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Robert Gastpar, Catharina Gross, Lydia Rossbacher, Joachim Ellwart, Julia Riegger and Gabriele Multhoff

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# The Cell Surface-Localized Heat Shock Protein 70 Epitope TKD Induces Migration and Cytolytic Activity Selectively in Human NK Cells<sup>1</sup>

Robert Gastpar,<sup>2\*</sup> Catharina Gross,<sup>2\*</sup> Lydia Rossbacher,<sup>\*</sup> Joachim Ellwart,<sup>†</sup> Julia Riegger,<sup>\*</sup> and Gabriele Multhoff<sup>3\*</sup>

**Profiling of surface-bound proteins uncovers a tumor-selective heat shock protein 70 (Hsp70) membrane expression that provides a target structure for human NK cells. Hsp70 peptide TKD (TKDNNLLGRFELSG; aa 450–463) was found to enhance the cytolytic activity of NK cells. In this study, we demonstrate that TKD-activated CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK cells are selectively attracted by Hsp70 membrane-positive tumor cells, and supernatants derived thereof. Hsp70 membrane-negative tumors failed to attract these NK cells. The capacity to migrate was associated with a substantial lytic activity against Hsp70-positive tumor cells. Because NK cell migration was independent of cell-to-cell contact, the involvement of a soluble factor was assumed. Interestingly, synthetic Hsp70 protein and Hsp70 peptide TKD, mimicking surface-bound Hsp70, initiates migration of NK cells in a concentration-dependent (1–5  $\mu$ g/ml), highly selective, and chemokine-independent manner. In summary, our results indicate that Hsp70 peptide TKD not only stimulates cytotoxicity but also chemotaxis in CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK cells. *The Journal of Immunology*, 2004, 172: 972–980.**

Unlike T and B cells, NK cells express neither clonally restricted, genetically diverse TCRs nor Abs. The interaction with target cells depends on an interplay between germline-encoded inhibitory receptors, with specificity for certain MHC class I alleles, and activating receptors whose ligands are less well characterized (1–3). Classical and nonclassical MHC class I molecules were characterized as interacting partners for killer cell Ig-like receptors, Ig-like transcripts, and C-type lectin receptors. According to the missing-self theory, target cells lacking MHC class I expression are susceptible to NK cell-mediated cytotoxicity, because they fail to provide the ligand for inhibitory receptors (1, 2, 4). We identified a heat shock protein 70 (Hsp70)<sup>4</sup> epitope (TKDNNLLGRFELSG; aa 450–463) on tumor cells as a recognition site for NK cells. To examine the role of membrane-bound Hsp70 in more detail, we investigated the ability of tumor cells with differential Hsp70 membrane expression pattern to attract effector cells. NK cells circulate in the peripheral blood or reside in the spleen and liver but are also present in thymus, bone marrow, and lymph nodes. Following viral and bacterial infections, NK cells accumulate in extrahematic organs (5–7). In addition, NK cells efficiently infiltrate murine tumors, especially those lacking host MHC class I molecules due to defects in transporter-

associated Ag processing or  $\beta_2$ -microglobulin genes (8, 9). In vitro, NK cell migration is inducible by several chemokines and cytokines including macrophage-inflammatory protein-1 $\alpha$ , IFN- $\gamma$ -inducible protein 10, TNF- $\alpha$ , and IL-2 (10–17). Chemokine knockout mice showed severe defects in the migration of NK cells into solid tumors (11). Apart from chemokines, less is known about additional stimuli, for the recruitment of NK cells. Therefore, in the present study we intended to identify tumor cell-derived factors involved in NK cell migration.

Several groups reported on a plasma membrane localization of molecular chaperones on tumor cells (18–20). We detected Hsp70, the major stress-inducible member of the HSP70 group on a number of different tumor cells, but not on normal tissues (21). By cell sorting via Hsp70-specific Ab, we generated colon carcinoma sublines with a stable high and low Hsp70 cell surface expression. Apart from Hsp70, the sublines exhibited an identical expression pattern of other cell surface markers, including MHC and adhesion molecules (22). The Hsp70 epitope exposed to the extracellular milieu could be identified as the amino acid sequence TKDNNLLGRFELSG (TKD; aa 450–463), which is part of the C-terminal domain of Hsp70 (23, 24). We identified NK cells as being relevant effector cells for the recognition of membrane-bound Hsp70 on tumor cells (21, 22, 25–27). Incubation of NK cells with Hsp70 peptide TKD results in an up-regulated CD94 expression that is associated with an enhanced lytic activity against Hsp70 membrane-positive tumor target cells (24). Binding studies revealed a firm interaction of Hsp70 and Hsp70 peptide TKD to CD94-positive NK cells (28). In this study, we studied the migratory capacity of Hsp70-activated NK cells toward Hsp70 membrane-positive and -negative tumor cells and supernatants derived thereof. In an effort to identify a tumor-derived soluble factor, different Hsp70-related peptides, including TKD, were tested as potential attractants for Hsp70-reactive effector cells.

## Materials and Methods

### Peptides and proteins

The following 14-mer peptides TKDNNLLGRFELSG (TKD; aa 450–463), TKDNNLLGKFELTG (Hsc70 14-mer; aa 450–463), and

\*Department of Hematology and Oncology, University Hospital Regensburg, Regensburg, Germany; and <sup>†</sup>Institute of Molecular Immunology, GSF National Research Center for Environment and Health, Munich, Germany

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<sup>2</sup> R.G. and C.G. contributed equally to this work.

<sup>3</sup> Address correspondence and reprint requests to Prof. Dr. Gabriele Multhoff, Department of Hematology and Oncology, University Hospital Regensburg, Franz-Josef Strauss Allee 11, D-93053 Regensburg, Germany. E-mail address: gabriele.multhoff@klinik.uni-regensburg.de

<sup>4</sup> Abbreviation used in this paper: Hsp70, heat shock protein 70.

AADNKSGLQFNLDG (DnaK 14-mer; aa 447–460) were produced by F-moc synthesis (fluorenylmethoxycarbonyl/*t*-butyl-based solid-phase peptide chemistry method; Bachem, Bubendorf, Switzerland), as previously described (24). The purity of each peptide was >96%.

Human rHsp70 protein was purchased from Stressgen (SPP-75; Victoria, British Columbia, Canada).

### Cell culture

The adherent growing human pancreatic carcinoma cell line Colo357 was separated into two sublines, Colo<sup>+</sup> and Colo<sup>-</sup>, by FACS according to their Hsp70 plasma membrane expression using an Hsp70<sup>FITC</sup> Ab specific for the epitope presented to the surface (clone cmHsp70.1; multimune, Regensburg, Germany). The resulting sublines exhibit either high (Colo<sup>+</sup>) or low (Colo<sup>-</sup>) Hsp70 plasma membrane expression. Similarly, the human colon carcinoma cell line CX2 was separated into sublines with stable high (CX<sup>+</sup>) and stable low (CX<sup>-</sup>) Hsp70 plasma membrane expression, as previously described (22). The cell lines were maintained mycoplasma free in RPMI 1640 medium containing 10% heat-inactivated FCS (Life Technologies, Eggenstein, Germany) supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 1 mM sodium pyruvate, and were harvested by treatment with trypsin/EDTA (all cell culture reagents were purchased from Life Technologies).

Supernatants were collected from tumor cells cultured for 48 h in a humidified atmosphere, at 37°C and 5% CO<sub>2</sub>. Then, supernatants were centrifuged at 300 × *g* to remove cell debris, and filtered through a 0.2-μm filter (Costar, Corning, NY).

### Selection of CD94-positive and CD94-negative cells

CD94-positive and -negative cells were selected from PBMC derived from healthy human donors using CD94-biotin Ab (HP3-D9; Ancell Immunology Research Products, Bayport, MN) and anti-biotin magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 1 × 10<sup>8</sup> cells/ml were incubated with 5 μg of biotin-conjugated CD94 Ab for 30 min at 4°C. After extensive washing with MACS buffer (PBS, 0.5% BSA, and 2 mM EDTA), cells were incubated with anti-biotin magnetic microbeads for 15 min at 4°C. Afterward, cells were washed and separated using a LS/VS column (Miltenyi Biotec).

PBL, CD94-positive, and CD94-negative cells were incubated for 4 days in RPMI 1640 medium (Life Technologies) supplemented with heat-inactivated FCS (10%) plus 100 IU/ml IL-2 and 2 μg/ml Hsp70 peptide TKD at 37°C, in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Migration assay

Migration assays were performed in a Transwell cell culture system (Costar; Corning) in triplicate. The upper and lower compartments were separated by a tissue culture polycarbonate membrane (polyvinyl-pyrrolidone free; Nucleopore, Pleasanton, CA), 6.5 mm in diameter, with a pore size of 5 μm. Tumor target cells (CX<sup>+</sup>, CX<sup>-</sup>, Colo<sup>+</sup>, Colo<sup>-</sup>), cell culture supernatants, and peptides were placed in a total volume of 600 μl of RPMI 1640 medium containing 10% FCS in the lower compartment. Then, 2 × 10<sup>6</sup> PBL or CD94-positively sorted cells stimulated with TKD were labeled with sodium [<sup>51</sup>Cr]chromate (100 μCi; NEN-DuPont, Boston, MA) for 2 h. After extensive washing, labeled cells were counted, and 0.2 × 10<sup>6</sup> cells were resuspended in 100 μl of RPMI 1640 medium supplemented with 10% FCS and carefully added to the upper compartment. rIL-15 (10 ng/ml; Immunex, Seattle, WA) served as a positive control for chemotaxis (29). After a 4-h incubation period in a humidified incubator at 37°C and 5% CO<sub>2</sub>, the cell suspension in the lower compartment was harvested, and radioactivity was measured on a gamma counter (Packard Instruments, Meriden, CT). The number of migrated cells was determined according to the following equation: % specific migration = (experimental value – spontaneous value)/(maximum value – spontaneous value) × 100.

### Flow cytometry

Directly conjugated Abs (CD3<sup>FITC</sup>, CD16/56<sup>PE</sup>, CD56<sup>FITC</sup> (BD Biosciences, Heidelberg, Germany); NKG2A<sup>PE</sup> (Immunotech Coulter, Marseille, France); CD94<sup>PE</sup> (Ancell); CD94<sup>FITC</sup> (BD Pharmingen, San Diego, CA)) were added to cell suspensions (1 × 10<sup>5</sup> cells), incubated for 20 min on ice, washed, and analyzed on a FACSCalibur instrument (BD Biosciences). The percentage of single- or double-positively stained cells was defined as the number of specifically stained cells minus the number of cells stained with an isotype-matched control Ab.

### Cytotoxicity assay

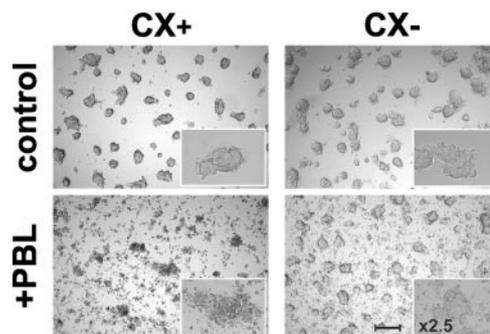
CX<sup>+</sup>, CX<sup>-</sup> and Colo<sup>+</sup>, Colo<sup>-</sup> tumor target cells (3 × 10<sup>3</sup>) were incubated for 1 h with sodium [<sup>51</sup>Cr]chromate (100 μCi; NEN-DuPont), extensively

washed, and plated with effector cells at indicated E:T ratio. After a 4-h incubation period at 37°C and 5% CO<sub>2</sub>, supernatants were harvested, and radioactivity was determined by gamma counting. The percentage of specific lysis was calculated according to the following equation: % specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. The spontaneous release was <15% for each target cell.

## Results

### TKD-stimulated PBL selectively infiltrate Hsp70-positive tumor cell clusters

Previously, we reported that incubation with Hsp70 peptide TKD and low-dose IL-2 increases the cytolytic activity of NK cells against tumor target cells expressing Hsp70 on their cell surface (24). The role of membrane-bound Hsp70 as a target structure for NK cells has been demonstrated using HLA-identical colon carcinoma sublines CX<sup>+</sup> and CX<sup>-</sup> that differ profoundly with respect to their capacity to express Hsp70 on the plasma membrane (22). To obtain further insight into the interaction of TKD-stimulated effector cells and Hsp70-positive tumor target cells, a long-term cocultivation experiment was set up. TKD-stimulated PBL were added to exponentially growing tumor cell subpopulations. After a 24-h incubation period, the morphology of cocultures and controls, omitting PBL, was analyzed by light microscopy (Fig. 1). The inserts represent a 2.5-fold higher magnification of a selected cell cluster. When cultivated in the absence of TKD-stimulated PBL, both Hsp70-positive CX<sup>+</sup> and Hsp70-negative CX<sup>-</sup> cells showed a similar morphology, forming adherent spheroidal cell clusters (control). In contrast, the morphology of CX<sup>+</sup> cells cocultured with TKD-stimulated PBL (+PBL) differed significantly from that of CX<sup>-</sup> cells, indicating that activated PBL were attracted by Hsp70-positive CX<sup>+</sup> tumor cells. Almost all CX<sup>+</sup> cell clusters were infiltrated by effector cells, whereas Hsp70-negative CX<sup>-</sup> cell clusters remained unaffected. In addition, a significantly reduced viability was observed in CX<sup>+</sup> cells compared with CX<sup>-</sup> cells after cocultivation with TKD-activated PBL (data not shown). These findings are in line with results from cytotoxicity assays showing that Hsp70-positive target cells are more susceptible to lysis mediated by TKD-stimulated effector cells, as compared with Hsp70-negative target cells (22, 25).



**FIGURE 1.** Infiltration of Hsp70-positive tumor cell clusters by TKD-activated PBL. Hsp70 membrane-positive CX<sup>+</sup> and -negative CX<sup>-</sup> cells (0.1 × 10<sup>6</sup> cells/well) were seeded in 96-well flat-bottom plates and grown for 48 h. Tumor cells were cocultured with either medium or TKD-stimulated PBL for another 24 h. Light-microscopic analysis is shown of tumor cells only (control; upper row) or tumor cells cocultured with TKD-activated PBL (+PBL; lower row) at a magnification of ×5 on a Zeiss (Oberkochen, Germany) Axiovert 25 microscope; small insets show a 2.5-fold magnification of one representative cell cluster. The scale bar in the lower right panel indicates 100 μm.

*TKD-stimulated PBL migrate to Hsp70 membrane-positive tumor cells and supernatants derived thereof*

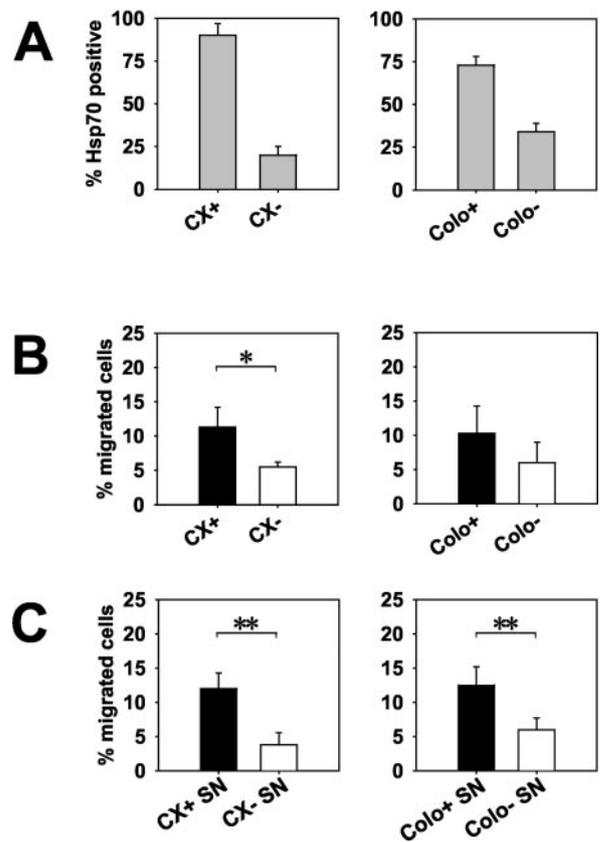
The increased sensitivity of Hsp70-positive tumor target cells mediated by TKD-stimulated NK cells has been demonstrated by using HLA-identical colon carcinoma sublines CX<sup>+</sup> and CX<sup>-</sup> with differential Hsp70 membrane expression. In this study, an additional tumor model system was established using the human pancreatic carcinoma cell line Colo357. Initially, Colo357 cells express Hsp70 on ~50% of the cells. Identical with the CX<sup>+</sup>CX<sup>-</sup> cell separation, Hsp70 high (Colo<sup>+</sup>)- and low (Colo<sup>-</sup>)-expressing sublines were generated by cell sorting via an Hsp70 mAb (22). Fig. 2A illustrates the percentages of Hsp70 positively stained cells in CX<sup>+</sup> (90%) and CX<sup>-</sup> (20%), and in Colo<sup>+</sup> (73%) and Colo<sup>-</sup> (34%) sublines after cell sorting. The migratory capacity of TKD-activated PBL was tested toward these different tumor sublines and against supernatants derived thereof. The cell density of the effector and tumor cells in the lower compartment, the pore size and pore distribution, and the coincubation time were optimized in kinetic studies (data not shown). In a first set of experiments, CX<sup>+</sup>, CX<sup>-</sup> and Colo<sup>+</sup>, Colo<sup>-</sup> cells, with differential Hsp70 membrane expression pattern, were cultured in the lower compartment for 48 h. Then, TKD-activated PBL were added carefully to the upper compartment and after a 4-h coincubation period, specific migration was measured. As depicted in Fig. 2B, migration of TKD-activated PBL, derived from five independent healthy human volunteers, toward Hsp70 plasma membrane-positive CX<sup>+</sup> (11%) and Colo<sup>+</sup> (10%) was higher as compared with target cells with low Hsp70 membrane expression (CX<sup>-</sup>, 5%; Colo<sup>-</sup>, 6%). Differences in migration toward CX<sup>+</sup>, CX<sup>-</sup> cells was more pronounced as compared with Colo<sup>+</sup>, Colo<sup>-</sup> tumor cells. This finding is due to the fact that differences in the Hsp70 cell membrane expression in the positive and negative sublines was also weaker in the Colo cell system. Migration to 10 ng/ml of the cytokine IL-15, serving as a positive control, was 15% (data not shown). This is in line with previously published data (29).

It has been shown that coincubation of IL-2-activated NK cells and K562 target cells results in effector-target cell conjugates that trigger chemokine secretion and chemoattraction of additional NK cells (30). To rule out chemoattraction induced by direct cell-to-cell contact, cell-free supernatants derived from 2-day tumor cell cultures, were used. Interestingly, the migration assay toward supernatants of Hsp70 membrane-positive CX<sup>+</sup> and Colo<sup>+</sup> cells resulted in significant migration (12%), whereas that toward CX<sup>-</sup> and Colo<sup>-</sup> cells did not induce migration (3 and 6%, respectively) (Fig. 2C). This suggests that migration of TKD-activated PBL might be induced by a soluble factor that is secreted equally well by Hsp70 membrane-positive CX<sup>+</sup> and Colo<sup>+</sup> tumor cells. Recent data provide evidence for a tumor-specific release of members of the HSP70 family (31), indicating that the soluble factor released by CX<sup>+</sup> and Colo<sup>+</sup> tumor cells might be Hsp70 or an HSP-related protein.

It is also important to note that incubation of PBL with low dose IL-2 plus Hsp70 peptide TKD is a prerequisite for the induction of the migratory capacity, because unstimulated PBL or PBL cultured with IL-2 alone did not migrate toward Hsp70 membrane-positive and -negative tumor target cells, or supernatants derived thereof (data not shown).

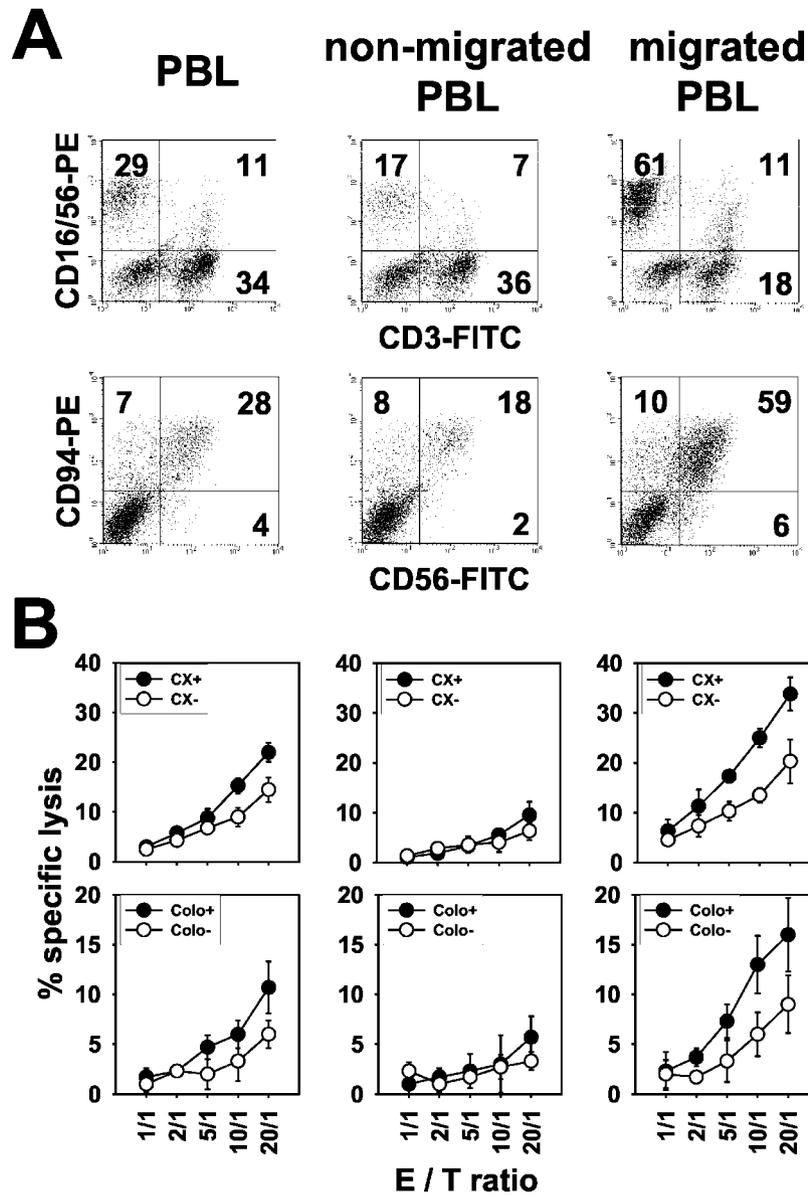
*CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK cells exhibit migratory and cytolytic activity against Hsp70 membrane-positive tumor cells*

According to the preceding findings, we were interested in the phenotype and functional properties of both the migrated and nonmigrated cell populations. Therefore, after a 4-h migration assay



**FIGURE 2.** TKD-activated PBL migrate toward Hsp70-positive target cells and supernatants (SN) derived thereof. **A**, Phenotypic characterization of the Hsp70 membrane expression on human colon carcinoma cell sublines CX<sup>+</sup> and CX<sup>-</sup> and human pancreatic carcinoma cell sublines Colo<sup>+</sup> and Colo<sup>-</sup>. The sublines were generated by FACS using Hsp70<sup>FITC</sup> Ab (clone cmHsp70.1), which recognizes the Hsp70 surface epitope. The data represent the mean percentage ( $\pm$ SEM) of Hsp70-positively stained cells of at least eight independent experiments. **B**, Migration of TKD-activated PBL to CX<sup>+</sup>, CX<sup>-</sup> and Colo<sup>+</sup>, Colo<sup>-</sup> tumor cells. Migration was tested in a Transwell system with two compartments separated by a polycarbonate membrane with a pore size of 5  $\mu$ m. In the lower compartment, CX<sup>+</sup>, CX<sup>-</sup> or Colo<sup>+</sup>, Colo<sup>-</sup> cells ( $0.2 \times 10^6$  cells per well) were grown for 48 h in 600  $\mu$ l of RPMI 1640 medium containing 10% FCS. PBL, stimulated with TKD (2  $\mu$ g/ml) plus low-dose IL-2 (100 IU/ml) for 4 days, were harvested, labeled with sodium [<sup>51</sup>Cr]chromate (100  $\mu$ Ci), and transferred into the upper chamber of the Transwell system. Following a 4-h coincubation period, radioactivity was determined in PBL that migrated to the lower chamber. Specific migration was calculated as described in *Materials and Methods*. Results are shown as the mean  $\pm$  SEM of at least five independent experiments. Statistically significant (Student's *t* test), \*,  $p < 0.05$ . **C**, In the next set of experiments, 600  $\mu$ l of cell-free culture supernatant (SN) of CX<sup>+</sup>CX<sup>-</sup> and Colo<sup>+</sup>Colo<sup>-</sup> tumor cells cultured for 48 h were administered in the lower compartment. Following a 4-h coincubation period with PBL, radioactivity was determined in the migrated cells of the lower chamber. Specific migration was calculated as described in *Materials and Methods*. Results are shown as the mean  $\pm$  SEM of at least five independent experiments. Statistically significant (Student's *t* test), \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .

toward supernatants of Hsp70 membrane-positive tumor cells, nonmigrated cells from the upper compartment and migrated cells from the lower compartment were collected and immunophenotyped. The initial population (PBL) consisted of 34% CD3<sup>+</sup> T cells, 11% CD3<sup>+</sup>CD16/56<sup>+</sup> NKT cells, and 29% CD3<sup>-</sup>CD16/56<sup>+</sup> NK cells (Fig. 3A). The nonmigrated cells showed no significant differences in the percentage of CD3<sup>+</sup> T cells (36%), and CD3<sup>+</sup>CD16/56<sup>+</sup> NKT cells (7%). However, the CD3<sup>-</sup>CD16/56<sup>+</sup>



**FIGURE 3.** NK cells migrate and exhibit lytic activity against Hsp70-positive tumor target cells. A 4-h migration assay was performed using TKD-stimulated PBL against supernatants of Hsp70-positive CX<sup>+</sup> tumor cells to determine the phenotype and the lytic activity of nonmigrated and migrated cells in comparison with the initial PBL population. *A*, Nonmigrated PBL (*middle panel*), and migrated cells (*right panel*) were harvested and analyzed by multiparameter flow cytometry using CD3<sup>FITC</sup>, CD16/56<sup>PE</sup>, CD56<sup>FITC</sup>, and CD94<sup>PE</sup> Abs, and a double staining was performed with CD94<sup>FITC</sup> and NKG2A<sup>PE</sup> Abs. The phenotype of each cell population was compared with the initial PBL population (*left panel*) that had been stimulated with TKD for 4 days. Numbers in the dot plot diagrams indicate percentage of positively stained cells for each quadrant. Data of one representative result of five independent experiments is shown. Increase in CD56<sup>+</sup>CD94<sup>+</sup> cells was always doubled in the migrated cell population if compared with the initial PBL population. *B*, Concomitantly, the cytolytic activity of the three effector cell populations (initial PBL, nonmigrated PBL, and migrated PBL) was tested in a standard 4-h chromium release assay. Specific lysis of the effector cells was assessed against CX<sup>+</sup> and Colo<sup>+</sup> tumor target cells with high Hsp70 surface expression (●) and CX<sup>-</sup> and Colo<sup>-</sup> tumor target cells with low Hsp70 surface expression (○). E:T ratios ranged from 1:1 to 20:1. Data represent the mean values of four independent experiments.

NK cell subpopulation shrank from 29 to 17%. In contrast, a significant increase in the percentage of CD3<sup>-</sup>CD16/56<sup>+</sup> NK cells was observed (from 29 to 61%) within the migrated cell population, whereas the amount of CD3<sup>+</sup> T cells was found to be reduced by half (from 34 to 18%). These results suggest that predominantly CD3<sup>-</sup>CD16/56<sup>+</sup> NK cells were attracted by supernatants derived from Hsp70 membrane-positive tumor cells.

Recently, we demonstrated an increase in cell surface density of the C-type lectin receptor CD94 after TKD stimulation, selectively on NK cells (28, 32). Furthermore, the enhanced lysis of tumor target cells with Hsp70 plasma membrane expression by TKD-

stimulated NK cells was blockable in the presence of CD94 Ab (32). This suggests an involvement of CD94 in the interaction of NK cells with Hsp70-expressing tumor cells. Therefore, the role of CD94 was studied in more detail with respect to the migratory capacity. Compared with the original population, the percentage of CD56<sup>+</sup>CD94<sup>+</sup> cells was markedly reduced in the nonmigrated cell population (from 28 to 18%). Concomitantly, a drastic increase of CD56<sup>+</sup>CD94<sup>+</sup> cells was found in the migrated cell population (from 28 to 59%; Fig. 3A). The results of five independent migration assays confirmed this finding; the amount of CD56<sup>+</sup>CD94<sup>+</sup> cells was always twice as high in the migrated cell population if

compared with the initial PBL and three to four times higher if compared with the nonmigrated cell population. No significant changes were observed in the  $CD56^+CD94^+$  (7 vs 8 vs 10%) and in the  $CD56^+CD94^-$  cell population (4 vs 2 vs 6%), indicating that predominantly  $CD56^+CD94^+$  NK cells were attracted by cell culture supernatants of  $CX^+$  cells. Multiparameter staining using  $CD94^{FITC}$ - and  $NKG2A^{PE}$ -specific Abs revealed that the migrated cells were double positive for  $CD94$  and  $NKG2A$ .

To correlate phenotype and function, cytotoxicity assays were performed using nonmigrated and migrated cells as effectors. The initial cell population (PBL) exhibited only a moderately increased lytic activity against Hsp70-positive  $CX^+$  and  $Colo^+$  tumor cells at an E:T ratio of 20:1 (Fig. 3B, left graph). Nonmigrated cells consisting predominantly of T cells showed a very weak lytic activity against all four tumor sublines (Fig. 3B, middle graph). Interestingly, the migrated cell population showed a very strong lytic activity against Hsp70-positive tumor target cells ( $CX^+$  and  $Colo^+$ ) and a weaker activity against Hsp70-negative tumor target cells ( $CX^-$  and  $Colo^-$ ) (Fig. 3B, right graph). Because this effect is associated with an increase in the percentage of  $CD3^-CD56^+CD94^+$  NK cells, we speculate that these effector cells might be responsible for migration and lytic activity.

To further confirm this hypothesis, functional tests were performed using MACS-sorted effector cell populations. Fig. 4 illustrates the phenotypic characterization of  $CD3$ -sorted PBL that had been stimulated with TKD for 4 days, directly after cell separation. The cytolytic activity of both cell populations was tested against Hsp70-positive tumor target cells ( $CX^+$ ,  $Colo^+$ , and K562). K562 cells are susceptible to NK cell-mediated lysis due to a missing MHC class I expression that might trigger inhibitory NK cell receptors, and due to the Hsp70 membrane expression as a trigger for  $CD94$  (our unpublished observation). The  $CD3^+$  T cell population ( $93 \pm 10\% CD3^+CD16/56^-$ ) showed no lytic activity (Fig. 4A). In contrast, the  $CD3^-$  cell population consisting of  $44 \pm 22\% CD3^-CD16/56^+$  NK cells lysed Hsp70-positive tumor target cells to a significant extent (Fig. 4A). Regarding these findings,  $CD3^+$  T cells were excluded as mediators of the cytolytic response against Hsp70-positive tumor target cells.

Preceding migration assays indicated that the phenotype of the migrated cells might be  $CD3^-CD56^+CD94^+$ . To analyze the role of  $CD94$  in more detail, TKD-stimulated PBL derived from three independent donors were separated by MACS using a  $CD94$  Ab. The  $CD94$ -depleted population ( $4 \pm 2\% CD56^+CD94^+$ ) showed a negligible lysis of Hsp70-positive tumor target cells (Fig. 4B), whereas the  $CD94$ -enriched population ( $58 \pm 9\% CD56^+CD94^+$ ) showed a strong lytic activity against  $CX^+$ ,  $Colo^+$ , and K562 cells. Compared with the  $CD3$  separation, following  $CD94$  enrichment, the cytolytic activity was slightly increased. The  $CD94$ -enriched cell population consists of  $CD3^-CD16/56^+$  NK cells,  $CD3^+CD16/56^+$  NKT cells, and  $CD3^+CD16/56^-$  T cells. To define which subpopulation is responsible for the Hsp70-mediated cytotoxicity,  $CD94^+$  cells were further separated into a  $CD94^+CD3^-$  ( $79 \pm 19\% CD56^+CD94^+$ ) and a  $CD94^+CD3^+$  cell subpopulation ( $70 \pm 7\% CD56^+CD94^+$ ). A strong lytic activity against Hsp70-positive  $CX^+$  cells was mediated by the  $CD94^+CD3^-$  population (Fig. 4C). Due to the fact that the  $CD3^+$  T cell population still contains  $28 \pm 8\% CD56^+CD94^+$  NK cells, a weak cytolytic activity was also seen in this population. In contrast to Hsp70-positive tumors, lysis of Hsp70-negative counterparts ( $CX^-$ ,  $Colo^-$ ) was  $<10\%$ .

Taken together, these results support our migration data showing that  $CD3^-CD56^+CD94^+$  NK cells are responsible for the migratory and lytic activity against Hsp70 membrane-positive tumor cells.

#### *Hsp70 protein and Hsp70 peptide TKD stimulate migration in $CD3^-CD56^+CD94^+$ NK cells*

Earlier, we have shown that not only Hsp70-positive tumor cells but also supernatants derived thereof were able to induce NK cell migration. Because supernatants of Hsp70 membrane-negative tumor cells were unable to induce migration, attraction appeared to be associated with Hsp70 membrane expression. Therefore, we speculate about a soluble factor that might be released by Hsp70 membrane-positive tumor cells.

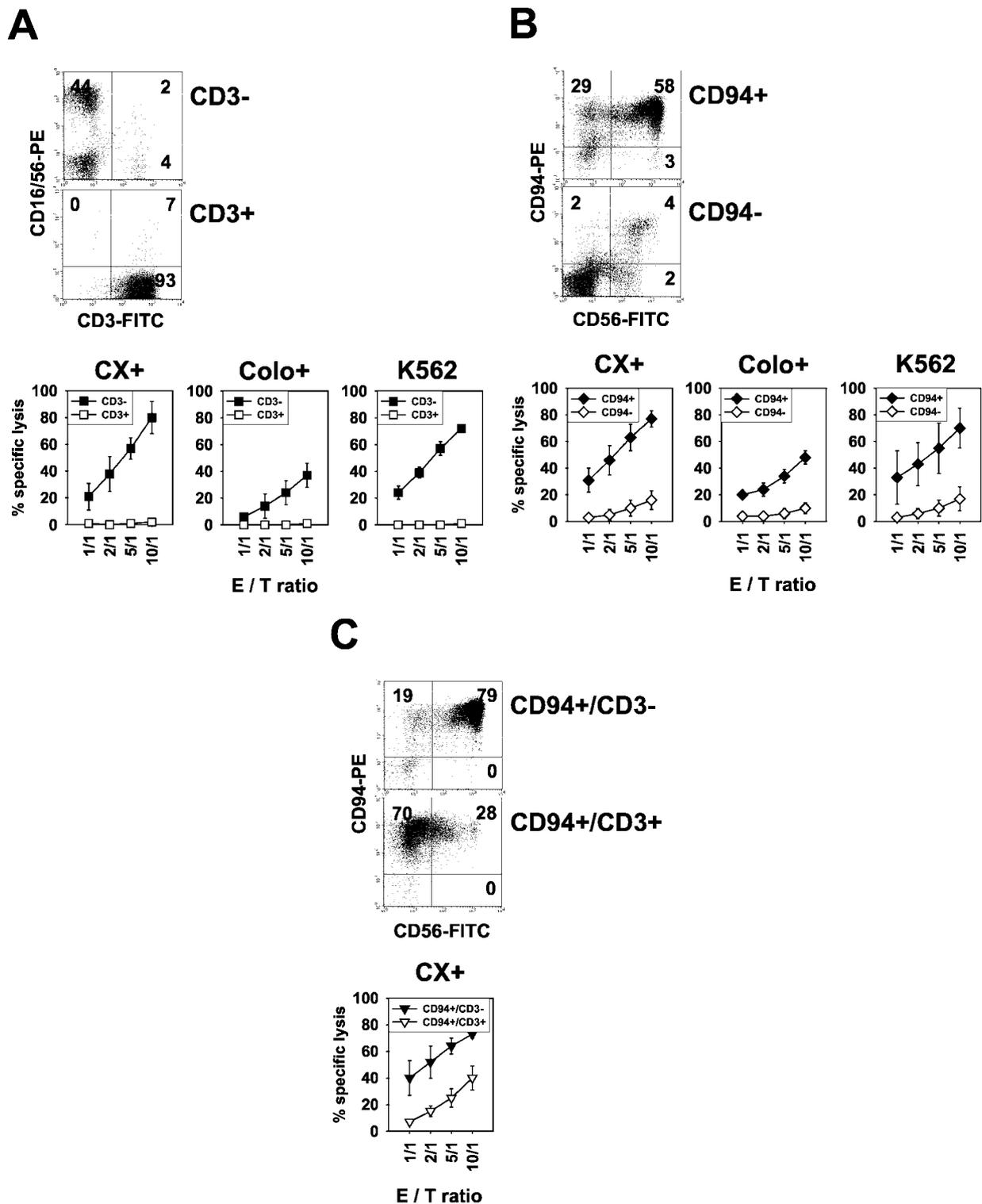
The Hsp70 epitope recognized by NK cells is identical with the 14-aa peptide TKD, residing in the C-terminal domain (24). Therefore, in addition to full-length Hsp70 protein, the cell surface-localized peptide TKD, mimicking the NK cell recognition site in a soluble form, was tested in migration assays. As shown in Fig. 5A, maximal migratory capacity of NK cells was detected if  $10 \mu\text{g/ml}$  Hsp70 protein diluted in fresh medium was subjected to the lower compartment. A comparable migration of NK cells was induced by  $1 \mu\text{g/ml}$  TKD peptide. In summary, these data indicate that Hsp70 protein and Hsp70 peptide TKD both exhibited similar chemotactic activity, which is comparable with that of supernatants derived from Hsp70 membrane-positive tumor cells.

In our previous experiments, the chemotactic factor was placed in the lower compartment, and the effector cells were placed in the upper compartment. To rule out chemokinesis or random cell mobility, the following experiments were performed. One criteria for directed chemotaxis is migration along a positive gradient. If the chemoattractant is equally distributed (no gradient), or in case of a negative gradient, active migration should therefore be inhibited. Fig. 5B demonstrates that migration of  $CD94$ -positive cells in response to  $1 \mu\text{g/ml}$  TKD was observed only in a positive TKD-gradient (16%). In the presence of equal TKD concentrations in the upper and lower compartments, migration was inhibited (5%; kinesis I). Similar results were seen in a negative gradient (3%; kinesis II). These results demonstrate that migration in response to TKD was rather chemotaxis than chemokinesis or random cell mobility.

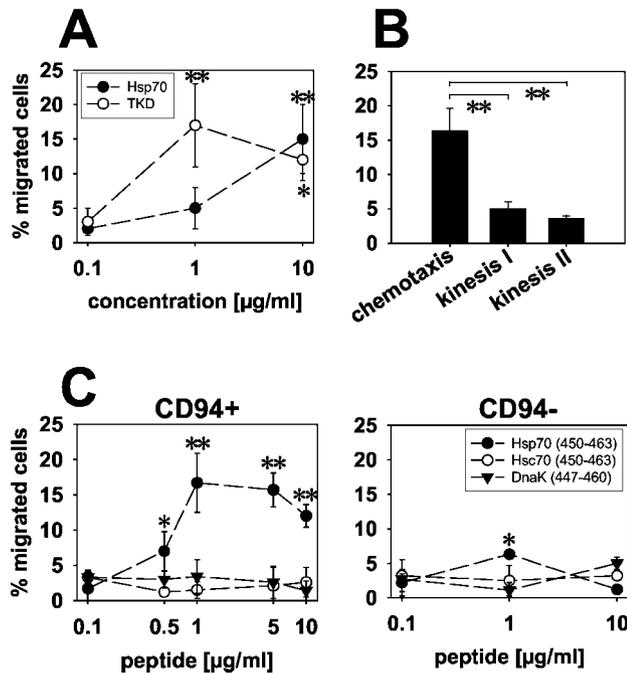
To determine whether TKD-related peptides also stimulate  $CD94$ -positive NK cells to migrate, two peptides derived from members of the HSP70 group (Hsc70<sub>450-463</sub> and DnaK<sub>447-460</sub>) were used at concentrations ranging from 0.1 to  $10 \mu\text{g/ml}$  (Fig. 5C). Interestingly, TKD-activated  $CD94$ -positive NK cells were selectively attracted by TKD, whereas no migration was induced by HSP70 peptides derived from Hsc70 and DnaK. Because Hsc70<sub>450-463</sub> (TKDNNLLGKFLTG) and DnaK<sub>447-460</sub> (AADNKSLGQFNLDG) differ from TKD (TKDNNLLGRFELSG) by 2- and 7-aa exchanges (indicated by bold type and underlining), respectively, the induction of migration mediated by TKD has to be considered to be specific. Furthermore, the migratory response to TKD was concentration dependent, showing an optimal concentration range between 1 and  $5 \mu\text{g/ml}$ . Concentrations  $>5 \mu\text{g/ml}$  resulted in a reduced migratory activity of NK cells. In contrast to  $CD94$ -positive NK cells,  $CD94$ -negative T cells showed no migration toward the HSP70 peptides at any concentration. Taken together, these findings demonstrate a concentration-dependent, specific attraction of  $CD94$ -positive NK cells by Hsp70 peptide TKD.

## Discussion

NK cells circulate in the blood vessel system but also reside in solid tissues. Mobilization, recruitment, and migration of NK cells and cytotoxicity are induced in response to a variety of inflammatory stimuli (7, 12, 14-16). Several mouse tumors, including B16



**FIGURE 4.** Hsp70-positive tumor target cells are lysed selectively by CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK Cells, but not by CD3<sup>+</sup> T cells. **A**, Separation of PBL of three independent donors in a CD3-negative (CD3<sup>-</sup>; upper dot plot) and CD3-positive (CD3<sup>+</sup>; lower dot plot) subpopulation by MACS using CD3 Ab. Separated cells were stimulated for 4 days with TKD (2 μg/ml) plus IL-2 (100 IU/ml) and phenotyped using CD3<sup>FITC</sup> and CD16/56<sup>PE</sup> Abs. The dot plot diagrams of one representative separation is illustrated. Numbers in the dot plot diagram represent the mean percentage values of positively stained cells of three experiments. Concomitantly, the cytolytic activity of both effector cell populations (CD3<sup>-</sup>, ■; CD3<sup>+</sup>, □) was tested in a standard 4-h chromium release assay. Specific lysis of effectors was assessed against Hsp70 membrane-positive CX<sup>+</sup>, Colo<sup>+</sup>, and K562 tumor target cells. E:T ratios ranged from 1:1 to 10:1. The mean ± SEM from three independent separations is shown. **B**, Separation of PBL of three independent donors in CD94-positive (CD94<sup>+</sup>; upper dot plot) and CD94-negative (CD94<sup>-</sup>; lower dot plot) subpopulation by MACS using CD94 Ab. TKD-stimulated subpopulations were phenotyped using CD56<sup>FITC</sup> and CD94<sup>PE</sup> Abs. Concomitantly, the cytolytic activity of both effector cell populations (CD94<sup>+</sup>, ◆; CD94<sup>-</sup>, ◇) was tested against Hsp70 membrane-positive CX<sup>+</sup>, Colo<sup>+</sup>, and K562 tumor target cells. **C**, The CD94-positive subpopulations were further separated in a CD3-negative (CD94<sup>+</sup>CD3<sup>-</sup>; upper dot plot) and CD3-positive (CD94<sup>+</sup>CD3<sup>+</sup>; lower dot plot) cell population by MACS using CD94 and CD3 Ab. TKD-stimulated effector cells were phenotyped separately using CD56<sup>FITC</sup> and CD94<sup>PE</sup> Abs. Concomitantly, the cytolytic activity of both effector cell populations (CD94<sup>+</sup>CD3<sup>-</sup>, ▲; CD94<sup>+</sup>CD3<sup>+</sup>, △) was tested against Hsp70 membrane-positive CX<sup>+</sup> tumor target cells. Due to low cell numbers, cytolytic activity against K562 cells could not be tested.



**FIGURE 5.** TKD induces migration selectively in CD94-positive NK cells. **A**, Migration of CD94-positive NK cells is inducible by human recombinant Hsp70 and TKD. CD94-positive NK cells were derived from MACS. Following stimulation with TKD (2 µg/ml) plus IL-2 (100 IU/ml) for 4 days, migration toward medium supplemented with Hsp70 (●) or TKD (○) was tested at concentrations ranging from 0.1 to 10 µg/ml. Results are shown as the mean ± SEM of three independent experiments. Statistically significant differences (Student's *t* test), \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ . **B**, TKD induces chemotaxis but not chemokinesis. Migration of CD94<sup>+</sup> NK cells, stimulated with TKD (2 µg/ml) plus IL-2 (100 IU/ml) for 4 days, was tested in a positive gradient (migration; 1 µg/ml TKD in the lower compartment), equal gradient (kinesis I; equal concentrations of 1 µg/ml TKD in the upper and lower compartments), or a negative gradient (kinesis II; 1 µg/ml TKD only in the upper compartment). Results are shown as the mean ± SEM of three independent experiments. Statistically significant differences between chemotaxis and chemokinesis (Student's *t* test), \*\*,  $p < 0.005$ . **C**, Migration of CD94-positive NK cells to TKD is concentration dependent and highly specific. TKD-activated CD94-positive NK cells were tested for their capacity to migrate toward TKD at concentrations ranging from 0.1 to 10 µg/ml TKD (left panel; ●). Two closely related peptides derived from Hsc70 (aa 450–463; ○) and DnaK (aa 447–460; ▲) were included as controls. All peptides were diluted in RPMI 1640 medium containing 10% FCS. Identical experiments were performed with the CD94-depleted T cell subpopulation (CD94<sup>-</sup>; right panel). Results are shown as the mean ± SEM of five independent experiments. Statistically significant differences compared with initial values (Student's *t* test), \*,  $p < 0.05$ ; \*\*,  $p < 0.005$ .

melanoma, MCA 102 sarcoma, lung carcinoma, MADB106 mammary carcinoma, and RMA-S lymphomas have been found to be infiltrated with NK cells (11, 33–36). In these models, attraction could be associated with chemokines, a large family of small proteins involved in lymphocyte trafficking, and cytokines (37, 38).

In the present study, we record on a cytokine- and chemokine-independent stimulus inducing selective migration of NK cells. Incubation of PBL with Hsp70 peptide TKD enhances the cytolytic activity against Hsp70-positive tumor target cells. Coincubation and migration assays revealed infiltration of TKD-stimulated PBL selectively into Hsp70 membrane-positive, but not into Hsp70 membrane-negative tumor cell clusters. Therefore, we hypothesized that predominantly Hsp70-positive tumor cells possess

chemotactic properties. To answer the question whether cell-to-cell contact or a soluble factor is responsible for migration, in additions to cells, supernatants of Hsp70 membrane-positive and -negative tumor cells were tested with respect to their capacity to induce migration. Our results indicate that a soluble factor is secreted by Hsp70 membrane-positive tumor cells. Recent data demonstrate Hsp70 can be released by tumor cells expressing Hsp70 on their cell surface (31, 39). Although the molecular nature of the chemotactic factor secreted by Hsp70 membrane-positive tumor cells remained elusive, we could demonstrate that NK cells were attracted by Hsp70 protein and TKD peptide in a tumor cell-free setting. Due to the fact that migration was found only toward a positive gradient, chemokinesis or random cell mobility appears to be unlikely.

A comparative phenotypic characterization of the migrated and nonmigrated cell population revealed that predominantly CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK cells but not CD3-positive T cells migrated toward supernatants of Hsp70 membrane-positive tumor cells. The C-type lectin receptor CD94 is covalently linked to members of the NKG2 family (40). CD94/NKG2A, an inhibitory, and CD94/NKG2C, an activating receptor, are recognizing HLA-E. It appeared that cell surface homeostasis of the inhibitory receptor is independent of functional signaling (41). CD94 Ab blocking (32), and binding studies (28) indicate that, apart from HLA-E, presenting leader peptides of HLA-A, -B, and -C molecules, Hsp70 and Hsp70 peptide TKD provide an additional positive ligand for an activating CD94 receptor. This finding is further supported by the fact that, although CX<sup>+</sup> and CX<sup>-</sup> tumor sublines are lacking HLA-E cell surface expression (42), Hsp70 membrane-positive tumor cells are lysed significantly better by CD94<sup>+</sup> migrated NK cells as compared with their Hsp70 membrane-negative counterparts. Cell sorting via CD3 and CD94 further confirmed these findings. Although, in addition to CD94, the expression of NKG2A was comparably up-regulated in the migrating cell population, these NK cells showed a strong cytolytic activity against Hsp70 membrane-positive target cells. Because, irrespective of the NK cell activity (inhibitory/activatory), NKG2A was always co-expressed with CD94 (data not shown), the specificity of this Ab for inhibitory NK cells is doubtful. Additional experiments will elucidate the molecular nature of the coreceptor for NK cells with Hsp70 reactivity.

Interestingly, migration of CD94-positive NK cells toward Hsp70 peptide TKD appears to be highly selective, because closely related HSP70 peptides with 2- or 7-aa exchanges were unable to stimulate migration. This observation is in line with our previous findings, that the stimulation of the cytolytic activity of NK cells is also restricted to TKD and Hsp70 protein, the major stress-inducible member of the HSP70 family. Other members of this group, including the constitutively expressed hsc70 (84% homology to Hsp70) or the *E. coli*-derived Hsp70 homolog DnaK (50% homology to Hsp70), failed to stimulate cytotoxicity (32). Furthermore, protein database BLAST search revealed that the 14-mer sequence TKD is a highly variable region within the Hsp70 sequence and thus is not found in any other protein sequence registered so far (43). In this study, the uniqueness of this sequence has been demonstrated with respect to its migratory function. However, TKD stimulation was found to be a prerequisite to generate sensitivity for the chemotactic signal. No migration was observed if the effector cells were not prestimulated with TKD plus low-dose IL-2.

The role of chemokines in the migration of TKD-activated CD3<sup>-</sup>CD56<sup>+</sup>CD94<sup>+</sup> NK cells could be ruled out for several reasons. No differences in the expression pattern of chemokine receptors was found following stimulation of NK cells with TKD

(data not shown). Differences in the chemokine secretion pattern correlating with Hsp70 membrane expression was also unlikely, because NK cell attraction was also possible with TKD peptide in a tumor cell-free setting. Because Hsp70 membrane-positive and -negative tumor sublines were generated by cell sorting of one parental tumor cell line, both sublines exhibited an identical expression of MHC and adhesion molecules (22).

Adoptive transfer of TKD-stimulated NK cells in tumor-bearing mice revealed that predominantly Hsp70-positive tumors were eliminated (44–46). In this report, we show directed migration of NK cells to Hsp70-positive tumor cells, and supernatants derived thereof, and to TKD peptide. Therefore, it was speculated that killing of Hsp70-positive tumors *in vivo* might be related to an enhanced migratory capacity of CD94-positive NK cells.

It is known that IL-2-activated NK cells can induce regression of established lung and liver tumors (47–50). However, the anti-tumor effect is often limited, because primary tumors and metastases are not efficiently infiltrated by NK cells. The prestimulation of CD94-positive NK cells with TKD in combination with low-dose IL-2 results in cytolytic active effector cells with the ability to migrate toward Hsp70-positive tumors that have to be defeated. Therefore, our findings might have further clinical implications with respect to the development of an NK cell-based cellular immunotherapy.

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