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Perforin and IFN-γ Are Involved in the Antitumor Effects of Antibody-Targeted Superantigens

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The bacterial superantigen staphylococcal enterotoxin A (SEA) is a potent inducer of cytokine production and cytotoxic T cell responses. To target a T cell attack against tumor cells we have genetically engineered a fusion protein of SEA and the Fab part of the tumor-reactive mAb C215. Injection of this Fab-SEA fusion protein to mice carrying lung metastases of the poorly immunogenic B16 melanoma transfected with the C215 Ag resulted in infiltration of cytokine-producing T cells, perforin-containing CTL, and a marked tumor elimination. Fab-SEA therapy induced substantial levels of IFN-γ and TNF-α in serum. In the present study we have characterized the molecular mechanisms of the antitumor effect induced by Fab-SEA treatment in vivo. Neutralization of cytokines by specific Abs demonstrated a major role for IFN-γ in the suppression of tumor growth. In addition, a minor contribution of TNF-α was recorded. Injections of Fab-SEA into normal mice induced strong CTL activity but failed to promote cytotoxic function in perforin knockout mice. Also, a markedly reduced therapy was noted in perforin knockout mice, implicating a role for CTL in Fab-SEA-mediated tumor eradication. The data suggest that Fab-SEA-targeted T cells may suppress tumor growth by both perforin-dependent cytotoxicity and local release of cytokines such as IFN-γ. The latter mechanism may have an important role in cytostatic effects against Ag-negative bystander tumor cells. *The Journal of Immunology,* **1998, 160: 5309–5313.**

The existence of tumor-specific T cells has been demonstrated in several experimental animal models and in some types of human tumors. However, the frequency of tumor-specific T cells is generally too low and insufficient to interfere with progressive tumor growth. Expansion of tumor-infiltrating T cells (TIL)[2] in vitro by culture in the presence of IL-2 has shown promising results in patients with metastatic melanoma (1–3). The majority of the infused TIL, however, are trapped in liver, lung, or spleen, resulting in poor accumulation of tumor-reactive T cells in the tumor area (4, 5). This means that large numbers of TILs need to be infused to ensure some tumor localization.

T cells have been demonstrated to possess various potent antitumor functions. One mechanism consists of the directional release of cytotoxic effector molecules upon specific interaction with the target cell. This eventually leads to cell lysis and apoptosis (6). Perforin is a granule protein capable of forming transmembrane pores in a Ca^{2+}-dependent manner, but fails to elicit nuclear damage (7, 8). Granzymes A and B, two granule proteins belonging to the serine protease family, may penetrate perforin-formed transmembrane pores and induce apoptosis or necrosis of the target cell (9, 10). Another mechanism of T cell-mediated lysis of tumor cells involves the release of growth-suppressive cytokines such as IFN-γ and TNF-α. Several tumors have been shown to be sensitive to the growth-suppressive effects of these cytokines but the therapeutic use is generally hampered by systemic side effects.

Staphylococcal enterotoxin A (SEA) belongs to a family of bacterial superantigens that has the capacity to activate a large proportion of T cells to mediate cytotoxicity and cytokine secretion (11–13). Superantigens bind to MHC class II molecules as unprocessed proteins and subsequently activate T cells expressing particular T cell receptor Vβ-chains (TCR Vβ) (14, 15). To target a local T cell attack against tumor cells, we have genetically engineered fusion proteins with SEA and the Fab fragment of the tumor-reactive mAb C215 (16, 17). Treatment with Fab-SEA fusion proteins has proven to be highly efficient in the elimination of B16-C215 melanoma metastases (18). In the present report we have characterized the molecular mechanisms by which Fab-SEA induces elimination of established B16-C215 lung metastases. The results clearly demonstrate that multiple T cell effector functions, including release of IFN-γ and perforin-mediated cytotoxicity, act to eliminate B16-C215 lung metastases in Fab-SEA-treated animals.

Materials and Methods

Animals

Female C57BL/6J (H-2^b, Mls1^b-2^b) were purchased from Bomholtgaard (Ry, Denmark) and were routinely used at the age of 8 to 12 wk. Perforin KO mice on C57BL/6 background were provided by Dr. T. Schopp (University of Lausanne, Lausanne, Switzerland) and were bred in our animal facility.

Cell lines

The murine B cell lymphoma cell line A20, the murine B16 melanoma (American Type Culture Collection, Rockville, MD), and the rat hybridomas R46A2 (American Type Culture Collection) and V1q (provided by Dr. B. Echtenacher, Regensburg, Germany) were cultured in R-medium (RPMI 1640 supplemented with 10% FCS (Life Technologies, Paisley, Scotland), 1 mM glutamine (HyClone Europe, Cramlington, U.K.), 5×10^{-5} M β-ME (ICN Biomedicals, Costa Mesa, CA), 0.2% $NaHCO_3$ (Seromed Biochrome, Munich, Germany), 1×10^{-2} M HEPES (HyClone

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[2] Abbreviations used in this paper: TIL, tumor-infiltrating T cells; SEA, staphylococcal enterotoxin A; KO, knockout; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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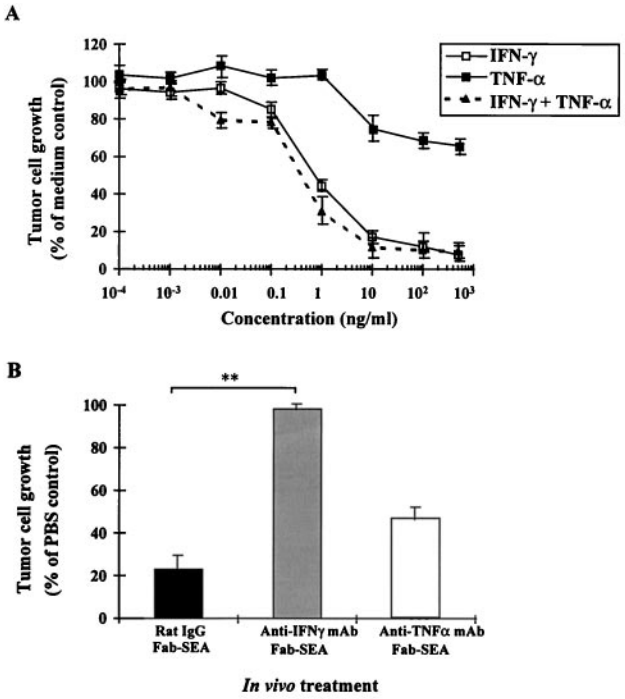

FIGURE 1. IFN-γ- and TNF-α-induced growth inhibition of C215-transfected B16 melanoma in vitro. *A*, C215-transfected B16 melanoma cells (1.7×10^3) were cultured in the presence or the absence of IFN-γ, TNF-α, or both cytokines. *B*, Mice were injected with 50 μg of Fab-SEA alone or in combination with 150 μg of anti-IFN-γ or 80 μg of anti-TNF-α mAbs i.p., and sera were collected. C215-transfected B16 melanoma cells (1.7×10^3) were cultured in the presence or the absence of serum from anti-IFN-γ- or anti-TNF-α-treated mice in vitro. Viability was determined in an MTT assay after 72 h. Statistical analysis was performed using Student's *t* test. * indicates $0.05 < p > 0.01$; ** indicates $0.01 < p > 0.001$. Mean values from triplicate cultures are shown. The SD in the assays was routinely <15%. Data are presented from one of three (*A*) and two (*B*) similar experiments.

Europe), 0, 1 mg/ml gentamicin (Biologic Industries, Kibbutz Beit Haemek, Israel), and 1×10^{-3} M sodium pyruvate (HyClone Europe)).

Reagents

Recombinant Fab-SEA was expressed in *Escherichia coli* K-12 UL635 (ara-14, xyl-7, ompT, T4R) as described previously (17). Rat anti-mouse IFN-γ, biotin-labeled rat anti-mouse IFN-γ, rIFN-γ, and rTNF-α were purchased from PharMingen (San Diego, CA). Neutralizing rat IgG1 anti-mouse IFN-γ and rat IgG2a anti-mouse TNF-α mAbs were purified from hybridoma supernatants on a protein G column according to a standard protocol. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO).

Tumor growth inhibition in vitro

C215-transfected B16 melanoma cells (1700 cells/well) were cultured in the presence or the absence of rIFN-γ, rTNF-α, or sera from Fab-SEA (30 μg/injection)-treated animals injected with PBS, R46A2, or V1q. The cell viability was analyzed in an MTT assay after 72 h according to a standard protocol.

Cytotoxicity assay

Cytotoxicity was measured against SEA-coated A20 cells (1 μg/ml), Fab-SEA-coated C215-B16 melanoma cells (1 μg/ml) using standard 4- and 20-h ^{51}Cr release assays (19). Calculations were conducted according to the formula: % specific lysis = 100 × (cpm experimental − cpm background release)/(cpm total release − cpm background release).

Serum cytokine analyses

Blood was drained 4 h after i.v. injections of Fab-SEA or PBS, both supplemented with 1% normal syngeneic serum. Cytokine content was analyzed using standard sandwich ELISA techniques. Absorbance was determined in an EL 312e Bio-Kinetics Reader (Bio-Tek Instruments, Winooski, VT).

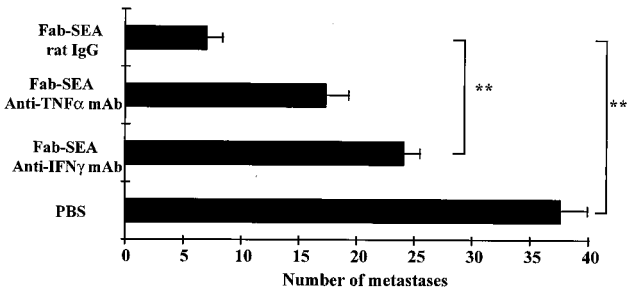

FIGURE 2. Tumor therapy after IFN-γ and TNF-α neutralization. Animals were inoculated with 10^5 Fab-SEA transfected B16 melanoma cells. On days 4 to 7, mice were treated with 150 μg of anti-IFN-γ or 75 μg of anti-TNF-α mAbs followed by 50 μg of Fab-SEA i.v. Lung metastases were counted on day 21. Statistical analysis was performed using the Mann-Whitney *U* test. * indicates $0.05 < p > 0.01$; ** indicates $0.01 < p > 0.001$. Each group contained seven animals. Data are presented from one of two similar experiments. The mean ± SEM from seven animals are shown.

In vivo therapy

C215-transfected B16 melanoma cells (1×10^5 in C57BL/6 mice, 5×10^4 in perforin KO mice) in 0.2 ml of PBS with 1% syngeneic mouse serum were inoculated i.v. into the tail vein. Treatment with four 0.05- to 50-μg daily i.v. injections of Fab-SEA was initiated on day 5. Mice were killed on day 21, and lung metastases were counted. To evaluate the role of cytokines in Fab-SEA tumor therapy we injected tumor-bearing animals with neutralizing anti-IFN-γ mAb (150 μg) or anti-TNF-α mAb (80 μg) 2 h before Fab-SEA therapy. Mice were sacrificed on day 21, and lung metastases were counted.

Results

IFN-γ and TNF-α inhibit B16-C215 tumor cell growth

We have previously demonstrated that Fab-SEA therapy induced the production of a panel of cytokines, induction of CTL activity, and inhibition of C215-transfected B16 melanoma lung metastases (18). Culture of C215-transfected B16 melanoma cells in the presence of IFN-γ and TNF-γ (Fig. 1*A*) inhibited tumor growth in a dose-dependent manner, confirming earlier reports that B16 melanoma cells are sensitive to IFN-γ and TNF-α (20, 21). IFN-γ completely blocked B16-C215 cell growth, while TNF-α mediated only a partial effect. When B16-C215 cells were grown in the presence of both IFN-γ and TNF-α, no statistically significant additive or synergistic effect was recorded. To investigate whether Fab-SEA-induced serum IFN-γ and TNF-α levels were sufficient to inhibit growth of C215-B16 melanoma in vitro, we collected serum from mice injected with the fusion protein alone or from mice injected with neutralizing mAb against IFN-γ and TNF-α 2 h before the Fab-SEA injection. Culture of C215-B16 melanoma in vitro with serum from Fab-SEA-treated animals resulted in a profound growth inhibition. Only minimal growth inhibition was observed after neutralization of the sera with anti-IFN-γ mAb or anti-IFN-γ plus TNF-α mAb (Fig. 1*B* and data not shown). In contrast, only a partial growth inhibition was observed when TNF-α bioactivity was neutralized (Fig. 1*B*).

To evaluate the relevance of Fab-SEA-induced TNF-α and IFN-γ in vivo, mice carrying B16-C215 melanoma cells were injected with neutralizing Abs against IFN-γ and TNF-α before each Fab-SEA injection. Treatment with Fab-SEA alone or with an isotype-matched control Ab produced a significant ($p < 0.01$) reduction (80%) of pulmonary metastases (Fig. 2). Neutralization of

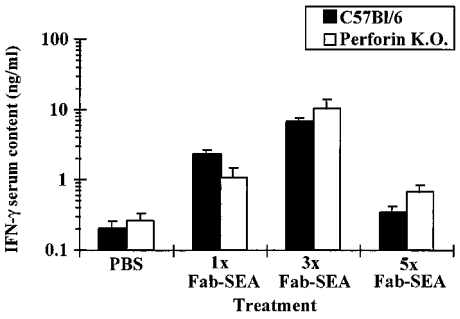

FIGURE 3. IFN-γ production in perforin KO animals. Perforin KO or normal C57BL/6 mice were injected with PBS or with one, three, or five doses of 50 μg of Fab-SEA. Four hours after the last injection serum was collected, and the IFN-γ concentration was determined. Mean values from each group containing three animals are shown. The SD was <15%. Data are presented from one of two similar experiments.

IFN-γ or TNF-α in vivo during Fab-SEA therapy resulted in only a partial and nonsignificant ($p > 0{,}05$) tumor reduction (Fig. 2). No major additive synergistic effect was noted when both IFN-γ and TNF-α were neutralized (data not shown). Treatment with anti-IFN-γ mAb significantly ($p < 0.01$) blocked the therapeutic efficacy of Fab-SEA protein. The effect of anti-IFN-γ mAb was more pronounced than that of anti-TNF-α mAb. These results clearly demonstrate that release of the tumoricidal cytokines IFN-γ and TNF-α participates in Fab-SEA-mediated elimination of B16-C215 melanoma metastases in vivo.

CTL activity, but not IFN-γ production, is dependent of perforin

Perforin-dependent cytotoxicity is generally an important effector function of Ag-specific $CD8^+$ T cells. Perforin KO mice were used to investigate whether local release of perforin is an important effector function during Fab-SEA therapy. Injection of Fab-SEA into normal C57BL/6 mice or perforin KO mice induced similar levels of IFN-γ and TNF-α (Fig. 3 and data not shown) and expanded TCR $V\beta3^+$ and $V\beta11^+$ $CD4^+$ and $CD8^+$ T cells (data not shown). This indicated that the C57BL/6 perforin KO mice

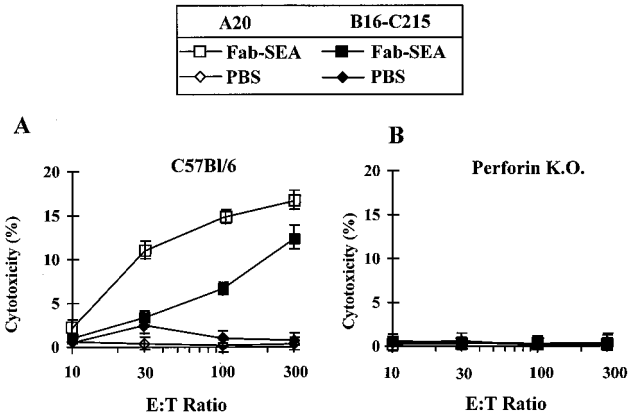

FIGURE 4. CTL activity in perforin KO and normal C57BL/6 mice. Fab-SEA (50 μg) was injected i.v. into perforin KO and C57BL/6 mice. After 48 h spleens were removed, and CTL activity was examined in a standard 4-h ^{51}Cr release assay. Cytotoxicity was measured against SEA-coated A20 cells after 4 h and against Fab-SEA-coated C215-transfected B16 melanoma cells after 20 h at various E:T cell ratios. Each experimental group contained two mice. The mean value from triplicate wells is shown. The SD in the assay was routinely <15%. Data are presented from one of two similar experiments.

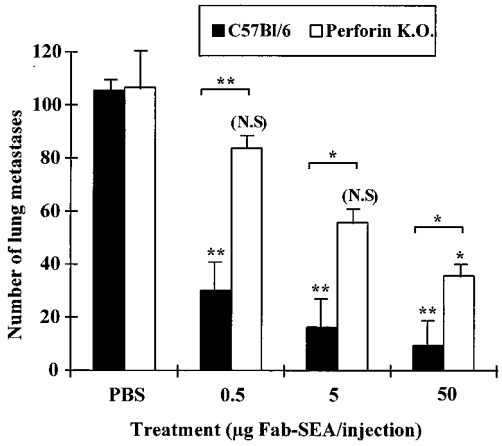

FIGURE 5. The role of perforin in tumor therapy. Animals were inoculated with 10^5 B16-C215 melanoma cells. On days 4 to 7, mice were treated with 0.5 to 50 μg of Fab-SEA i.v. Lung metastases were counted on day 21. Statistical significance was analyzed by the Mann-Whitney *U* test. * indicates $0.05 < p > 0.01$; ** indicates $0.01 < p > 0.001$. Each group contained seven animals. Data are presented from one of two similar experiments. The mean ± SEM are shown.

mounted a normal immune response when challenged with Fab-SEA. Stimulation with Fab-SEA in wild-type C57BL/6 mice elicited a strong CTL response against both the murine B cell lymphoma A20 and the B16-C215 melanoma (Fig. 4*A*). In contrast, splenocytes from Fab-SEA-treated perforin KO mice failed to mount a CTL response against the A20 and C215-B16 melanoma cells (Fig. 4*B*). These data demonstrate that the perforin pathway participates in Fab-SEA-mediated killing of the relevant target cell B16-C215 in vitro.

An optimal antitumor response is dependent on perforin

To examine whether local release of perforin in the tumor area was important in the elimination of tumor cells, perforin KO mice and normal C57BL/6 mice were inoculated with C215-transfected B16 melanoma cells, and the number of lung metastases was recorded after Fab-SEA treatment. Treatment with a low dose of Fab-SEA (0.5 μg) resulted in a marginal and nonsignificant ($p > 0.05$) tumor reduction in perforin KO mice, whereas a strong antitumor response ($p < 0{,}01$) was seen in normal mice (Fig. 5). Dose escalation demonstrated a significantly reduced activity of Fab-SEA at all tested doses in perforin KO mice compared with that in normal mice ($0.05 < p > 0.001$) (Fig. 5). These data clearly suggest that the perforin pathway has an important role in Fab-SEA-based cancer immunotherapy.

Discussion

In vivo injection of Fab-SEA results in cytokine production, T cell proliferation, and induction of CTL activity (18). The prominent appearance of SEA-reactive cytokine-producing TIL is recorded in the lung metastases after treatment with Fab-SEA i.v. (22, 23). In vivo elimination of $CD4^+$ or $CD8^+$ T cells with depleting mAb has demonstrated that both T cell subsets contribute to the therapeutic efficacy (22). This was recently confirmed in CD4 and CD8 KO mice, in which $CD4^+$ T cells were essential to induce infiltration of leukocytes into the tumor area, whereas $CD8^+$ T cells

were required for CTL effector functions and optimal production of IFN-γ.[3]

In the present study we have characterized the molecular mechanisms for Fab-SEA eradication of B16-C215 melanoma lung metastases in vivo. We now show that the growth of B16-C215 in vivo is strongly influenced by the level of IFN-γ produced during Fab-SEA therapy. The high sensitivity to IFN-γ and moderate sensitivity to TNF-α is in conformity with earlier studies using various B16 sublines and other melanoma cell lines (20, 21). Neutralization with anti-IFN-γ mAb in vivo markedly reduced the efficacy of Fab-SEA treatment. This suggests that IFN-γ is produced locally in an amount sufficient to interfere with tumor growth. However, one cannot rule out that systemic production of IFN-γ also contributes by promoting activation and differentiation of Th1 cells and CTL (24–26). Local secretion and diffusion of cytokines in the tumor area are suggested to be an important mechanism to avoid progressive growth and escape of tumor Ag-negative tumor cells. Indeed, preliminary experiments using a mixture of B16 melanoma cells transfected with the C215 Ag or an irrelevant Ag resulted in maintained therapeutic efficacy (data not shown). This suggests that the presence of a fraction of Ag-negative cells in a heterogeneous tumor, which seems to occur in most human cancers, do not counteract successful Fab-SEA therapy.

A major pathway of cell-mediated cytotoxicity involves targeted release of perforin from cytoplasmic storage. Perforin-mediated killing is rapid and may eliminate certain tumor cells (27, 28). In vivo studies in perforin KO mice have clearly demonstrated that perforin-mediated cytotoxicity plays a prominent role in the elimination of noncytopathic viruses such as LCMV (27, 29), in controling the growth of certain tumors, i.e., fibrosarcoma (27), and in eliminating intracellular bacteria (30). Perforin-deficient mice have normal numbers of $CD8^+$ T cells and NK cells. However, these mice fail to mediate $CD8^+$ T cell-dependent cytotoxicity against allogeneic and virus-infected target cells as well as NK cell-mediated cytotoxicity against the YAC-1 target cells (27, 31). In conformity with these studies, we failed to detect any T or NK cell (data not shown)-mediated cytolytic activity in the perforin KO mice. A markedly impaired therapeutic efficacy was recorded in perforin KO mice, indicating that this mechanism is important in our system. Perforin-mediated lysis by $CD8^+$ CTL most likely contributes mainly in the eradication of Ag-positive tumor cells. This conclusion is substantiated by the reduced efficacy seen in CD8-deficient mice, whereas depletion of NK cells has shown a marginal influence on the reduction of established B16-C215 metastases (data not shown). However, since perforin-mediated lysis is rapid, and CTL may be recycling in the tumor, this mechanism may have importance for blocking progressive tumor expansion.

The Fas system constitutes an additional pathway of cell-mediated cytotoxicity that has been implicated in the elimination of certain target cells (32). The Fas system also has a key role in vivo to maintain immune homeostasis by controlling cell expansion and deletion (33, 34). Recent studies clearly demonstrate that most cells of the immune system express Fas and/or Fas ligand constitutively or after activation (35). Earlier experiments in SCID mice inoculated with C242-positive colon carcinoma cells clearly demonstrated that apoptosis was induced in tumor infiltrates by C242 Fab-SEA-activated T cells (36). It was suggested that this pathway may contribute to Fab-SEA-induced elimination of colon cancer cells. Although Fas ligand is up-regulated on SEA-reactive T cells after Fab-SEA treatment in vivo (data not shown), this mechanism seems to be of minor importance in our tumor model, since C215-B16 melanoma cells express only marginal amounts of Fas in vitro and in vivo (data not shown). Moreover, preliminary experiments in *lpr* and *gld* mice, which have a dysfunctional Fas/Fas ligand pathway, resulted in intact therapeutic efficacy. This suggests that perforin-mediated, but not Fas-mediated, cytotoxicity is relevant for Fab-SEA-induced tumor regression.

Fab-SEA-induced $CD4^+$ and $CD8^+$ TILs have been found to produce high levels of IFN-γ and TNF-α locally in the tumor area (22). Since TNF-α is known to play a major role in the initiation of an immune response (37), it is tempting to speculate that Fab-SEA-activated $CD4^+$ T cells contribute during the early phase of the response by producing proinflammatory cytokines. The subsequent recruitment of $CD8^+$ T cells may be required for superinduction of IFN-γ and secretion of cytotoxic proteins such as perforin and granzymes (9, 10). Several specific CTL epitopes on human melanomas, eliciting strong CTL responses, have been identified (38, 39). Moreover, several human melanoma cell lines, such as HS294T, have been shown to be sensitive to the growth-suppressive effects of IFN (40–42). Thus, human melanoma cells are sensitive to both perforin-deficient CTL cytotoxicity and the growth inhibitory effects induced by IFN-γ. Therefore, development of Fab-SEA-based clinical protocols optimized to induce high levels of IFN-γ and perforin in TIL may be attractive for successful elimination of small established tumor cell aggregates such as human melanoma cells residing as micrometastases after surgical removal of the primary tumor.

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