activated perforin-positive lymphocytes could serve as effector cells in BCG-immunotherapy.

Discussion

Since the first report on the treatment of recurrent superficial bladder cancer with BCG (1), clinical trials have confirmed that BCG is an extremely effective biological response modifier in the treatment of this tumor (2, 3). Therefore, during the last years, efforts increased to elucidate the immunological mechanisms underlying this therapy. BCG and BCG-induced cytokines have been proposed as inducers of lymphocyte-mediated cytotoxicity against bladder tumor cells (14, 15, 27, 43, 44).

We have previously shown that PBMCs stimulated with BCG or IL-2 effectively kill bladder tumor cells *in vitro*. Whereas LAK cells can be induced by IL-2 alone, BAK cells require additional cytokines (*e.g.,* IFN-γ) as well as monocytes and $CD4^+$ T cells during the stimulation period (14, 27). Furthermore, LAK cells have been described as a heterogeneous population of cytolytic cells, including T-cell and NK cell subsets (45). In contrast, the cytolytic activity of BAK cells is largely confined to $CD3^-/CD8^+/CD56^+$ NK cells (14, 28). To gain more insight into the antitumor mechanisms operative during BCG immunotherapy, we have now defined the lytic pathways predominantly used by these two potential cytotoxic effector cell populations.

It has been shown that perforin and FasL are the key molecules in lymphocyte-mediated cytotoxicity (16) with some accessory function of TNF (46). We used different inhibitors of cell-mediated cytotoxicity, *i.e.,* EGTA/MgCl₂, CMA, and an inhibitory anti-FasL mAb (NOK-1) to identify the major lytic pathways used by BAK and LAK cells, respectively. In contrast to the receptor-mediated apoptosis induced by FasL, perforin-mediated lysis does not require the existence of a specific receptor but depends on the interaction of perforin with phosphorylcholine residues on the target cell surface (47). Killing via granule exocytosis/perforin is Ca^{2+}-dependent and can be inhibited with CMA (35). In contrast, FasL-mediated lysis has been described to be operative in the absence of Ca^{2+} (19, 48).

When we coincubated BAK and LAK cells, respectively, with T-24 or Jurkat in the presence of CMA or EGTA/MgCl₂, lysis of target cells was drastically abrogated (Figs. 3 and 4). This means that effector cell-mediated killing of target cells is strongly dependent on Ca^{2+} and indicates that perforin is by far
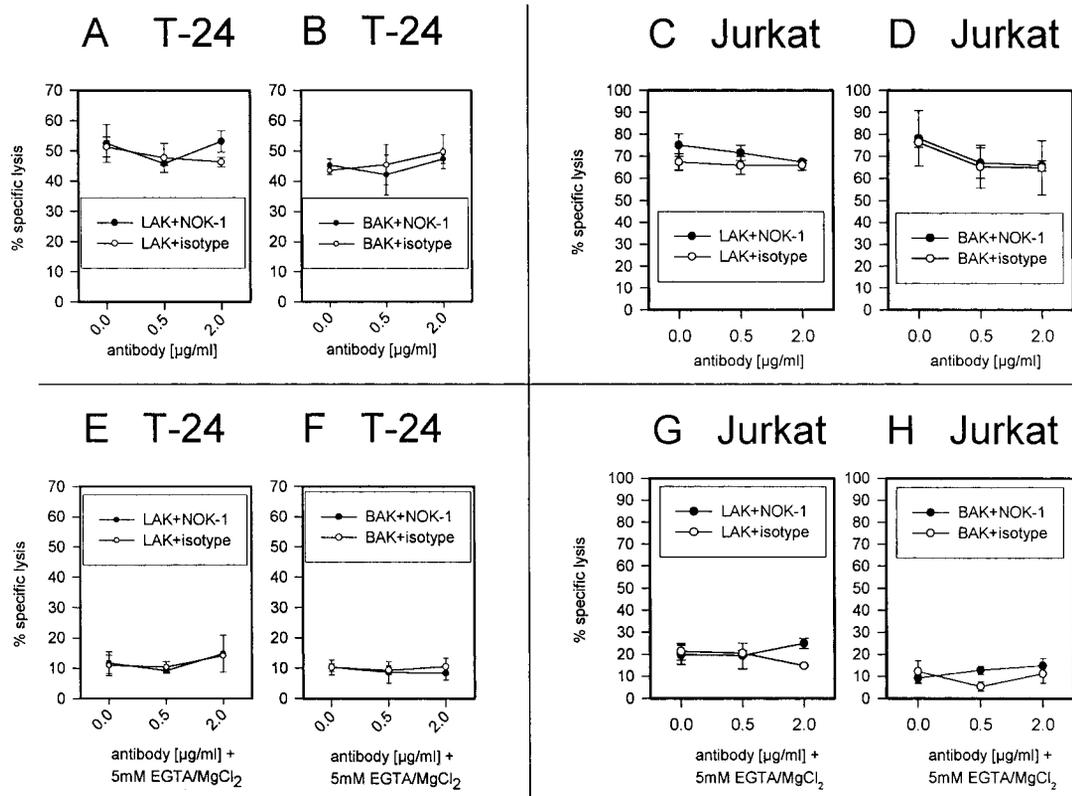
Fig. 4 BAK and LAK cells use Fas-independent cytolytic mechanisms to kill FasL-sensitive Jurkat and T-24. PBMCs were stimulated with IL-2 (*A, C, E,* and *G*) or BCG (*B, D, F,* and *H*) for 7 days. Cytolytic activity against T-24 and Jurkat was tested in a 4-h chromium release assay in the presence of anti-FasL mAb or an isotype control. To prevent the alternative use of calcium-dependent lytic pathways, EGTA/MgCl$_2$ was added where indicated (*E–H*). The E:T ratio was 40:1, experiments were done in triplicate, and results are shown as mean ± SD. In an 18-h assay, no inhibition of cell-mediated cytotoxicity was observed after the addition of NOK-1 (data not shown).

the most important lytic principle in LAK and BAK cell-mediated killing of T-24 and Jurkat. However, a minor contribution of other lytic pathways cannot be excluded. Therefore, experiments were designed to check for the involvement of FasL-induced killing.

We used mAb NOK-1 and the metalloproteinase inhibitor KB8301 to analyze FasL expression on BAK and LAK cells. However, with these reagents we were unable to demonstrate reliable and significant expression of FasL on the cell surface.[4] Nevertheless, target cell lysis dependent on the interaction of FasL and Fas is potentially possible because PBMC stimulated with BCG or IL-2 express FasL mRNA (not shown) and sFasL protein (Fig. 1). In addition, T-24 as well as Jurkat expresses significant amounts of Fas. When we coincubated T-24 bladder tumor cells and Jurkat with recombinant FasL or Chinese hamster ovary cells expressing membrane-bound FasL, both targets were shown to be susceptible to FasL-mediated killing (Fig. 5). Furthermore, killing was reversible on the addition of anti-FasL mAb NOK-1. These results demonstrate that NOK-1 is a suitable antibody for the inhibition of lysis mediated by sFasL and cell-membrane-bound FasL. Thus, a lack of inhibition is not attributable to insufficient blocking capacity of the mAb used. However, in subsequent experiments, lysis of T-24 and Jurkat by BAK

and LAK cells, respectively, could not be inhibited with NOK-1 (Fig. 4, *A–D*). To consider a possible alternative use of perforin, which could circumvent the inhibition with NOK-1, we also performed inhibition with anti-FasL mAb in the presence of EGTA/MgCl$_2$. However, also with this experimental setup, we obtained no evidence for the involvement of FasL-mediated killing (Fig. 4, *E–H*). Together with our flow cytometric data showing no reduced Annexin V binding of T-24 and Jurkat on the addition of NOK-1 during a 12-h coincubation with BAK and LAK, respectively, we conclude that killing of both targets occurs without a significant contribution of the FasL/Fas pathway. This was further confirmed by our observation that killing of Jurkat was also not altered in the presence of an inhibitory antibody to Fas receptor (ZB4), which blocks triggering of the receptor on the target cell. Nevertheless, we would like to point out that we have analyzed killing by BAK cells at day 7, the time point at which BAK cells display strongest cytotoxicity (14). Thus, although overall cytotoxicity is lower at earlier time points, FasL-mediated lysis might be detectable at these early time points (*e.g.,* days 3–4).

Recently, Shemtov *et al.* (13) showed that LAK cells lyse T-24 bladder tumor cells and lead to the release of T-24-derived DNA fragments into the supernatant. DNA fragmentation can be
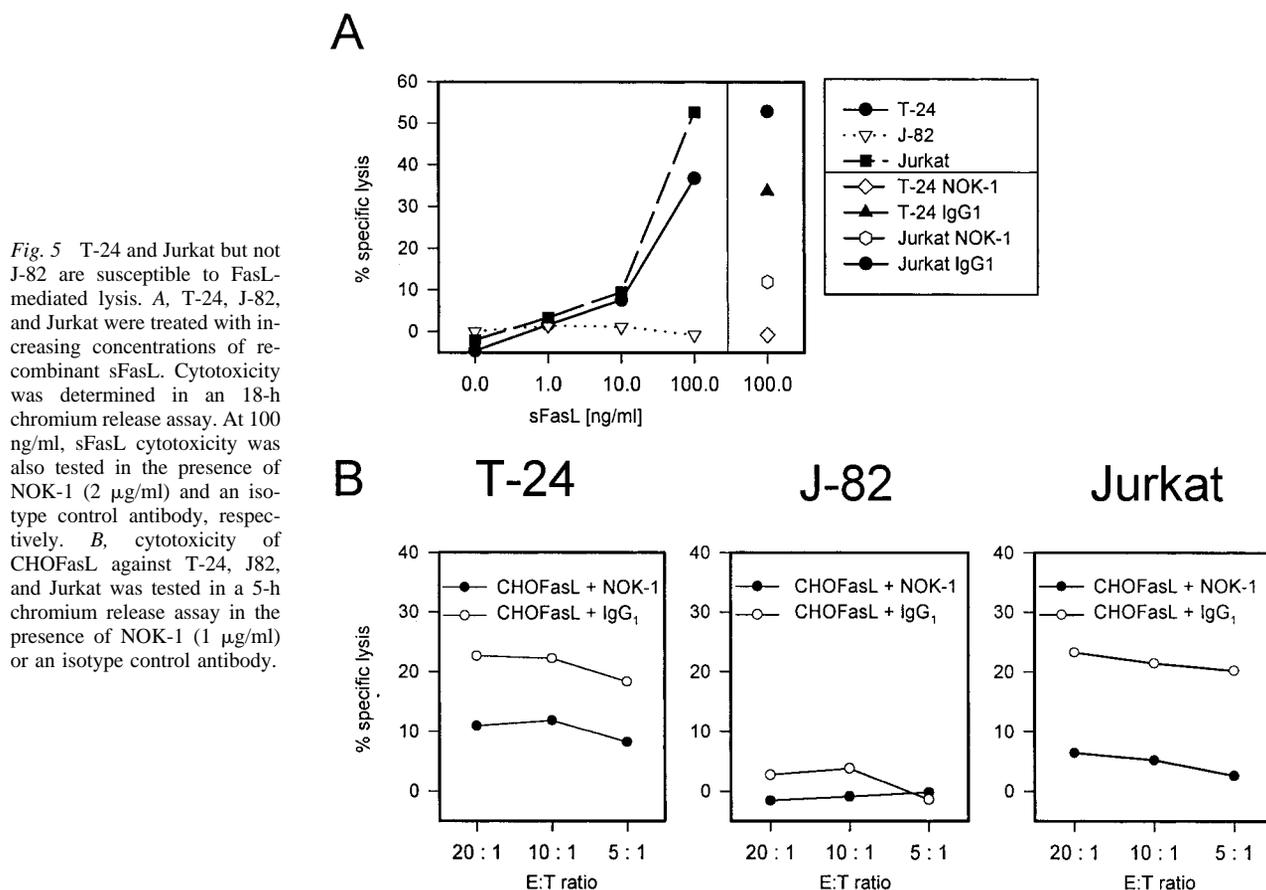
A

Fig. 5 T-24 and Jurkat but not J-82 are susceptible to FasL-mediated lysis. *A,* T-24, J-82, and Jurkat were treated with increasing concentrations of recombinant sFasL. Cytotoxicity was determined in an 18-h chromium release assay. At 100 ng/ml, sFasL cytotoxicity was also tested in the presence of NOK-1 (2 μg/ml) and an isotype control antibody, respectively. *B,* cytotoxicity of CHOFasL against T-24, J82, and Jurkat was tested in a 5-h chromium release assay in the presence of NOK-1 (1 μg/ml) or an isotype control antibody.

B

attributable to the activity of FasL or perforin/granzyme because both pathways finally lead to degradation of target cell DNA (16). Whereas Fas ligation directly leads to DNA fragmentation via caspases, perforin-mediated killing leads to cell necrosis by pore formation, and subsequently, target cell apoptosis is induced by granzymes (49). Thus, we confirm the data of Shemtov *et al.* (13) and furthermore show that lysis of T-24 occurs via perforin/granule exocytosis.

Using knock-out mice, it has been shown that in CTLs, perforin and FasL are the major cytotoxic principles (18, 20, 21, 50). *In vivo*, perforin seems to be important for the killing by NK cells, especially in tumor control (22). *In vitro*, the lytic pathways of NK cells differ with respect to their maturation stage, phenotype or mode, and state of activation. Perforin and FasL were reported to be the key effector molecules of mature NK cells (23–26, 51).

For the first time in our study, we have analyzed the killing mechanism of PBMCs stimulated with a cellular biological response modifier (BCG). The predominant lytic principle of these BAK cells was shown to be perforin. In a separate report, we demonstrated that most of the cytotoxic activity of BAK cells can be attributed to the CD3$^-$/CD8$^+$/CD56$^+$ subpopulation of lymphocytes (28). Therefore, our data support the notion that NK cells activated with the biological response modifier BCG predominantly use the granule exocytosis pathway for target cell lysis. In human

NK cells stimulated with phorbol 12-myristate 13-acetate/ionomycin (23) or IL-2 (24), a minor contribution of the FasL/Fas pathway was reported. Oshimi *et al.* (25) observed a significant induction of FasL-mediated target cell apoptosis by freshly isolated, unstimulated human CD16$^+$ NK cells. The degree of apoptosis correlated with the amount of Fas expressed by the various targets tested (25). However, we could not find any contribution of the FasL pathway to the killing by BAK cells in our system. A possible reason for this difference is the fact that BAK cells are generated by stimulation of PBMCs via a complex network of cellular (*e.g.,* CD4$^+$ cells and monocytes) and humoral factors (*e.g.,* IL-2, IFN-γ, IL-12) (14).[4] In contrast, in the above mentioned studies of Montel *et al.* (23), Medvedev *et al.* (24) and Oshimi *et al.* (25), purified unstimulated NK cells or NK cells stimulated with phorbol 12-myristate 13-acetate/ionomycin or IL-2 have been used. These differences in the mode of activation might lead to a differential use of lytic pathways. In addition, according to Mori *et al.* (52), the differential use of killing mechanisms is also influenced by the activation status of the target cell. Similar to us, these authors found perforin to be the major killing mechanism of NK cells. FasL did not significantly contribute to the NK-mediated lysis of various FasL-sensitive tumor targets. Only after IFN-γ treatment of normally FasL-insensitive HT-29 cells was a minor contribution of the FasL pathway was observed. However, in
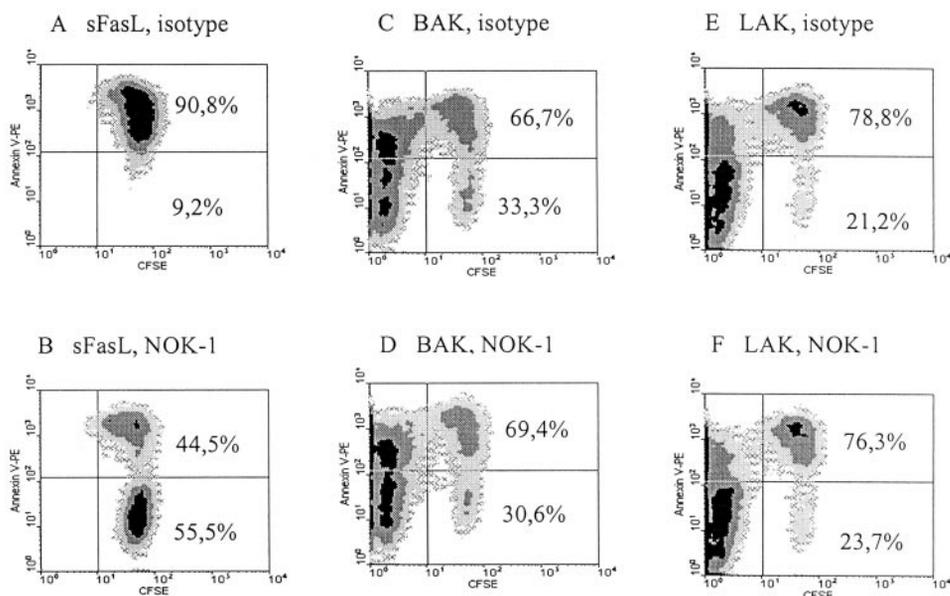
Fig. 6 Loss of target cell membrane integrity induced by BAK and LAK cells is not attributable to FasL/Fas interaction. Jurkat target cells were labeled with the green fluorescent dye CFSE. One hundred ng/ml sFasL (A and B), BAK cells (C and D), and LAK cells (E and F) at an effector target ratio of 2:1 were added, and after 12-h coincubation, cells were harvested. Annexin V-PE binding was determined on a FACS-Calibur (Becton Dickinson). CFSE-positive Jurkat target cells (top and bottom right area) can be distinguished from CSFE-negative effector cells (top and bottom left area). The percentage of Annexin V-positive Jurkat (top right) and Annexin V-negative living Jurkat (lower right) was determined.

Fig. 7 Expression of perforin in bladder tissue. Frozen sections were obtained from cold cup biopsies, and immunoenzymatic detection of perforin was performed using the alkaline phosphatase antialkaline phosphatase technique (counterstain hematoxylin). Bladder tissue before initiation of BCG therapy (A) and 24 h after the sixth instillation (B) is shown. Note the increased number of perforin-positive cells in the suburothelial stroma and in the urothelial layer after BCG therapy.
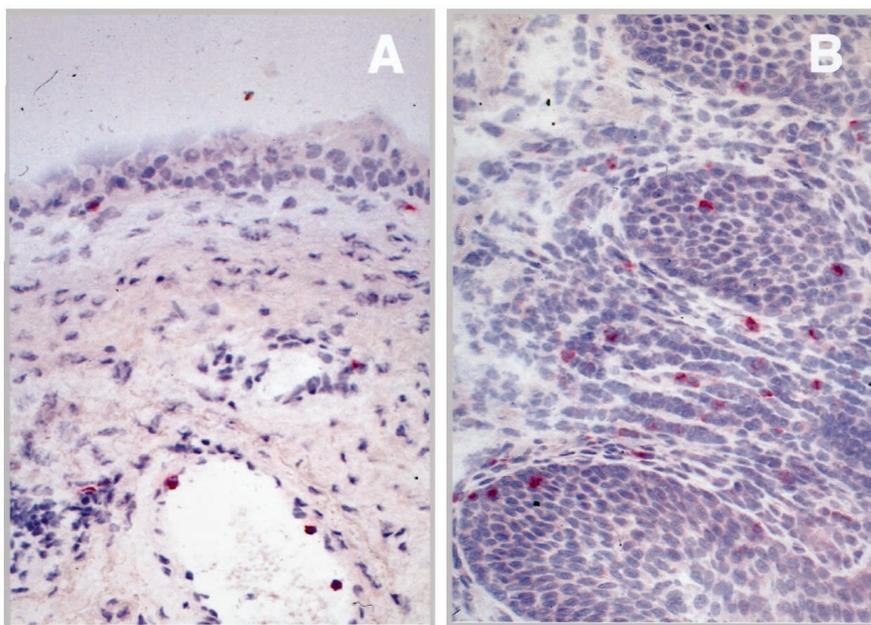
our experiments with FasL-insensitive J-82 bladder tumor cells, we found no difference between cytokine stimulated and unstimulated target cells.

In summary, we have used BCG and IL-2, two compounds that are thought to mediate antitumor effects during BCG-immunotherapy of bladder cancer, to generate tumor-cytolytic cells *in vitro*. Stimulated lymphocytes expressed perforin and FasL. However, effector cells did not use the Fas pathway to kill FasL-sensitive targets. Instead, most of the cytotoxicity could be attributed to perforin. Most interestingly, for the first time,

perforin expression could also be demonstrated in the bladder urothelium of patients after BCG-therapy. On the other hand, the induction of BAK cells has thus far only been demonstrated *in vitro*. The phenotype of the effector lymphocytes activated during BCG immunotherapy *in vivo* still needs to be defined. Mouse studies, which address this issue, are presently underway in our lab.

In conclusion, our data support the hypothesis that perforin-mediated killing serves as a potent effector mechanism during BCG immunotherapy because even tumor targets with

inappropriate expression of Fas- and FasL-insensitive tumors would not escape destruction (53).

Acknowledgments

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