Targeting membrane heat-shock protein 70 (Hsp70) on tumors by cmHsp70.1 antibody

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Immunization of mice with a 14-mer peptide TKDNNLLGRFELSG, termed "TKD," comprising amino acids 450–461 ($aa_{450–461}$) in the C terminus of inducible Hsp70, resulted in the generation of an IgG1 mouse mAb cmHsp70.1. The epitope recognized by cmHsp70.1 mAb, which has been confirmed to be located in the TKD sequence by SPOT analysis, is frequently detectable on the cell surface of human and mouse tumors, but not on isogenic cells and normal tissues, and membrane Hsp70 might thus serve as a tumor-specific target structure. As shown for human tumors, Hsp70 is associated with cholesterol-rich microdomains in the plasma membrane of mouse tumors. Herein, we show that the cmHsp70.1 mAb can selectively induce antibody-dependent cellular cytotoxicity (ADCC) of membrane Hsp70$^+$ mouse tumor cells by unstimulated mouse spleen cells. Tumor killing could be further enhanced by activating the effector cells with TKD and IL-2. Three consecutive injections of the cmHsp70.1 mAb into mice bearing CT26 tumors significantly inhibited tumor growth and enhanced the overall survival. These effects were associated with infiltrations of NK cells, macrophages, and granulocytes. The Hsp70 specificity of the ADCC response was confirmed by preventing the antitumor response in tumor-bearing mice by coinjecting the cognate TKD peptide with the cmHsp70.1 mAb, and by blocking the binding of cmHsp70.1 mAb to CT26 tumor cells using either TKD peptide or the C-terminal substrate-binding domain of Hsp70.

immunotherapy | syngeneic tumor model | tumor antibody dependent cellular cytotoxicity | epitope mapping | surface antigen

Although the combination of mAbs with standard therapies plays a pivotal role in the treatment of cancer (1–4), the therapeutic success of this strategy is restricted by the availability of tumor-specific antibodies. Global profiling of the surface proteome of human tumors has revealed an abundancy of stress proteins in the plasma membrane (5, 6). Herein, we describe the generation of a mouse mAb directed against a 14-mer peptide TKDNNLLGRFELSG (TKD) of the major stress-inducible heat-shock protein 70 (Hsp70, Hsp70-1, Hsp72, HspA1A #3303), which is present on the cell surface of human tumor cell lines (7). Screening of a large number of primary human tumor biopsies and the corresponding normal tissues has indicated that carcinomas, but none of the tested normal tissues, frequently present Hsp70 on their cell surface (8, 9). Moreover, a membrane Hsp70$^+$ tumor phenotype has been found to be associated with a significantly decreased overall survival in patients with lower rectal and lung carcinomas. The expression of this molecule might therefore serve as a negative prognostic marker (9) in these patient groups. It has been hypothesized that membrane Hsp70 might support the spread of distant metastasis or might confer resistance to standard therapies (10).

The TKD sequence, which is exposed to the extracellular milieu of tumors, resides in the C-terminally localized oligomerization domain of the Hsp70 molecule (11). Furthermore, this TKD peptide in combination with low-dose IL-2 has been found to stimulate the migratory and cytolytic activity of NK cells against membrane Hsp70$^+$ tumor cells (12). In contrast to other commercially available Hsp70 antibodies, the cmHsp70.1 mAb uniquely identifies the membrane form of Hsp70 on viable tumor cells with an intact plasma membrane in vitro.

We have recently shown that the cmHsp70.1 mAb also binds to the CT26 mouse colon tumor cells in vivo (13). Herein, we demonstrate that consecutive injections of the cmHsp70.1 mAb into mice bearing CT26 tumors can significantly reduce the mass of membrane Hsp70$^+$ tumors and increase overall survival during therapy via the induction of antibody-dependent cellular cytotoxicity (ADCC). The in vitro ADCC activity could be further enhanced by using TKD/IL-2–activated NK cells as effector cells instead of unstimulated mouse spleen cells. These findings suggest that membrane Hsp70 could serve as a unique immunotherapeutic target for a broad spectrum of different tumor entities.

Results

Monoclonal Antibody cmHsp70.1 Binds to Membrane Hsp70$^+$ Human and Mouse Tumors. The epitope of the cmHsp70.1 mAb, which was generated by immunizing mice with the 14-mer peptide TKD, was confirmed by SPOT analysis and peptide blocking studies (Fig. S1). Viable human tumor cell lines, such as colon (CX2), breast (MDA436, MCF-7), and lung (A549) carcinomas and malignant melanomas (Malme, Mel Ei, Mel Ho, Parl, A375, Sk Mel29) bind cmHsp70.1 mAb, but not the Hsp70-specific SPA810 mAb (Fig. 1*A*). The cmHsp70.1 mAb also stains immortalized endothelial cells (EA.hy926, HMEC), but not their nontransformed, isogenic counterparts (primary ECs) (Fig. 1*B*, *Upper* two panels). Similar to the human tumor cell lines, single-cell suspensions of primary human gastrointestinal and pancreatic tumor samples (n = 229) also frequently (more than 40% of all tested tumor cases) bind cmHsp70.1 mAb, whereas the corresponding reference tissues are always membrane Hsp70$^-$.

With respect to mouse tumors, the cmHsp70.1 mAb binds to CT26 colon (61%) (Fig. 1 *C* and *D*) and highly malignant B16F10 mouse melanoma cells (74%) (Fig. 1*B*, *Lower Right*), whereas only a minor population of the isogenic, low-malignant counterpart B16F0 (14%) (Fig. 1*B*, *Lower Left*), 1048 pancreatic carci-

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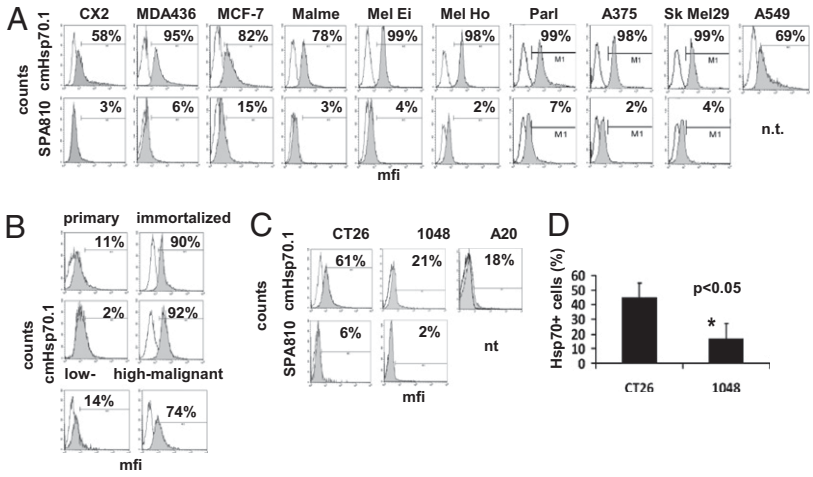

Fig. 1. Representative flow cytometric histograms of membrane Hsp70 expression on human and mouse tumor cell lines. (*A*) Human CX2 (colon), MDA436 (breast), MCF-7 (breast), Malme, Mel Ei, Mel Ho, Parl, A375, Sk Mel29 (all malignant melanomas), A549 (lung) carcinoma, (*B*) primary and immortalized endothelial cells, and (*C*) mouse CT26 (colon), 1048 (pancreas), A20 (B lymphoma), B16F0 (low-malignant melanoma), and (*B*) B16F10 (high-malignant, metastatic melanoma), tumor cell lines were analyzed by flow cytometry using either cmHsp70.1-FITC (*Upper*) or SPA810-FITC (*Lower*) mAb. Results are expressed as log green fluorescence intensity vs. relative cell numbers. The IgG1 isotype-matched control is indicated in white and membrane Hsp70 staining in gray histograms. The whole staining procedure was performed at 4 °C and only viable, 7-AAD$^-$ cells were gated upon and analyzed. (*B*) Comparison of the membrane Hsp70 expression in isogenic human and mouse cells. Human primary endothelial cells (ECs, *Top* and *Middle*) were compared with their corresponding immortalized partner cell lines EA.hy926, a fusion product of human umbilical vein ECs (HUVEC) with A549 lung carcinoma cells, and HMEC, which was derived by a transfection of primary ECs with SV40 large T antigen, were stained with cmHsp70.1 mAb, as described above. Furthermore, the low-malignant mouse melanoma cell line B16F0 (*Left*) was compared with the high-malignant, metastatic tumor cell line B16F10. The IgG1 isotype-matched control is indicated in white and membrane Hsp70 staining using cmHsp70.1 mAb in gray histograms. (*D*) The flow cytometric analysis of CT26 and 1048 tumor cell lines using cmHsp70.1-FITC mAb was repeated six times. The differences in membrane Hsp70 positivity in CT26 and 1048 tumor cell lines was significant (*$P < 0.05$).

noma (21%) (Fig. 1 *C* and *D*), and A20 B-lymphoma cells (18%) (Fig. 1*C*) are membrane Hsp70$^+$. The staining of cytosolic Hsp70 was excluded in all experiments, as only viable, 7-AAD$^-$ tumor cells with intact plasma membranes were gated and analyzed.

Monoclonal Antibody cmHsp70.1 Initiates ADCC in Membrane Hsp70$^+$ Tumors in Vitro. Measurements using fluorescence-conjugated marker beads revealed that ≈10,000 Hsp70 molecules are present on the plasma membrane of CT26 mouse tumor cells (13). Despite this relatively low surface density, 50 μg/mL cmHsp70.1 mAb could induce significant ADCC-mediated killing of CT26 carcinoma cells by unstimulated mouse spleen effector cells at E:T ratios ranging from 50:1 to 6.25:1 (Fig. 2*A*) ($P < 0.05$). The 1048 carcinoma cells that contained only a small proportion of Hsp70$^+$ cells were not sensitive to ADCC (Fig. 2*A*). As a control, the capacity of other mouse IgG1 antibodies (SPA810, Ox7.11) and the cmHsp70.1 Fab fragment to induce ADCC was assessed and compared with that of cmHsp70.1 mAb. As shown in Fig. 2*B*, neither SPA810 mAb nor cmHsp70.1 Fab induced any significant ADCC against membrane Hsp70$^+$ CT26 tumor cells. Similar negative findings were obtained if mouse BW cells (hybrid cross between New Zealand Black and White mice) transfected with theta (56% membrane theta$^+$ cells) were used as target cells for ADCC (Fig. 2*C*). In the same experiment, cmHsp70.1 mAb induces significant ADCC in CT26 colon adenocarcinoma cells (60% membrane Hsp70$^+$ cells) (Fig. 2*C*).

To determine whether preactivating mouse spleen cells with TKD (2 μg/mL) plus IL-2 (100 IU/mL) improves the killing of membrane Hsp70$^+$ CT26 cells in vitro, ADCC experiments were repeated using unstimulated and preactivated effector cells. The stimulation of mouse spleen cells with TKD/IL-2 significantly increased the proportion of CD49b$^+$ NK cells and CD25$^+$ cells (Table 1) ($P < 0.05$) and the lysis of CT26 cells (Fig. 2*D*) ($P < 0.01$). An element of this increase in cytolysis could be explained by a direct killing of membrane Hsp70$^+$ tumor cells by TKD/IL-2–activated NK cells (12), as it was apparent in the absence of the

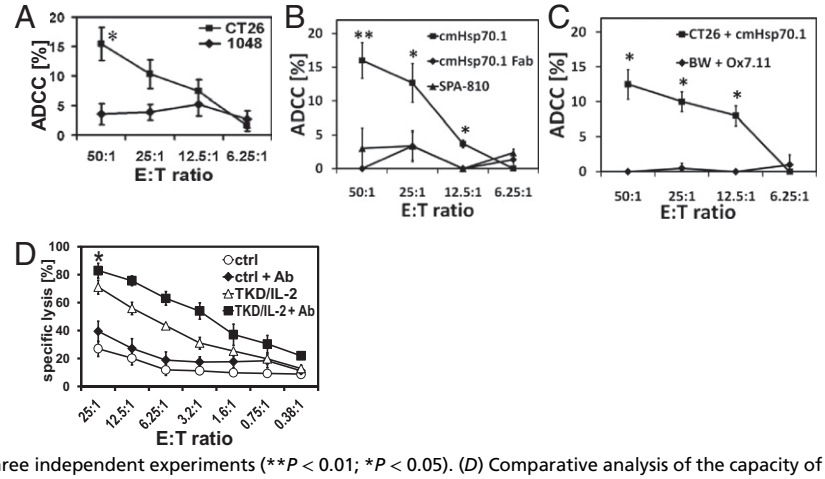

Fig. 2. Comparative analysis of ADCC using different IgG1 mAbs and cmHsp70.1 Fab fragment. (*A*) In vitro ADCC of membrane Hsp70$^+$ mouse CT26 colon (60%, filled squares) and membrane Hsp70$^-$ 1048 pancreatic carcinoma cells (filled diamonds), using 50 μg/mL Hsp70.1 mAb and unstimulated mouse spleen cells at E:T ratios ranging from 50:1 to 6.25:1. (*B*) In comparison with cmHsp70.1 mAb (filled squares), no significant ADCC was induced in mouse CT26 colon carcinoma cells (61% cmHsp70.1$^+$) using the non-binding IgG1 mAb SPA810 (filled circles) or cmHsp70.1 Fab fragment (filled triangles). (*C*) The IgG1 Ox7.11 mAb, which detects the theta antigen on 56% of the BW mouse tumor cells, does not induce ADCC in BW mouse tumor cells (filled triangles). Specific ADCC was measured using 50 μg/mL antibody or Fab fragment, respectively; unstimulated mouse spleen cells at E:T ratios ranging from 50:1 to 6.25:1 were used as effector cells. Specific lysis mediated by the direct cytotoxic effect of NK cells in the absence of cmHsp70.1 mAb was subtracted. The phenotypes of the effector cells are summarized in Table 2. Data are means ± SE of at least three independent experiments (**$P < 0.01$; *$P < 0.05$). (*D*) Comparative analysis of the capacity of unstimulated (ctrl, open circles; ctrl+Ab, filled diamonds) and TKD (2 μg/mL) plus IL-2 (100 IU/mL) preactivated (TKD/IL-2, open triangles; TKD/IL-2+Ab, closed squares) mouse spleen cells to kill CT26 carcinoma cells. The ADCC experiment was performed either in the absence (open symbols) and presence (+Ab; closed symbols) of 50 μg/mL cmHsp70.1 mAb. Lysis is mediated by ADCC in the presence of cmHsp70.1 mAb and by a direct cytotoxic effect of mouse NK cells in the absence of mAb, at E:T ratios ranging from 25:1 to 0.38:1. Data are means ± SE of at least three independent experiments. Lysis of activated effector cells in the absence and presence of cmHsp70.1 mAb was significantly different (*$P < 0.05$, all E:T ratios). ADCC was calculated using the formula: percent of specific lysis = (experimental release − spontaneous release)/(maxiumum release − spontaneous release) × 100.

Table 1. Proportion (%) of marker-positive cells in unstimulated and TKD/IL-2 preactivated mouse spleen cells

Antigen	Proportion of antigen-positive cells (%)	
	Unstimulated	TKD/IL-2–stimulated
CD8 (T cells)	11.2 ± 1.3	13.8 ± 3.4
CD4 (T cells)	21.1 ± 0.9	15.4 ± 5.9
CD205 (granulocytes)	6.4 ± 3.3	12.0 ± 5.4
CD11c (APC)	6.4 ± 3.2	8.4 ± 5.5
Ly6G/Ly6C (Gr-1)	8.7 ± 4.7	7.5 ± 3.0
B220 (B cells)	62.9 ± 5.4	61.0 ± 5.3
CD11b (APC)	14.6 ± 2.9	18.2 ± 4.9
CD49b (NK cells)	12.8 ± 4.7	22.5 ± 4.0*
CD25 (activation marker)	6.9 ± 4.7	9.7 ± 7.1*

*$P < 0.05$, corrected for multiple testing.

cmHsp70.1 mAb (Fig. 2*D*). However, the presence of cmHsp70.1 mAb further enhanced the cytolytic activity of unstimulated and TKD/IL-2–stimulated mouse spleen cells against membrane Hsp70$^+$ CT26 cells. The differences in the killing of CT26 tumor cells by TKD/IL-2–activated mouse spleen cells in the presence and absence of cmHsp70.1 mAb can be viewed in a movie which illustrates two major findings: the targeted migration of effector cells toward membrane Hsp70$^+$ tumor cells, which is enhanced in the presence of the cmHsp70.1 mAb and the concerted attack of tumor cells by effector cells (Movie S1).

ADCC in Tumor-Bearing Mice. An intraperitoneal injection of 2.5×10^4 CT26 mouse colon tumor cells suspended in 100 μL PBS resulted in rapidly growing tumors with a tumor take of 100%. A comparative phenotyping of cultured CT26 and single-cell suspensions derived from CT26 tumor-bearing mice on day 14 revealed the proportion of membrane Hsp70$^+$ cells to be significantly greater in the latter (46.2 ± 9%, $n = 6$ vs. 69.8 ± 14%, $n = 7$; $P < 0.05$).

Based on our observation that the cmHsp70.1 mAb initiates ADCC in membrane Hsp70$^+$ CT26 cells in vitro, the capacity of this antibody to induce tumor killing in CT26 tumor-bearing mice was evaluated. The tumor weights in mice that received two and three consecutive intravenous injections of cmHsp70.1 mAb (20 μg per injection) on days 3, 5, and 7 were significantly lower than those in mice receiving an isotype-matched control antibody (1.7 ± 0.63 g vs. 0.59 ± 0.32 g and 0.44 ± 0.29 g, respectively, $P < 0.05$) (Fig. 3*A*).

Immunohistochemical studies of consecutive CT26 tumor sections following one to three injections of cmHsp70.1 mAb revealed a dramatic increase in F4/80$^+$ macrophages and Ly6G/Ly6C$^+$ granulocytes, and a moderate increase in Ly49b$^+$ CD56$^+$ NK cells within the tumor (Fig. 3*B* and Table 2). CD3$^+$ T cells began to infiltrate tumor tissue from day 21 onwards (Table 2).

Growth curves of CT26 tumors after subcutaneous injection of 1×10^6 cells after one and three intravenous injections of cmHsp70.1 mAb (20 μg per injection) on days 4, 7, and 10 revealed that three repeated injections of cmHsp70.1 mAb resulted in a significant growth delay (Fig. 3*C*) ($P < 0.05$), which correlated with an increased overall survival (Fig. 3*D*) ($P < 0.05$). In line with these findings, overall survival was also greater in mice with intraperitoneal CT26 tumors (Fig. 4*A*, filled squares) ($n = 24$, $P < 0.0001$) than their IgG1 isotype-matched control antibody treated counterparts (Fig. 4*A*, open circles) ($n = 14$). In contrast, an identical treatment regimen had no significant effect ($P = 0.310$) on the survival of mice bearing A20 B-cell lymphomas, which lack membrane Hsp70 expression (Fig. 4*B*). Furthermore, the decrease in tumor weight after three intravenous injections of cmHsp70.1 mAb was associated with a significant increase in serum levels of Hsp70 on day 14 (154 ± 41.7 pg/mL vs. 1,434.5 ± 786 pg/mL, $n = 4$, $P < 0.01$), as measured by ELISA.

TKD Peptide Is the Target for ADCC. Coinjection of cmHsp70.1 mAb (20 μg per injection) with an excess of Hsp70 peptide TKD (50 μg per injection) into CT26 tumor-bearing mice (intraperitoneally) on days 3, 5, and 7 completely reversed the antitumoral effect of the antibody therapy ($P < 0.02$) (Fig. 5*A*). This finding indicated that the TKD peptide, which contains the epitope of the cmHsp70.1 mAb, competes with membrane Hsp70 on the cell surface of mouse tumors for binding in vivo.

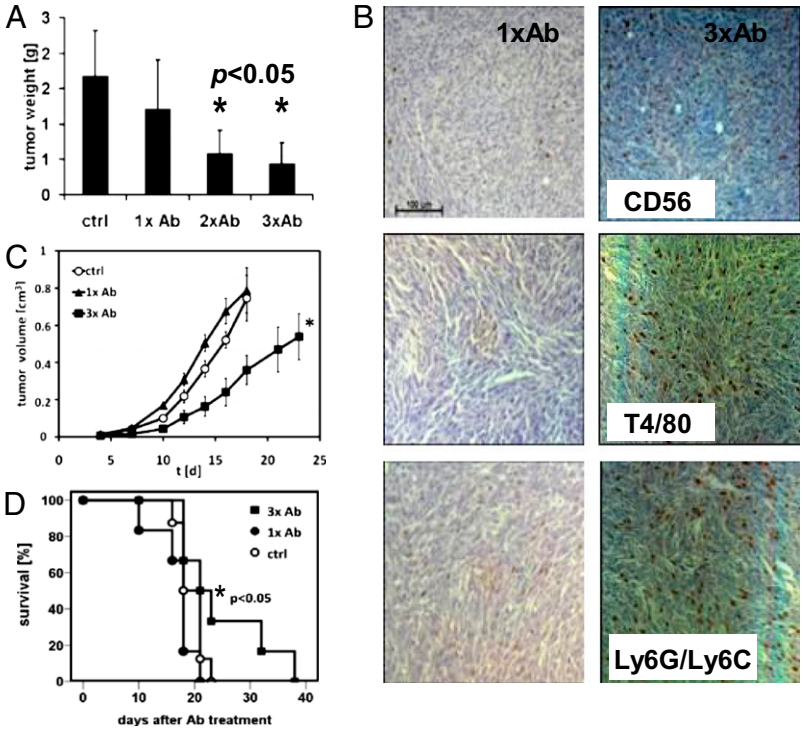

Fig. 3. Reduction in tumor weight and the delay of CT26 tumor growth in BALB/c mice after one to three injections of cmHsp70.1 mAb is associated with an infiltration of immunocompetent effector cells. (*A*) Two and three consecutive injections of cmHsp70.1 mAb (intravenously) result in a significant reduction in tumor weight (*$P < 0.05$). The cmHsp70.1 mAb (20 μg per injection) was injected intravenously on days 3, 5, and 7 following intraperitoneal injection of 2.5×10^4 CT26 tumor cells. Mice were killed on day 14 and tumor weights were determined. Data are means of six to nine animals (*$P < 0.05$). (*B*) Representative photomicrographs of CT26 tumor sections after one (1 × Ab; *Left*) and three consecutive injections of cmHsp70.1 mAb (3 × Ab; *Right*) on days 3, 5, and 7 (20 μg per injection). Infiltration of NK cells (CD56; *Top*), monocytes (T4/80; *Middle*) and granulocytes (Ly6G/Ly6C; *Bottom*) was determined on consecutive sections of CT26 tumors derived from mice on day 14. Semiquantitative data are summarized in Table 2. (Scale bar, 100 μm.) (*C*) Three (filled square) but not one (filled triangle) injections of cmHsp70.1 mAb (i.v.) result in a significant growth delay of subcutaneously injected CT26 tumors (*$P < 0.05$). The cmHsp70.1 mAb (20 μg per injection) was injected intravenously on days 4, 7, and 10 following subcutaneous injection of 1×10^6 CT26 tumor cells. Tumor weight was measured in each mouse every second day after the last antibody injection (*$P < 0.05$ for all time points from day 10 onwards). (*D*) Control mice (open circles) and mice that were injected only once with mAb cmHsp70.1 (filled circles, day 5) became moribund from day 18 onwards, whereas mice that were injected three times (filled squares, day 4, 7, 10) with cmHsp70.1 mAb showed a significant increase in overall survival (*$P < 0.05$). Each datapoint represents measurements of six to nine mice.

Table 2. Semiquantitative analyses of the lymphocytic and granulocytic infiltration of CT26 tumors after one to three injections of the cmHsp70.1 mAb

Marker	Treatment with cmHsp70.1 mAb			
	Ctrl	1×	2×	3×
CD3ε (T cells)	—	—	—	+
Ly49b/CD56 (NK cells)	+	+	++	+++
F4/80 (macrophages)	++	++	++	+++
Ly6G/Ly6C (Gr-1) (granulocytes/macrophages)	+	++	++	+++

BALB/c mice were injected (intraperitoneally) with CT26 tumor cells (2.5×10^4) on day 0 and injected with cmHsp70.1 mAb (20 μg per injection) on days 3, 5, and 7. Mice were killed on day 21 and at least six consecutive tumor sections (5 μm) were examined immunohistochemically using antibodies directed against T cells (CD3ε), NK cells (Ly49b), monocytes (F4/80), and granulocytes (Ly6G/Ly6C). The results indicate the number of infiltrating cells within a defined tumor section of 1 cm^2; —, no infiltration (<10); +, weak infiltration (10–50); ++, intermediate infiltration (50–200); +++, strong infiltration (<200). Representative images of consecutive tumor sections stained with Ly49, CD56, F4/80, and Ly6G/Ly6C antibodies are illustrated in Fig. 3*B*.

The specificity of the interactions was further confirmed by determining whether the binding of cmHsp70.1 mAb to the cell surface of cultured CT26 tumor cells could be blocked by the TKD peptide, which represents the immunogen (Fig. S1), but not by a 14-mer scrambled NGL(NGLTLKNDFSRLEG) peptide consisting of the same amino acid residues in a different order. The proportion of membrane $Hsp70^+$ cells decreased in a concentration-dependent manner from 59% (white graph) to 44% (gray graph; 12.5 μg/mL) and from 60% (white graph) to less than 15% (gray graph; 25 μg/mL) (Fig. 5*B*). In contrast, no inhibition in binding was apparent when the same concentrations of NGL peptide were used for the blocking experiments (Fig. 5*B*, *Right*). As a control, the binding of cmHsp70.1 mAb to CT26 cells was also significantly inhibited using the C-terminal substrate binding domain of Hsp70 ($P < 0.05$) (Fig. 5*C*). All blocking studies were performed at 4 °C because of the rapid internalization of cmHsp70.1 mAb at higher temperatures (13).

Discussion

Hsp70 mediates the stability of tumor cells following environmental stress (14, 15), and it is commonly regarded as an intracellular molecule. However, it is now apparent that a membrane

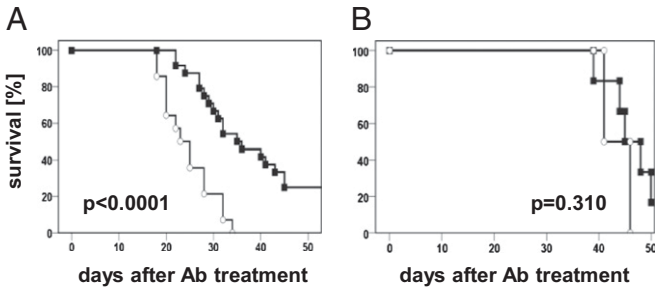

Fig. 4. (*A*) Kaplan-Meyer curves of overall survival of mice treated with an isotype-matched control antibody or cmHsp70.1 mAb on days 3, 5, and 7 after intraperitoneal injection of 2.5×10^4 CT26 tumor cells (20 μg per injection). The overall survival of mice (3 × Ab cmHsp70.1, filled squares; $n = 24$) treated with cmHsp70.1 mAb was significantly higher than that of animals (ctrl, open circles; $n = 14$) that received the IgG1 isotype-matched control antibody ($P < 0.0001$). (*B*) In contrast, the cmHsp70.1-mAb treatment (3 × Ab cmHsp70.1, filled squares) had no significant effect on the survival of mice bearing membrane $Hsp70^-$ A20 lymphomas ($n = 12$) compared with mice receiving the IgG1 isotype-matched control antibody (ctrl, open circles; $n = 4$, $P = 0.310$).

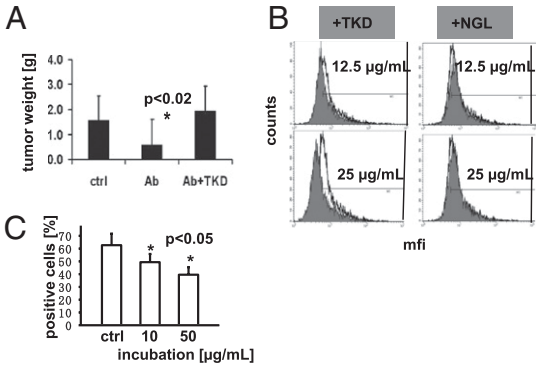

Fig. 5. (*A*) Coinjection of an excess of the Hsp70 peptide TKD with the cmHsp70.1 mAb completely inhibits the significant antitumoral effect of the latter (*$P < 0.02$). The cmHsp70.1 mAb (20 μg per injection) was coinjected intravenously on days 3, 5, and 7 together with 50 μg TKD following intraperitoneal injection of 2.5×10^4 CT26 tumor cells; media, $n = 21$; Ab, $n = 22$; Ab+TKD, $n = 21$. Mice were killed on day 14 and tumor weights were determined. (*B*) Binding of cmHsp70.1-FITC mAb to CT26 tumor cells was inhibited by the coincubation with an excess of TKD peptide. As a control, the scrambled NGL peptide was used. Tumor cells were coincubated either with cmHsp70.1-FITC mAb (5 μg/mL; white histogram) or with cmHsp70.1-FITC mAb (5 μg/mL) plus TKD (gray histogram; *Left*) or NGL peptide (gray histogram; *Right*) at concentrations of 12.5 and 25 μg/mL, respectively. The data illustrate one representative experiment out of three independent experiments, all of which show similar results. (*C*) Binding of cmHsp70.1-FITC to CT26 tumor cells was inhibited significantly (*$P < 0.05$) by the coincubation with the C-terminal substrate-binding domain of Hsp70 in a concentration dependent manner (10 and 50 μg/mL).

form of Hsp70 is frequently expressed on a broad variety of human tumors, but not on the corresponding normal tissues. This membrane expression can be specifically detected using the cmHsp70.1 mAb, which has been generated using the human 14-mer TKD sequence as an immunogen. Because the human and murine TKD sequences only differ in one amino acid (16) (human T**K**DNNLLGRFELSG; mouse T**R**DNNLLGRFELSG), we also screened mouse tumor cell lines for their capacity to express the membrane form of Hsp70. Similar to human tumors (7, 8), the mouse colon tumor cell line CT26 (17) was also membrane $Hsp70^+$. The depletion of cholesterol from the plasma membrane reduced the membrane density of Hsp70 on CT26 mouse colon tumor cells (Fig. S2), whereas changes in the salt concentration or the pH—indicative of receptor-mediated associations—had no effect. These data demonstrate that the membrane form of Hsp70 in mice also appears to be located in cholesterol-rich microdomains (18, 19). Although the cmHsp70.1 mAb can specifically detect membrane Hsp70 expression on viable tumor cells, other commercially available Hsp70-specific antibodies cannot.

We have previously reported that a membrane $Hsp70^+$ phenotype serves as a negative prognostic marker for patients with lower rectal and lung carcinomas (9). Herein, we have demonstrated that immortalized, oncogenic-transformed and highly metastatic tumors, but not their primary counterparts and low-malignant cells, present Hsp70 on their cell surface. The study therefore assessed the capacity of membrane Hsp70 to act as a tumor-specific target for antibody-mediated killing of highly aggressive tumors using a syngeneic CT26 mouse-colon tumor model. Despite the relatively low density of Hsp70 molecules that are presented on the cell surface of CT26 mouse tumor cells (≈10,000 per cell), and that IgG1 has a low capacity to induce ADCC (20) and complement-dependent cytotoxicity (21) in mice, the cmHsp70.1 mAb mediates specific killing in membrane $Hsp70^+$ CT26 tumors. In contrast, other IgG1 control antibodies directed against theta or the Fab fragment of cmHsp70.1 mAb had no such effect. Furthermore, binding of cmHsp70.1 mAb to membrane $Hsp70^+$ tumors did not enhance the intracellular Hsp70 levels

(Fig. S2), and thus a cmHsp70.1 mAb-based therapy is not likely to enhance protection of tumors against Hsp70-mediated apoptosis.

We have previously demonstrated that the incubation of lymphocytes with Hsp70 peptide TKD in the presence of low-dose IL-2 results in an enhanced cytolytic and migratory capacity of NK cells toward membrane Hsp70$^+$ tumor cells in vitro and in vivo (12, 22). The direct cytolytic effects of TKD/IL-2–activated NK cells against membrane Hsp70$^+$ mouse tumors were clearly detectable in the current study, as has previously been described for human tumors (12, 23, 24). In a clinical phase I trial, the tolerability, feasibility and safety of adoptively transferred, autologous TKD/IL-2–activated NK cells has been shown in patients having colorectal and lung carcinomas (23). Here, we show that the in vitro cytotoxic effects of TKD/IL-2–activated NK cells against membrane Hsp70$^+$ tumor cells can be further improved by the addition of cmHsp70.1 mAb. This process is most likely mediated by ADCC. The cmHsp70.1 mAb-induced killing of membrane Hsp70 CT26 tumor cells involves an enhanced migratory capacity of effector cells and a direct cytotoxic attack.

Three intravenous injections of relatively low amounts of unconjugated cmHsp70.1 mAb into tumor-bearing mice induced an infiltration of innate immune cells and significantly reduced the growth of CT26 tumors. The finding that the membrane Hsp70-positivity of CT26 tumors derived from mice autopsies was greater than that of in vitro cultured CT26 cells might explain the cmHsp70.1 mAb-mediated ADCC effect.

We have also previously reported that membrane Hsp70$^+$ tumors actively release Hsp70 surface-positive lipid vesicles (25), which have the biophysical characteristics of exosomes (26, 27) and that these can attract activated, but not resting NK cells. The current study found a significant increase in circulating Hsp70 in those mice in which tumor growth was inhibited. Whether this serum Hsp70 originates from exosomes or from necrotic tumor material has not yet been elucidated. Furthermore, ongoing studies are evaluating whether the administration of low-dose IL-2 into tumor-bearing mice might further improve the antitumoral effect of cmHsp70.1 mAb via the in vivo activation of mouse NK cells.

Remarkably, three consecutive intravenous injections of relatively low amounts of cmHsp70.1 mAb not only delayed the growth of subcutaneous- and intraperitoneal-residing CT26 tumors, but also significantly prolonged the survival of the mice. The Hsp70-specificity of this approach is supported by the finding that cmHsp70.1 mAb had no effect on tumor growth or the survival of mice bearing membrane Hsp70$^-$ A20 B-cell lymphomas. Moreover, coincubating membrane Hsp70$^+$ tumors with an excess of TKD peptide or the C-terminal substrate-binding domain and cmHsp70.1 mAb blocked the antibody binding in vitro, and TKD peptide also completely reversed the antitumoral effect in vivo. These data confirm that the TKD peptide sequence represents the recognition site of cmHsp70.1 mAb (Fig. S1).

As radiochemotherapy has been shown to enhance the cell-surface density of Hsp70 on tumors (10, 28–30), we speculate that a combined approach consisting of an Hsp70 mAb-based immunotherapy, which involves activated NK cells, as has been shown for a Her2-targeted ADCC (31), might provide a previously unexplored strategy to improve the clinical outcome of patients undergoing standard radiochemotherapy or with distant metastases. This proposition is in line with the observation that a metastasis-free survival rate of patients can be associated with an enhanced NK cell activity (32). The clinical relevance of our data are further supported by published observations on the ADCC activity of trastuzumab in metastatic breast cancer patients (33). In this study, the in vitro ADCC activity toward Her2 overexpressing tumor cells, which was quantitatively comparable to that which was seen against Hsp70 membrane-positive tumor cells in the current study, could be correlated to the short-term antitumor responses in trastuzumab-treated breast cancer patients (33).

Materials and Methods

Human Tumor Cell Lines, Mouse Tumor Cell Lines, and Primary Cells. Human tumor cell lines: CX2 (colon), MCF-7, MDA436 (breast), and A549 (lung) carcinoma cell lines (Tumorbank Deutsches Krebsforschungszentrum, Heidelberg, Germany), Malme, Mel Ei, Mel Ho, Parl, A375 and Sk Mel29 malignant melanomas (J. Johnson, Institute of Immunology, Ludwig-Maximilians-Universität Munich, Germany) (34). Mouse tumor cell lines: CT26 (colon, CT26.WT; ADCC CRL-2638, BALB/c) (17), 1048 (pancreatic), A20 (B-cell lymphoma, BALB/c) (35), B16F0 (low malignant, C57BL/6), B16F10 (high malignant, C57BL/6), BW transfected with the theta antigen (E. Kremmer, Helmholtz-Zentrum München, Munich, Germany). Cells were cultured in RPMI 1640 or DMEM supplemented with 10% (vol/vol) heat-inactivated FCS, 2 mM L-glutamine, 1 mM sodium-pyruvate and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin) at 37 °C in 5% (vol/vol) CO_2. Single-cell suspensions were derived by short-term (less than 1 min) treatment with 0.25% (wt/vol) Trypsin-0.53 mM EDTA.

Primary macrovascular HUVECs and their isogenic EC counterparts EA.hy.926, which results from a fusion of HUVECs with the epithelial lung-carcinoma cell line A549, and HMEC, which were obtained by a transfection of primary microvascular ECs with the coding region for the simian virus 40A gene product (SV40) large T antigen, were cultured in ECGM medium supplemented with Supplement Mix (Sigma Aldrich). Cell-culture reagents were purchased from Life Technologies and Sigma Aldrich.

Tumor specimens and corresponding normal tissues were obtained from patients at the University Regensburg, Germany between February 2002 and January 2004. Fresh biopsy material was washed in antibiotic (penicillin/streptomycin) containing DMEM and single-cell suspensions were prepared by mincing the tissue and forcing it through a sterile mesh. The corresponding normal tissue was derived from the same patients at a distance of at least 0.2 cm from the tumor. The study was approved by the Institutional Review Board of the Medical Faculty of the University Hospital Regensburg, Germany and all patients included in the study provided signed informed consent.

Flow Cytometry and Blocking of Binding. The membrane Hsp70 phenotype on tumor cells was determined by flow cytometry using either the FITC-conjugated cmHsp70.1 mAb (IgG1; Multimmune GmbH), which is directed against the extracellular exposed sequence of membrane Hsp70 or the SPA810 mAb (IgG1; Stressgen via Assay Designs). Briefly, after incubation of viable cells (0.2×10^6 cells) with the primary antibodies for 30 min at 4 °C and following two washing steps, 7-AAD$^-$ viable cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). An isotype-matched (IgG1) control antibody was used to determine nonspecific binding to cells. Blocking of the antibody binding was performed by coincubating viable tumor cells (0.2×10^6 cells) using cmHsp70.1-FITC mAb (5 μg/mL) and an excess of TKD or scrambled NGL peptide (12.5 and 25 μg/mL) or the C-terminal substrate binding domain of Hsp70 (aa 383–548, 10 and 50 μg/mL).

The proportion of lymphocyte subpopulations, monocytes, granulocytes, and their expression of the activation marker CD25 (α chain of the IL-2 receptor) was determined using FITC/PE-labeled mAb directed against CD4, CD8, CD205, CD11c, Ly6G/Ly6C (Gr-1), B220, CD11b, CD49b, CD56 and CD25 (BD Biosciences).

Animals. BALB/c mice were obtained from an animal breeding colony (Harlan Winkelmann) and maintained in pathogen-free, individually ventilated cages (Tecniplast). Animals were fed with sterilized, laboratory rodent diet (Meika) and were used for experiments between 6 and 12 wk of age. All animal experiments were approved by the "Regierung von Oberbayern" and were performed in accordance with institutional guidelines of the Klinikum rechts der Isar, Technische Universität München.

Stimulation of Mouse Spleen Cells for ADCC. Freshly isolated BALB/c mouse spleen cells (5×10^6 cells/mL) were cultured in RPMI medium 1640 containing 10% (vol/vol) FCS alone (unstimulated) or medium containing low-dose IL-2 (100 IU/mL) plus TKD peptide (2 μg/mL) (Bachem) at 37 °C for 4 d. TKD is a GMP-grade 14-mer peptide of the C-terminal substrate binding domain of human Hsp70 (TKDNNLLGRFELSG, $aa_{450–463}$), which is known to selectively induce the reactivity of human NK cells against membrane Hsp70$^+$ tumor cells (12). The TKD equivalent region in the mouse (TRDNNLLGRFELSG) exhibits only one conservative amino acid exchange at position 2 (K-R) and this sequence stimulates mouse NK cells, even in the absence of IL-2 (16).

ADCC and Blocking Assays. ADCC was measured using a standard 4 h ^{51}Cr-release assay (36, 37). For blocking, labeled target cells were preincubated with the cmHsp70.1 mAb, the IgG1 isotype-matched control mAbs, SPA810 mAb, the theta-specific Ox7.11 (50 μg/mL, each), or the cmHsp70.1 Fab fragment (50 μg/mL). The degree of ADCC-dependent cytotoxicity was calculated, from which the lysis mediated by NK cells in the absence of cmHsp70.1 mAb/isotype control was subtracted. The spontaneous release for each target cell ranged

between 10 and 15%. Complement-dependent cytotoxicity was performed using identical experimental conditions, but in the absence of effector cells.

Intraperitoneal and Subcutaneous Injection of Tumor Cells. Tumor cells were thawed from a common frozen stock and cultured in vitro for 2 to 3 d before use. Next, 2.5×10^4 CT26 or 8×10^5 A20 cells (35) were injected intraperitoneally. For the growth-delay experiments, 1×10^6 CT26 cells were injected subcutaneously (neck) in BALB/c mice using a 1-mL plastic syringe and a 22-gauge needle. The injection was visually controlled using a 7× Stereomicroscope (Zeiss). The cmHsp70.1 mAb was injected either once on day 4 or on days 4, 7, and 10 after subcutaneous tumor injection and the tumor volume was determined every second day using a caliper and confirmed using ultrasound (GE Healthcare).

Injection of Antibodies and the 14-mer Hsp70 Peptide TKD. For the immunotherapeutic approach, mice were injected with unconjugated cmHsp70.1 mAb (i.v., 20 μg mAb per injection) or an IgG1 isotype-matched control antibody on days 3, 5, and 7 after the injection of CT26 cells (i.p., 2.5×10^4). For the inhibition assays, 20 μg cmHsp70.1 mAb was coinjected with an excess of the TKD peptide (TKDNNLLGRFELSG; 50 μg/mL per injection; purity >97%, EMC Microcollections GmbH) on days 3, 5, and 7 after an intraperitoneal tumor cell injection.

Autopsy. Control mice and cmHsp70.1 mAb treated mice were killed by craniocervical dislocation. The peritoneal cavity was macroscopically inspected for tumor dissemination and the primary tumors were excised in total, and their weights determined.

Immunohistochemistry. After weighing, tumors were cut into 4-mm thick pieces, fixed in Bouin's solution containing 71.5% (vol/vol) picric acid, 23.8% (wt/vol) formaldehyde, 4.7% (vol/vol) acetic acid, and embedded in paraffin. Consecutive section-pairs of the tumors (5 μm) were prepared from the ventral margin of each piece for a distance of 250 μm. The morphology of the excised tumors was visualized using standard H&E and Masson-Goldner staining. Nuclei were costained in 1% (wt/vol) Mayer's Hematoxylin (Dako). For the immunohistochemistry, endogenous peroxidase activity was blocked using freshly prepared 1% (vol/vol) H_2O_2 containing 0.1% (wt/vol) NaN_3. For the detection of effector cells, sections were heated for 30 min at 97 °C and then incubated with anti-NK cell (clone DX5, 1:25, rat–anti-mouse CD49b, IgM; Biozol; clone 12F11, 1:100, rat–anti-mouse CD56; BD Biosciences), anti-T cell (clone 145–2C11, 1:50, hamster–anti-mouse CD3ε, IgG; Biolegend; clone SP7, 1:100, rabbit–anti-goat CD3; Abcam), anti-macrophage (clone BM8, 1:50, rat–anti-mouse F4/80, IgG2a; ACRIS Antibodies GmbH, 1:50), anti-granulocyte/macrophage (clone RB6-8C5, 1:50, rat–anti-mouse Gr-1 Ly6C/Ly6G, IgG2bκ; Biolegend) mAbs or the appropriate isotype-matched control reagent overnight at 4 °C. After washing, sections were incubated for 2 h at room temperature with a rabbit anti-rat or rabbit anti-hamster HRP-conjugated secondary polyclonal antibody preparations as appropriate (Dako) followed, after washing, by diaminobenzidine (Dako) as the chromogen. Sections were counter stained with 1% (wt/vol) Mayer's Hematoxylin (Dako) for 30 s and analyzed on an Axiovert 25 microscope (Zeiss).

Statistical Analysis. Comparative analysis of in vitro data was undertaken using a nonparametric log-rank test (Mann-Whitney). Survival times were estimated from Kaplan-Meyer curves by log-rank test (38).

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