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Apoptosis of a Human Melanoma Cell Line Specifically Induced by Membrane-Bound Single-Chain Antibodies

Concepción de Inés,* Björn Cochlovius,† Stefanie Schmidt,* Sergey Kipriyanov,* Hans-Jürgen Rode,* and Melvyn Little†‡*

CD28 is a key regulatory molecule in T cell responses. Ag-TCR/CD3 interactions without costimulatory signals provided by the binding of B7 ligands to the CD28R appear to be inadequate for an effective T cell activation. Indeed, the absence of B7 on the tumor cell surface is probably one of the factors contributing to the escape of tumors from immunological control and destruction. Therefore, to increase the immunogenicity of tumor cell vaccines, we have expressed anti-CD3 and anti-CD28 single-chain Abs (scFv) separately on the surface of a human melanoma SkMel63 cell line (HLA-A*0201). A mixture of cells expressing anti-CD3 with cells expressing anti-CD28 resulted in a marked activation of allogeneic human PBL in vitro. The apparent induction of a Th1 differentiation pathway was accompanied by the proliferation of MHC-independent NK cells and MHC-dependent CD8+ T cells. PBL that had been cultured with transfected SkMel63 tumor cells were able to specifically induce apoptosis in untransfected SkMel63 cells. In contrast, three other tumor cell lines expressing HLA-A*0201, including two melanoma cell lines, showed no significant apoptosis. These results provide valuable information for both adoptive immunotherapy and the generation of autologous tumor vaccines. The Journal of Immunology, 1999, 163: 3948–3956.

T here are several means by which tumor cells can overcome an immune attack. These include a high rate of proliferation, the invasion of normal tissue, and a high metastatic potential. Moreover, cancer cells are poor immunogens. The concentration of tumor-associated Ag is often very low, and the cells usually lack costimulatory molecules for T cell activation (1–3).

One of the recent strategies for provoking an immune reaction has been to train the patients’ own T cells to destroy cancer cells. Optimal stimulation of T cells first requires the engagement of the TCR/CD3 complex. This leads to a series of signal transduction events that are critical for the activation of T cell functions during an immune response. However, a costimulatory signal is required to initiate cytotoxic activity, and this is usually provided by the interaction of the CD28 molecule on the surface of T cells with members of the B7 ligand family on the surface of APC. Activation of T cells in the absence of a costimulatory signal may result in an inability to produce IL-2 leading to anergy (4–6).

In the present study, which is aimed at the development of an autologous tumor vaccine, we have transformed tumor cells with DNA coding for scFv1 against either CD3 (7) or CD28. ScFv represent the smallest functional fragment of an Ab that maintains the complete specificity of the whole Ab. They are heterodimers composed of VH and VL domains bound together and stabilized by a flexible peptide linker (8–10). The scFv carried a secretion signal peptide at their N-terminus and were bound to a membrane-binding peptide at their C-terminus for anchoring them to the cell surface. In this study, we show that these surface-bound scFv were able to induce the appropriate signals in vitro for T cell stimulation and cytolytic activity. Evidence is also presented for the induction of a Th1 differentiation pathway, and we demonstrate the proliferation of MHC-independent NK cells and MHC-dependent CD8+ T cells. Finally, we show that it is possible to induce the specific DNA fragmentation of an untransfected human melanoma cell line with PBL that had been incubated with a mixture of the same cells transfected with anti-CD3 and anti-CD28 scFv, respectively.

Materials and Methods

Eukaryotic expression constructs

DNA coding for the anti-CD3 scFv (7) and anti-CD28 scFv was a kind gift of Dr. S. Kipriyanov and Dr. S. Schmidt (Recombinant Antibody Group, German Cancer Research Center, Heidelberg, Germany), respectively. They were cloned into the retroviral expression vector p50-M-x-neo (pMESV), as described elsewhere (11).

Cell culture

Media, sodium pyruvate, nonessential amino acids, and vitamins were from Life Technologies (Eggenstein, Germany); antibiotics were from Seromed (Berlin, Germany); and FCS was from PAA Laboratories (Linz, Austria). The murine fibroblasts GP+E-86 (12) and the human melanoma cell line SkMel63 (kindly supplied by Dr. A. Knuth, Nordwest-krankenhaus, Frankfurt/Main, Germany) were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. The murine colon adenocarcinoma CT26 (American Type Culture Collection ATCC, Manassas, VA) was grown in MEM containing 5% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, and vitamins. The human melanoma cell lines SkMel25, BLM, MML-Ib, MML-I, and COLO44; the human colon adenocarcinoma SW480; the human cervical carcinoma HeLa (all from ATCC); and the human cell lines Jurkat (CD3+ T lymphoma), HPB-ALL (CD28+ T lymphoma), and K562 (erythroleukemic) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All cell lines were grown at 37°C in a humidified 5% CO2 air atmosphere.
Stable cell transfections

Transfection of GP+E-86 cells was performed with 20 μg of each pMESV-scFv construct by the calcium phosphate precipitation method (13). The SkMel63 cell line was transfected with 20 μg of each EcoRI-linearized pMESV-scFv construct using the polylysine/DNA transfection method, as described previously (14). In all cases, cells were selected in 1 mg/ml genticin sulfate (G418; Life Technologies) for 14 days at 37°C, and the G418-resistant cells were cloned by limiting dilution in the presence of this antibiotic.

Immunofluorescence staining

The intracellular expression of scFv was visualized by indirect immunofluorescence. The cells were grown on eight-well multistest slides (ICN Biomedicals, Eschwege, Germany), washed with PBS, and fixed with PBS/2% formaldehyde for 30 min at room temperature (RT). The cells were then permeabilized with PBS/1% Triton X-100 for 10 min at RT, washed three times in PBS, and incubated in PBS/20% FCS for 30 min at RT. After incubation, the cells were stained with the following primary Ab diluted 1/100 in PBS/20% FCS for 1 h at 37°C: polyclonal rabbit serum A (Ab) (15) or anti-μ-myc mouse mAb 9E10 (Cambridge Research Biochemicals, Wiltshire, DE), and washed three times in PBS. Cells were developed for 45 min at 37°C with FITC-conjugated affinity-purified goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) or FITC-conjugated affinity-purified goat anti-mouse IgG (Dianova, Hamburg, Germany) diluted 1/2000 in the same buffer. After washing three times in PBS, photographs were taken using a Zeiss Axioshot fluorescence microscope and Kodak (Rochester, NY) Ektachrome film.

The extracellular expression of the scFv was determined by incubating the live cells with 1100 anti-α-tubulin rat mAb YOL1/34 (16) (Harlan, Sera-Lab, Belton, U.K.), washed three times in PBS, and incubated with 1200 of FITC-conjugated affinity-purified goat anti-rat IgG (Dianova). In all cases, the incubations were performed for 30 min at 37°C.

Cell surface analysis

Cells were plated onto 13-mm tissue culture coverslips (Sarstedt, Newton, NC) at a density of 2 × 10⁵ and cultured for 24 h at 37°C. The medium was removed and the coverslips were incubated with 2 × 10⁶ Jurkat, HPB-ALL, or K562 cells for 2 h at 37°C. Finally, the cells were washed three times with 2 ml PBS and fixed with PBS/2% formaldehyde for 1 h at RT.

Extraction of cellular membrane scFv by temperature-inducible phase partitioning with Triton X-114 and Western blotting

The membrane-anchored scFv were solubilized by phase partitioning with the nonionic detergent Triton X-114, as described (17), but with some modifications. A total of 60–120 × 10⁶ cells was suspended in 1 ml of ice-cold PBS/1 mM PMSF/10 mM EDTA and disrupted with a glass-glass Potter homogenizer. The cell homogenate was centrifuged at 5,850 × g for 10 min at 4°C. The supernatant (membrane phase) was removed and the coverslips were incubated with 2 ml PBS/0.2% Triton X-114 at 37°C for 30 min at RT. The cells incubated with PBS/0.2% Triton X-114 were considered in all calculations. The membrane phase was centrifuged at 4°C for 10 min. The supernatant (nonmembrane phase) was removed from the Triton X-114 solution, and the Triton X-114 was added to a final concentration of 1%. The solution was vigorously mixed, kept on ice for 20 min, and incubated at 28°C for 10 min. The phase separation was accelerated by centrifuging the sample at 4,600 × g for 5 min at RT. Finally, the proteins from the aqueous and detergent phases were precipitated with 10 vol of acetone at −20°C for 1 h and pelleted at 13,000 × g for 10 min. The scFv were identified by SDS-PAGE on 12% polyacrylamide gels under reducing conditions (18). ScFv on nitrocellulose filters were revealed using mAb 9E10 or serum A as first Ab and the respective second Ab coupled to HRP using diaminobenzidine as substrate.

Cell proliferation assay

Untransfected SkMel63 cells as well as those transfected with anti-CD3 or anti-CD28 scFv were irradiated (150 Gy) and added in triplicate to 96-well round-bottom microtiter plates (Greiner, Solingen, Germany) at a density of 1 × 10⁶ cells/well. In the case of both SkMel63 transfectants, 5 × 10⁶ or 1 × 10⁷ anti-CD3 SkMel63-transfected cells were mixed with 5 × 10⁶ or 1 × 10⁷ anti-CD28 SkMel63-transfected cells, respectively. The cells were then mixed with human PBL (E:T ratio of about 1:2) obtained from an allogeneic healthy donor (HLA-A*0201), as described (19). These cells were grown at 37°C for 3 days and then pulsed with 1 μCi [3H]thymidine (6-b-[3H]thymidine; Amershams, Buckinghamshire, U.K.) per well during the last 18 h of culture. The [3H]thymidine uptake was determined by liquid scintillation counting. Three independent experiments were performed in triplicate, and the proliferation of PBL was expressed as the mean cpm corrected for that of PBL incubated alone under the same conditions.

Flow cytometry analysis

The human PBL were grown in 24-well plates (Falcon) under the same conditions as described above. They were then collected and incubated at 4°C for 1 h with 50 μl of anti-CD3 (OKT3), anti-CD4 (OKT4), anti-CD8 (OKT8), anti-β2 (OKT9), and HNK-1 (anti-human NK cells; T. Abo and C. Balch, University of Alabama, Birmingham, AL) mAb. The cells were then washed three times with 2 ml PBS and incubated with 1:10 diluted pMESV-scFv construct by the calcium phosphate precipitation method, as described above (17). The scFv on nitrocellulose filters were incubated for 45 min at 37°C, washed three times with PBS, and incubated with 1:1000 diluted affinity-purified goat anti-mouse IgG (Amersham, Amersham International, U.K.) diluted 1/5000 for 30 min at 4°C. Cells were washed again and suspended in PBS containing 0.5 μg/ml propidium iodide (Sigma-Aldrich, Deisenhofen, Germany) to exclude dead cells. The fluorescence intensity corrected for that of PBL incubated under the same conditions but without primary Ab was determined using a Coulter (Palo Alto, CA) FACScan analyzer for three independent experiments.

Analysis of human cytokine production

The cytokine production of PBL and PBL depleted of CD4⁺ T cells after stimulation for 3 days, as described above, was determined by flow-cytometric analysis. To deplete PBL of CD4⁺ T cells, six-well plates (Falcon) were incubated at 5 ml/well with the OKT4 mAb diluted 1/100 in BC buffer (34 mM NaHCO₃, 3 mM NaH₂PO₄, pH 9.6) for 24 h at 4°C. The plates were washed three times in PBS, incubated in PBS/10% FCS for 1 h at 37°C, and washed once in PBS. The B cells were then added to the plates at a density of 5 × 10⁶ cells/well and incubated for 2.5 h at 37°C. PBL depleted of CD4⁺ T cells were gently removed from the plates. The cytokines were assayed by intracellular staining according to the manufacturer’s instructions using carboxyfluorescein-conjugated mAb against human IL-2, IL-4, or IFN-γ (R&D Systems, Wiesbaden-Nordenstadt, Germany). The human PBL were grown in 24-well plates, as described above, employing 15 × 10⁶ cells/well and 30 × 10⁵ SkMel63 cells/well at 37°C for 3 days. The preactivated human PBL were then incubated with a panel of target cells (30 × 10⁴ cells/well) for 4–5 h at 37°C in culture medium alone (control cells) or with human PBL that had been activated for 3 days, as described above. They were washed again and incubated with PBS/0.2% Triton X-100 for 30 min at 4°C. Some control cells (without incubation with PBL) were incubated in culture medium alone under the same conditions to evaluate their viability. The plate was centrifuged, and the radioactivity present in the supernatant (fragmented DNA) and pellet (intact DNA) was determined by liquid scintillation counting. The percentage of DNA fragmentation was obtained from the ratio of fragmented to total DNA. The data from control cells incubated with PBS/0.2% Triton X-100 were considered in all calculations, and they were very similar to control cells not incubated with this buffer. Three individual experiments were made in triplicate.

Alternatively, the DNA fragmentation in target cells was quantified using a Cell Death Detection ELISA kit (Boehringer Mannheim, Mannheim, Germany). The human PBL were grown in 24-well plates, as described above, employing 15 × 10⁶ cells/well and 30 × 10⁵ SkMel63 cells/well at 37°C for 3 days. The preactivated human PBL were then incubated with a panel of target cells (30 × 10⁴ cells/well) for 4–5 h. Cells incubated in culture medium alone under the same conditions were used as controls. The target cells were washed twice with PBS and they were processed according to the manufacturer’s instructions. Three independent experiments were made in duplicate.

Results

Analysis and selection of stable transfected cells

To express anti-CD3 and anti-CD28 scFv on the surface of tumor cells, we first cloned their genes into the retroviral expression vector p50-M-x-neo (pMESV) (11). After transfection into the human melanoma cell line SkMel63, cells with stably integrated scFv genes were selected with the neomycin marker. We also transfected the murine fibroblast cell line GP+E-86 to compare the transformation efficiencies, because this cell line has already been successfully used to express an anti-phOx scFv on the cell surface (11).
Figure 1. Immunofluorescence localization of anti-CD3 and anti-CD28 scFv on the cell surface. Viable GP-E+86 cells were stained with mAb YOL1/34. Untransfected cells (A); cell surface expression of anti-CD3 scFv (B); cell surface expression of anti-CD28 scFv (C). Bar = 20 μm.

Positive cells were examined by indirect immunofluorescence. Intracellular expression of anti-CD3 or anti-CD28 scFv was detected by fixing and permeabilizing the cells, followed by incubation with serum A, which recognizes the N-terminal epitope of secreted scFv on Western blots (15). A strong fluorescence appeared to be associated with membranes of the endoplasmic reticulum surrounding the nucleus and sometimes with Golgi vesicles throughout the cytoplasm that appeared as bright points (not shown).

Immunofluorescence analysis of cell surface expression of the scFv
A marker peptide recognized by the mAb YOL1/34 (16) is present in the linker joining the VH and VL domains. Staining with this Ab, we observed a strong immunofluorescence of the transfected GP-E+86 cells (Fig. 1), but not with the transfected SkMel63 cell line. These differences may be due to the particular morphological characteristics of the two cell lines. For example, conformational changes of membrane proteins and topographic rearrangements have been found in cancer cells (22). Alternatively, the scFv may be expressed on the surface in amounts too low for detection.

The scFv are anchored on the cell surface and are functional
To test the attachment of the scFv to cellular membranes, the integral membrane proteins were extracted with Triton X-114. Proteins from both the aqueous (cytosolic proteins) and the detergent phase (membrane proteins) were resolved by Western blotting using the mAb 9E10. The detergent phase of the transfected cells contained anti-c-myc-stained scFv proteins with apparent m.w. of about 35.6 and 34 kDa. Similar proteins, but only in very low concentrations, were observed in the aqueous phases. It seems unlikely that the two bands correspond to proteins with different degrees of glycosylation because the variable domains are rarely glycosylated (23). Two possible explanations are: 1) proteolytic degradation of the scFv and 2) the existence of processed and unprocessed scFv due to incomplete cleavage of the IL-6R leader peptide during secretion. To address the second possibility, we repeated the same experiment using serum A as the first Ab. Similar results were obtained as for detection with anti-c-myc, except for an additional protein with an apparent m.w. between 31 and 32 kDa that was mainly in the aqueous phases. It therefore appears that the two detergent-soluble scFv proteins arise from proteolytic degradation, presumably due to cleavage of the C-terminal cytoplasmic tail sequence (not shown).

To confirm that the scFv were indeed on the surface of the transfected SkMel63 cells, they were incubated with Jurkat (CD3+ T lymphoma), HPB-ALL (CD28+ T lymphoma), or K562 erythroleukemic cells (CD3-CD28-). As controls, scFv-transfected GP-E+86 cells, untransfected cells, and cells presenting the irrelevant anti-phOx scFv were used. A dense rosette of Jurkat cells formed around the transfected anti-CD3 GP-E+86 or SkMel63 cells, but only a few were bound to the same cells transfected with anti-CD28 scFv. Similarly, a dense rosette of HPB-ALL cells formed around the transfected GP-E+86 or SkMel63 cells presenting the anti-CD28 scFv, but only a few were bound to the same cells transfected with anti-CD3 scFv (Fig. 2). Further controls using untransfected cells and GP-E+86/anti-phOx cells showed very few bound Jurkat or HPB-ALL cells. Little or no binding of any transfected cells to K562 cells was seen. This indicates that the scFv are not only present on the cellular surface, but are functional and bind the respective Ag expressed on other cell surfaces.

Anti-CD3 and anti-CD28 scFv displayed on the surface of SkMel63 cells stimulate proliferation of human PBL
To test the ability of SkMel63 cells transfected with anti-CD3 or anti-CD28 scFv to stimulate T cell proliferation in vitro in the absence of exogenous cytokines, each one or a mixture of both was incubated for 3 days with allogeneic human PBL from a healthy donor.

Untransfected SkMel63 cells gave rise to a small increase in T cell proliferation, presumably because they are recognized as alloantigens by PBL. Cells carrying anti-CD3 induced a significant increase in the proliferation of PBL, but cells carrying anti-CD28 were much less effective. The largest increase in proliferation was achieved when both transfecants were cocultured with PBL. Moreover, this increase in lymphocyte proliferation was clearly demonstrated to be due to the synergistic effect of both transfecants when the PBL were cocultured with the anti-CD3 and anti-CD28 SkMel63 transfecants using the same number of cells as for each transfecant alone (Fig. 3).

SkMel63 transfecants induce Th1 cytokine production
To determine which subset of T lymphocytes was activated in response to the different SkMel63 transfecants, human PBL were analyzed by flow cytometry after 3 days of incubation for expression of CD3, CD4, and CD8 markers. As shown in Fig. 4, a significant fraction of the cells displayed these Ag on their surface after incubation, either alone (Fig. 4A), or together with untransfected SkMel63 cells (Fig. 4B), cells transfected with anti-CD3 (Fig. 4C), cells transfected with anti-CD28 (Fig. 4D), or with both transfecants together (Fig. 4E). Interestingly, a significant additional subpopulation of CD4+ cells appeared when PBL were stimulated with the transfected cells (Fig. 4, C, D, and E). This could also be observed to a lesser extent with untransfected cells, probably due to their recognition as alloantigens by PBL (Fig. 4B).
These data suggest that a CD4+ T cell differentiation had occurred, resulting in a possible Th1 or Th2 response. To test this further, the production of human IL-2, IL-4, and IFN-γ was monitored 3 days after incubation of the human PBL with the transfected and untransfected melanoma cells (Fig. 5). The highest production of human IL-2 and IFN-γ was seen when human PBL were activated by both SkMel63 transfecants. A significant production of both cytokines was also observed for human PBL activated with the anti-CD3-transfected cells alone. In contrast, untransfected cells or cells transfected with anti-CD28 induced only low amounts of these cytokines. Practically no human IL-4 production was detected in any of the incubations. The relatively high amounts of human IL-2 and IFN-γ produced suggest a predominantly Th1-type response (Fig. 5A). This finding was supported by experiments performed under the same conditions using PBL depleted of CD4+ cells (Fig. 5B). These PBL expressed much lower amounts of intracellular human IL-2 and IFN-γ.

SkMel63 transfecants induce cytolytic activity of PBL

To test whether the SkMel63 transfecants were able to induce cytolytic responses in activated human PBL, DNA fragmentation studies were conducted (see Materials and Methods). The DNA of target cells (untransfected SkMel63) was pulse labeled with [3H]thymidine, and the target cells were incubated for 4–5 h with prestimulated PBL at an E:T ratio of about 1:2. Target cells incubated with culture medium alone were used as controls. As shown...
in Fig. 6A, the induction of DNA fragmentation in target cells was only significant with PBL that had been incubated with both Sk-Mel63 transfectants or with the anti-CD3 transfectant alone. The highest percentage of DNA fragmentation was found after stimulation with both transfectants.

To investigate the relative amounts of NK and CD8 T cells, human PBL incubated for 3 days with the untransfected or transfected SkMel63 cells were analyzed by flow cytometry using OKT8, OKT9, and HNK-1 mAb. The expression of the TnR was also monitored, because this gives an approximate measure of relative T cell activation. After 3 days of incubation with untransfected cells, the amounts of the CD8 T and NK cells were approximately equal. A significant increase in the number of NK cells appeared after incubation with the anti-CD3 transfectant, and this incubation also showed the highest T cell activation. On the whole, however, no marked changes had occurred (Table I). To determine whether the transfectants were able to induce the proliferation of CD8 T cells after a second stimulation, human PBL were incubated with the SkMel63 variants for 5 days (first stimulation), removed and cultured alone without any stimulation for

**FIGURE 4.** Analysis of stimulated PBL by flow cytometry. Human PBL were cultured for 3 days alone (A), with untransfected SkMel63 cells (B), with SkMel63 cells transfected with anti-CD3 scFv (C), with SkMel63 cells transfected with anti-CD28 scFv (D), or with a 50:50 mixture of both transfectants (E). They were then collected and stained with anti-CD3 (OKT3), anti-CD4 (OKT4), or anti-CD8 (OKT8) mAb. In all cases, the PBL were incubated with the secondary Ab alone as a control.
another 5 days, and finally incubated again in the presence of SkMel63 transfectants for 2 days (second stimulation). No CD8\(^+\) T or NK cells were present in the incubation with either the untransfected cells or the anti-CD28 transfectant. A small number of both cell types was found in the incubation with the anti-CD3 transfec-
tant and a much larger number in the incubation with both trans-
fectants. A significant T cell activation as monitored by TnR ex-
pression was shown only for the incubation with both

Table I. Relative amount of human CD8\(^+\) T cells increases after a secondary stimulation with anti-CD3 and anti-CD28 SkMel63 transfectants

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD8(^+) T cells</td>
<td>NK</td>
</tr>
<tr>
<td>Untransfected</td>
<td>28.3(^b)</td>
<td>27.8</td>
</tr>
<tr>
<td>SkMel63/anti-CD3</td>
<td>24.3</td>
<td>40.0</td>
</tr>
<tr>
<td>SkMel63/anti-CD28</td>
<td>25.4</td>
<td>30.9</td>
</tr>
<tr>
<td>Both SkMel63 transfectants</td>
<td>22.1</td>
<td>33.3</td>
</tr>
</tbody>
</table>

*Human PBL were stimulated with untransfected SkMel63 cells or with the different SkMel63 transfectants for 3 days or
7 days (first stimulation for 5 days; no stimulation for 5 days; second stimulation for 2 days). CD8\(^+\) T cells, NK cells, and TnR expression was analysed by flow cytometry.

*Percentage of fluorescent-positive cells. The experiment was performed at least three times, and a representative result is shown. The data were corrected by subtracting the fluorescence intensity from PBL cultured alone.

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and untransfected SkMel63 cells, respectively, was evaluated by ELISA (Table II). The DNA fragmentation induced by PBL incubated alone for 3 days was similar to that induced by PBL preincubated with untransfected SkMel63 cells (data not shown). In all cell lines expressing HLA-A*0201 (the human melanoma cell lines SkMel63, BLM, and SkMel25, and the human colon adenocarcinoma SW480), DNA fragmentation was only significant for the untransfected SkMel63 target cells. PBL that had been incubated with a mixture of both SkMel63 transfectants (anti-CD3 and anti-CD28) gave the highest amounts of DNA fragmentation. Relatedly high amounts were also achieved using PBL that had been incubated only with anti-CD3-transfected SkMel63 cells. However, twice as many anti-CD3-transfected cells were used compared with incubations with both transfectants, which were a 50:50 mixture. A much lower DNA fragmentation in SkMel63 target cells was observed when they were incubated with PBL prestimulated with untransfected SkMel63 cells or with the anti-CD28 SkMel63 transfectant. No significant increases in DNA fragmentation were observed after a second round of stimulation (Table III). DNA fragmentation of the SkMel63 target cells incubated with PBL prestimulated with both SkMel63 transfectants was markedly higher than observed for any of the other cell lines. A smaller amount of DNA fragmentation was observed with PBL prestimulated with the anti-CD3 SkMel63 transfectant. No changes in DNA fragmentation were observed with PBL reincubated with untransfected SkMel63 cells or with the anti-CD28 SkMel63 transfectant. The results of incubating PBL with other cell lines expressing the HLA-A*0201 haplotype (the human melanoma cell lines SkMel25 and BLM, and the human colon adenocarcinoma SW480) were similar to those obtained using PBL prestimulated for only 3 days. In all cases, PBL reincubated with untransfected SkMel63 or SkMel63 transfected with anti-CD28 scFv did not give rise to any significant DNA fragmentation. Furthermore, the very large nonspecific reaction of the HLA-A*0201-negative cell lines previously seen after incubation with PBL stimulated for only one round was now reduced, in many cases, to almost baseline levels.

**Discussion**

Human and experimental tumors often express Ag that can be recognized by T lymphocytes. However, malignant tumors are evidently tolerated and ultimately progress to kill their host. One cause of tolerance may be a lack of costimulatory molecules for T cell activation. Several strategies for experimental tumor therapy have focused on transfecting tumor cells with members of the B7

### Table II. Induction of DNA fragmentation in tumor cells by PBL after one round of stimulation

<table>
<thead>
<tr>
<th>Target</th>
<th>HLA-A*0201 Expression</th>
<th>Untransfected(^a) SkMel63</th>
<th>Anti-CD3/SkMel63</th>
<th>Anti-CD28/SkMel63</th>
<th>Both SkMel63 Transfectants (50:50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkMel63</td>
<td>+</td>
<td>147.6 ± 4.0(^b)</td>
<td>607.2 ± 5.1</td>
<td>134.0 ± 5.1</td>
<td>670.0 ± 2.8</td>
</tr>
<tr>
<td>BLM</td>
<td>+</td>
<td>99.0 ± 2.0</td>
<td>137.4 ± 3.0</td>
<td>89.0 ± 3.6</td>
<td>130.0 ± 0.2</td>
</tr>
<tr>
<td>SkMel25</td>
<td>+</td>
<td>107.0 ± 2.3</td>
<td>115.0 ± 4.8</td>
<td>101.0 ± 2.4</td>
<td>104.3 ± 5.8</td>
</tr>
<tr>
<td>MML-Iib</td>
<td>-</td>
<td>181.0 ± 5.7</td>
<td>532.0 ± 16.0</td>
<td>255.0 ± 18.0</td>
<td>617.0 ± 32</td>
</tr>
<tr>
<td>MML-I</td>
<td>-</td>
<td>461.0 ± 31.0</td>
<td>600.0 ± 9.2</td>
<td>277.0 ± 17.0</td>
<td>507.0 ± 20.0</td>
</tr>
<tr>
<td>COLO44</td>
<td>-</td>
<td>170.0 ± 5.4</td>
<td>545.0 ± 16.0</td>
<td>80.7 ± 10.0</td>
<td>636.0 ± 37.5</td>
</tr>
<tr>
<td>SW480</td>
<td>+</td>
<td>97.4 ± 4.3</td>
<td>99.2 ± 5.2</td>
<td>98.0 ± 0.6</td>
<td>97.4 ± 5.2</td>
</tr>
<tr>
<td>HeLa</td>
<td>-</td>
<td>131.0 ± 5.4</td>
<td>338.0 ± 3.8</td>
<td>123.0 ± 3.8</td>
<td>370.0 ± 15.4</td>
</tr>
<tr>
<td>CT26</td>
<td>-</td>
<td>329.4 ± 0.8</td>
<td>770.6 ± 41.2</td>
<td>194.1 ± 0.0</td>
<td>847.0 ± 20.0</td>
</tr>
</tbody>
</table>

\(^a\) Human PBL were stimulated with untransfected SkMel63 cells or with the different SkMel63 transfectants for 3 days. The fragmented DNA in target cells induced by preactivated PBL was quantified by ELISA.

\(^b\) Percentage of fragmented DNA in target cells. The absorbance of fragmented DNA in control target cells (without incubation with human PBL) was 0.02 ± 0.002, 0.21 ± 0.022, 0.22 ± 0.036, 0.17 ± 0.000, 0.065 ± 0.000, and 0.09 ± 0.000 (human melanoma cell lines SkMel63, BLM, SkMel25, MML-Iib, MML-I, and COLO44, respectively), 0.21 ± 0.001 (human colon adenocarcinoma SW480), 0.13 ± 0.004 (human cervical carcinoma HeLa), and 0.17 ± 0.007 (murine colon adenocarcinoma CT26) and was considered to be 100%. The experiment was performed at least three times in duplicate.

### Table III. Induction of DNA fragmentation in tumor cells by PBL after two rounds of stimulation

<table>
<thead>
<tr>
<th>Target</th>
<th>HLA-A*0201 Expression</th>
<th>Untransfected(^a) SkMel63</th>
<th>Anti-CD3/SkMel63</th>
<th>Anti-CD28/SkMel63</th>
<th>Both SkMel63 Transfectants (50:50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SkMel63</td>
<td>+</td>
<td>155.7 ± 7.5(^b)</td>
<td>271.4 ± 2.1</td>
<td>134.3 ± 2.0</td>
<td>500.0 ± 2.7</td>
</tr>
<tr>
<td>BLM</td>
<td>+</td>
<td>100.0 ± 2.0</td>
<td>103.0 ± 0.8</td>
<td>91.0 ± 1.0</td>
<td>84.0 ± 2.0</td>
</tr>
<tr>
<td>SkMel25</td>
<td>+</td>
<td>97.0 ± 2.0</td>
<td>106.0 ± 8.0</td>
<td>70.0 ± 1.7</td>
<td>92.0 ± 1.3</td>
</tr>
<tr>
<td>MML-Iib</td>
<td>-</td>
<td>107.3 ± 9.1</td>
<td>154.5 ± 3.6</td>
<td>96.4 ± 9.1</td>
<td>134.5 ± 9.0</td>
</tr>
<tr>
<td>MML-I</td>
<td>-</td>
<td>128.0 ± 1.4</td>
<td>300.0 ± 12.0</td>
<td>162.0 ± 6.0</td>
<td>320.0 ± 3.8</td>
</tr>
<tr>
<td>COLO44</td>
<td>-</td>
<td>90.0 ± 1.5</td>
<td>92.3 ± 2.3</td>
<td>84.6 ± 2.3</td>
<td>90.0 ± 5.4</td>
</tr>
<tr>
<td>SW480</td>
<td>+</td>
<td>106.7 ± 1.7</td>
<td>130.8 ± 2.5</td>
<td>104.2 ± 2.5</td>
<td>114.2 ± 10.0</td>
</tr>
<tr>
<td>HeLa</td>
<td>-</td>
<td>126.0 ± 0.0</td>
<td>272.0 ± 6.0</td>
<td>132.0 ± 2.6</td>
<td>232.0 ± 2.8</td>
</tr>
<tr>
<td>CT26</td>
<td>-</td>
<td>84.2 ± 1.7</td>
<td>88.2 ± 3.5</td>
<td>78.8 ± 1.0</td>
<td>78.8 ± 1.2</td>
</tr>
</tbody>
</table>

\(^a\) Human PBL were stimulated with untransfected SkMel63 cells or with the different SkMel63 transfectants for 7 days (see Table I). The fragmented DNA in target cells induced by preactivated PBL was quantified by ELISA.

\(^b\) Percentage of fragmented DNA in target cells. The absorbance of fragmented DNA in control target cells (without incubation with human PBL) was 0.07 ± 0.001, 0.10 ± 0.013, 0.10 ± 0.010, 0.11 ± 0.013, 0.05 ± 0.002, and 0.13 ± 0.002 (human melanoma cell lines SkMel63, BLM, SkMel25, MML-Iib, MML-I, and COLO44, respectively), 0.12 ± 0.004 (human colon adenocarcinoma SW480), 0.05 ± 0.001 (human cervical carcinoma HeLa), and 0.17 ± 0.003 (murine colon adenocarcinoma CT26) and was considered to be 100%. The experiment was performed at least three times in duplicate.
family (24, 25). However, the transfected cells did not always induce an immune response (26, 27). A possible explanation was thought to be low levels of MHC class I molecules, although relatively high amounts were expressed by one of the nonimmunogenic cell lines (27).

In our own experiments, we were able to show a significant increase of T cell proliferation, as measured by thymidine incorporation after stimulation with only the anti-CD28-transfected SkMel63 tumor cells. However, we found no significant increase in the amount of IL-2 production, and only one of the nine tumor cell lines tested showed any increased amounts of DNA fragmentation. Other authors have described CD8-mediated increases in T cell proliferation (28, 29), but lysis of tumor cells has usually been accomplished by also engaging the CD3 signaling pathway using bispecific Ab that link up the CD3 molecule to a target Ag or using T cells that have been prestimulated with cytokines (30–34).

An immune response to unmodified tumor cells can be induced by transfected tumor cells (35, 36). The goal of the present work has been to increase the interaction and recognition of tumor cells with T cells by transfecting some of them with the gene for a membrane-binding anti-CD3 scFv and others with the gene for a membrane-binding anti-CD28 scFv. PBL cocultured with both SkMel63-transfected variants gave a marked increase in lymphocyte proliferation and produced cytokines typical for Th1 lymphocytes (human IL-2 and IFN-γ). After depleting the PBL of CD4+ T cells, they produced much lower amounts of these cytokines. PBL incubated only with the anti-CD3 transfectant also showed a significant response. However, comparisons should take into account that the incubations with both transfecteds contained only half the number of anti-CD3- and anti-CD28-transfected cells used for incubations with only one transfectant. For example, a reproducible synergistic effect on T cell proliferation was observed using the same cell number for each transfectant both combined and alone (Fig. 3). The IL-2 and IFN-γ cytokines are known to participate in the activation and tumoricidal activity of NK and CTL cells (32, 37). After 3 days of incubation, these cytokines appeared to have induced an effective cytolytic activity against untransfected SkMel63 cells (Fig. 6). Measurements of activated T cells by TnR expression showed that the amounts of CD8+ T cells after 3 days of incubation with all of the SkMel63 variants were fairly similar, whereas the number of NK cells was highest after incubation with the anti-CD3 transfectant. However, a dramatic loss of NK cells occurred on prolonged incubation. A similar loss of CD8+ T cells was observed in all cases, with the notable exception of PBL incubated with both transfecteds in which the amount of CD8+ T cells rose to 47% (Table I). Furthermore, only these PBL were able to induce any significant DNA fragmentation in untransfected SkMel63 cells.

The induction of apoptosis in the SkMel63 target cells did not appear to be caused by unspecifically activated T cells in an allogeneic setting because no DNA fragmentation was observed in any of the other three human tumor cell lines expressing the HLA-A*0201 haplotype. Furthermore, the large nonspecific reactions seen using HLA-A*0201-negative cells largely disappeared on using PBL that had been subjected to two rounds of stimulation. Interestingly, incubations of PBL with only the anti-CD28 scFv transfectant was not sufficient to induce a specific T cell cytotoxicity. However, in combination with anti-CD3 scFv transfectant, a clear synergistic effect on the stimulation of T cells specifically recognizing the untransfected target tumor cells was observed.

An explanation for the specific induction of apoptosis in the SkMel63 tumor cells could be the recognition of a tumor-associated Ag. For example, the peripheral blood of an HLA-A*0201 healthy donor was shown to contain tyrosinase-specific CTL that could be induced to lyse melanoma cells (38). Although the tyrosinase autoantigen is present in all melanocytes, CTL recognizing a peptide of this protein are apparently not subject to clonal deletion. It seems unlikely, however, that this same Ag is responsible for the T cell response to the SkMel63 cells, because no DNA fragmentation was induced in two other human melanoma cell lines expressing the HLA-A*0201 haplotype. The apoptosis of the target SkMel63 cells could also possibly be induced by a response of the PBL to more than one tumor-associated Ag.

Our results indicate that a mixture of tumor cells, some carrying anti-CD3 scFv and some carrying anti-CD28 scFv, may provide an effective means of inducing stimulated T cells in vitro for adoptive immunotherapy or for direct gene transfer into tumors in vivo. These and similarly modified tumor cells may be potent activators for producing a cytolytic T cell response in vivo. The question as to whether a combination of tumor cells bearing anti-CD3 and anti-CD28 can be used as a vaccine will shortly be investigated in animal experiments.

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References