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Inhibition of Human Tumor-Infiltrating Lymphocyte Effector Functions by the Homophilic Carcinoembryonic Cell Adhesion Molecule 1 Interactions[1]

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Efficient antitumor immune response requires the coordinated function of integrated immune components, but is finally exerted by the differentiated effector tumor-infiltrating lymphocytes (TIL). TIL cells comprise, therefore, an exciting platform for adoptive cell transfer (ACT) in cancer. In this study, we show that the inhibitory carcinoembryonic Ag cell adhesion molecule 1 (CEACAM1) protein is found on virtually all human TIL cells following preparation protocols of ACT treatment for melanoma. We further demonstrate that the CEACAM1 homophilic interactions inhibit the TIL effector functions, such as specific killing and IFN-γ release. These results suggest that CEACAM1 may impair in vivo the antitumor response of the differentiated TIL. Importantly, CEACAM1 is commonly expressed by melanoma and its presence is associated with poor prognosis. Remarkably, the prolonged coincubation of reactive TIL cells with their melanoma targets results in increased functional CEACAM1 expression by the surviving tumor cells. This mechanism might be used by melanoma cells in vivo to evade ongoing destruction by tumor-reactive lymphocytes. Finally, CEACAM1-mediated inhibition may hinder in many cases the efficacy of TIL ACT treatment of melanoma. We show that the intensity of CEACAM1 expression on TIL cells constantly increases during ex vivo expansion. The implications of CEACAM1-mediated inhibition of TIL cells on the optimization of current ACT protocols and on the development of future immunotherapeutic modalities are discussed. *The Journal of Immunology,* **2006, 177: 6062–6071.**

Melanoma is a malignancy of melanocytes (pigment-producing cells) located predominantly in the skin, but also found in the eyes, ears, gastrointestinal tract, leptomeninges, and oral and genital mucous membranes. The global incidence of melanoma has been rapidly increasing during the last 20 years (1, 2) and is considered to be the most common fatal malignancy of young adults (3). Complete surgical resection remains the primary mode of therapy for localized lesions, whereas numerous adjuvant modalities have been investigated for benefit in metastatic disease. So far, mainly the immune-based modalities, such as immunomodulatory agents, adoptive cellular transfer (ACT),[3] and vaccinations, still hold some promise.

ACT immunotherapy is based on the ex vivo selection of tumor-reactive lymphocytes followed by their activation and numerical expansion before reinfusion to the autologous tumor-bearing host (4). ACT protocols use T cells with antitumor reactivity, such as tumor-infiltrating lymphocytes (TIL) (5, 6) or other cell types such as NK cells (7, 8) or dendritic cells (9). In murine models, TIL ACT approach mediated the regression of established cancers and provided some important principles to guide human studies (10–12). This modality has been further improved by prior host immunosuppression (13, 14), systemic administration of cytokines to support the transferred cells (15), concurrent immunization with inflammatory formulations of the tumor Ag recognized by the transferred cells (16), and by genetic modification of the adoptively transferred cells (17). Recently, the results of a clinical trial of TIL treatment in 35 patients with metastatic melanoma were published. Noticeably, up to 50% of the patients exhibited significant clinical response with some of the patients remarkably achieving complete response (18). Nevertheless, although these results are encouraging, this modality is still far from its full potential. The development of improved TIL ACT therapy depends on acute delineation of the molecular interactions between TIL and tumors.

The carcinoembryonic Ag cell adhesion molecule 1 (CEACAM1) is a transmembrane glycoprotein that belongs to the carcinoembryonic Ag family (part of Ig-superfamily), which encompasses several forms of proteins with different biochemical properties, all encoded on chromosome 19q13.2 (19). The CEACAM proteins share a common basic structure of sequentially ordered different Ig-like domain(s), and are able to interact with each other (20). For example, CEACAM1 interacts homophilically with CEACAM1 (21, 22) and heterophilically with CEACAM5 but not with CEACAM6 (23). CEACAM1 is broadly distributed on a variety of epithelial cells and immune cells. Many different functions were attributed to the CEACAM1 protein. It was shown that the CEACAM1 protein exhibits antiproliferative properties in carcinomas of colon (24) and prostate (25). Additional data support the

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[3] Abbreviations used in this paper: ACT, adoptive cellular transfer; TIL, tumor-infiltrating lymphocyte; CEACAM1, carcinoembryonic Ag cell adhesion molecule; MCSP, melanoma-associated chondroitin sulfate proteoglycan; PI, propidium iodide; MFI, median fluorescence intensity.

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central involvement of CEACAM1 in angiogenesis (26), metastasis (27), and insulin clearance (28). CEACAM1 also has a role in the modulation of innate and adaptive immune responses. We have previously provided substantial evidence that CEACAM1 homophilic interactions inhibit NK-mediated killing independently of MHC class I recognition (29). This novel mechanism plays a pivotal role in the inhibition of activated decidual lymphocytes in vitro and most likely in vivo during prenatal CMV infections (30). CEACAM1 homophilic interactions are probably important in some cases of metastatic melanoma, because increased CEACAM1 expression was observed on NK cells derived from some patients compared with healthy donors (29). Indeed, a strong association of CEACAM1 expression on primary cutaneous melanoma lesions with the development of metastatic disease and poor survival was recently reported (27). In addition, we have recently demonstrated the cardinal role of CEACAM1-mediated inhibition in maintaining NK self-tolerance in TAP2-deficient patients (31, 32). Other reports (33, 34) have shown that CEACAM1 engagement either by TCR cross-linking with mAb or by *Neisseria gonorrhoeae* Opa proteins inhibits T cell proliferation.

In this study, we demonstrate that the inhibitory CEACAM1 protein is stably expressed on activated TIL cells following ACT preparation protocol. Furthermore, we show that the CEACAM1 is functional and inhibits crucial effector TIL functions, such as cytotoxicity and IFN-γ release. These results suggest that homophilic CEACAM1 interactions impair in vivo the antitumor response of the differentiated effector intratumoral lymphocytes. Moreover, this inhibitory mechanism might hinder in many cases the efficacy of TIL-based ACT treatment of malignancies. Finally, prolonged coincubation of reactive TIL cells with their target melanomas resulted in increased CEACAM1 expression by the surviving tumor cells. Thus, the surviving melanoma cells emerge more resistant following the attack by the tumor-reactive lymphocytes. This mechanism might be used by melanoma cells in vivo to evade destruction by tumor-reactive lymphocytes.

Materials and Methods

Cells

The cell lines used in this study were the MHC class I-negative 721.221 EBV-transformed B cell lymphoma human cell line, the murine thymoma BW cell line lacking expression of α- and β-chains of the TCR and the JKF6 (directed against MART1-derived peptide 27–35 in complex with HLA-A*02), L2G2 (directed against a gp100-derived peptide 209–217 in complex with HLA-A*02), and L2D8 (directed against a gp100-derived peptide 209–217 in complex with HLA-A*02) TIL clones. The melanoma cell lines used were 526mel (HLA-A*02, -A*03, -B*15, -Cw*03), 624mel (HLA-A*02, -A*03), and 938mel (HLA-A*0101, -A*2402, -B*0702, -B*0801, -Cw*0701, -Cw*0702). Primary culturing of MEL001 (HLA-A*01, -A*30, -B*07, -B*08, -Cw*07) and MEL009 (HLA-A*02, -A*03, -B*1501, Cw*03) was performed from surgically removed specimens as described previously (35).

TIL cultures

The generation of TIL with specific reactivity against tumor Ags was adopted precisely as published by Dudley et al. (35). All work with human tissues was approved by an institutional review board according to the Declaration of Helsinki.

Antibodies

The following Abs were purchased from DakoCytomation: Kat4c (anti-CEACAM1, -CEACAM5, -CEACAM6, and -CEACAM8), rabbit polyclonal anti-CEACAM, FITC-anti-CD3, FITC-anti-CD4, FITC-anti-CD16, PE-anti-CD8, and RPE-Cy5-anti-CD3. The following Abs were purchased from BD Pharmingen: PE-anti-CD56, APC-anti-CD56, biotinylated anti-hIFNγ mAb 4S.B3, purified anti-hIFNγ mAb HIB42, biotinylated anti-mIL-2 mAb JES6-5H4, and purified anti-mIL-2 mAb JES6-1A12. Other Abs used in this study were as follows: anti-CEA mAb and FITC-anti-HLA-A2 (Serotec), anti-PE-melanoma-associated chondroitin sulfate proteoglycan (MCSP) (Miltenyi Biotec), anti-CD99 mAb 12E7, anti-β_2-microglobulin mAb BBM-1, and the specific anti-CEACAM1 mAb 5F4 (33). Rabbit polyclonal Abs against purified ubiquitin were used as the control. FITC-conjugated goat anti-mouse $F(ab')_2$ (Jackson ImmunoResearch Laboratories) and FITC-conjugated goat anti-human $F(ab')_2$ (Jackson ImmunoResearch Laboratories) were used as secondary detection Abs.

Ig-fusion protein

The Ig-fusion protein used in this study, CEACAM1-Ig, was cloned, generated in COS-7 cells, and purified on a protein G column as described previously (29). The Ig-fusion proteins were used in FACS staining experiments. In each staining, 5 μg of Ig-fusion protein were used and detected by FITC-labeled goat anti-human secondary reagent.

Generation of transfectants

The 721.221 cells expressing CEACAM1 (.221/CCM1) or HLA-A2 (.221/A2) proteins were generated as described previously (29). The CEACAM1 cDNA was recloned into pcDNA3 vector with a hygromycin-resistant gene (Invitrogen Life Technologies) using the same primers and restriction sites used previously (29). The new construct was permanently transfected into .221/A2 cells (electroporation) to generate stable .221/A2/CCM1 cells.

IFN-γ secretion and cytotoxicity assays

IFN-γ secretion was assayed by coincubation of TIL cells with viable nonirradiated target cells in various E:T ratios for overnight period. The amount of IFN-γ secretion in the culture supernatant was detected with standard sandwich ELISA protocol. The cytotoxic activity of TIL against various targets was assayed in 5-h [^{35}S]methionine release assays, as described previously (29). Redirected killing experiments were performed as described previously (29). When antigenic peptides were used to induce specific TIL-mediated killing, target cells were preincubated with 1 mM of the appropriate peptide for 2 h in a humidified incubator followed by three consecutive washes. In experiments in which rabbit polyclonal Abs were included, the final concentration was 20 μg/ml. In all cytotoxicity assays performed, spontaneous release did not exceed 20% of maximal release.

Coculture assays

Twenty thousand melanoma cells were plated in a 48-well plate and allowed to adhere for 4 h. TIL cells were then added at an E:T ratio of up to 10:1. At indicated time points, supernatants were collected and floating cells were removed followed by careful washes of adherent cells with PBS. Adherent cells were then trypsinized, and viability was monitored by propidium iodide (PI) staining. In all days, $<$10% of the adherent melanoma cells were stained by PI. Cells were double-stained for CEACAM1 using Kat4c-FITC mAb and for MCSP using anti-MCSP-PE mAb to distinguish between the surviving melanoma cells ($MCSP^+$) from traces of TIL cells ($MCSP^-$). Floating cells were similarly double-stained daily to evaluate CEACAM1 on TIL cells ($MCSP^-$).

Results

Expanded TIL cultures express the CEACAM1 protein

The use of TIL cells in ACT therapy requires a tremendous amount of lymphocytes. Alas, the quantity of TIL cells obtained directly from a surgically removed tumor specimen is practically negligible. To generate a sufficient number of active TIL for efficient immunotherapy, primary TIL cultures are further activated and expanded as described previously (35). We have successfully generated several human TIL bulk cultures, obtained from surgically removed melanoma lesions, and massively expanded them as described (35). Noteworthy, some of the TIL cultures were derived from primary melanoma lesions, whereas the rest were derived from various melanoma metastases. The TIL bulk cultures were stained for CEACAM1 expression using either the Kat4c mAb (data not shown) or the CEACAM1-specific 5F4 mAb (Fig. 1*A*). Remarkably, all cells in any of the bulk TIL cultures tested expressed the CEACAM1 protein in similar levels (Fig. 1*A*). There were no significant differences in CEACAM1 expression levels among bulk TIL cultures derived either from primary melanoma lesions (see representative TIL-001 in Fig. 1*A*) or from various melanoma metastases (see representative TIL-008 in Fig. 1*A*). In addition, several TIL clones with defined antigenic reactivity were

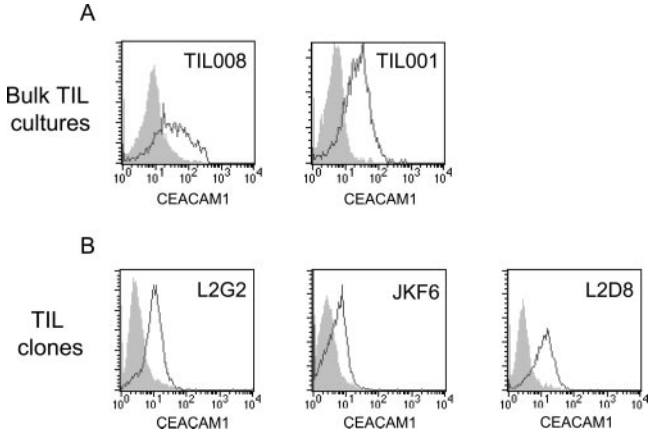

FIGURE 1. Expression of CEACAM1 on expanded TIL cultures. The figure shows the CEACAM1 expression intensity on (*A*) expanded bulk TIL cultures and on (*B*) expanded TIL clones. Background staining (shaded) is with secondary FITC-conjugated $F(ab')_2$ goat anti-mouse Abs. Expression of CEACAM1 (empty) was detected with the 5F4 mAb by flow cytometry. The figure shows one representative experiment of five performed.

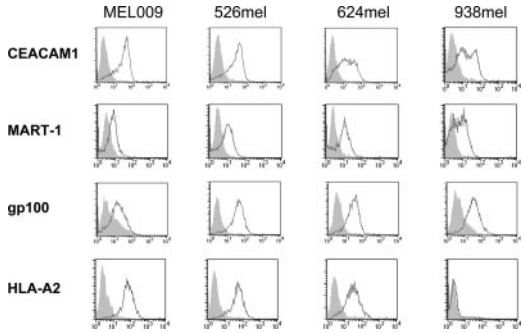

FIGURE 3. Expression of various proteins by different melanomas. Staining of melanoma cell lines 526mel, 624mel, and 938mel, and the primary melanoma cells MEL009 for expression of CEACAM1, MART-1, gp100, and HLA-A2, as indicated in each row, was performed with the appropriate mAbs. Flow cytometry staining results (empty histograms) are overlaid on the background staining with secondary FITC-conjugated $F(ab')_2$ goat anti-mouse Abs (shaded histograms). Figure shows one representative experiment of five performed.

tested, and similar CEACAM1 expression levels were observed (Fig. 1*B*).

Engagement of CEACAM1 inhibits the redirected killing activity of TIL

The specific effect of CEACAM1 engagement on general killing activity of TIL cells was initially tested on redirected killing activity. The murine P815 mastocytoma cells were used as target cells for both human TIL bulk cultures and clones. Briefly, the P815 cells express Fc receptors able to bind the Fc portion of various mouse Abs directed against human Ags, thus the Ag binding site of the Ab remains free. After preincubation of the P815 cells with various Abs, human TIL cells are added and specific cross-linking of their Ags occurs (36). The P815 cells lack expression of human MHC class I complexes, which explains their resistance to specific lysis by human T cells. Indeed, when no Abs were included in the assay, no significant killing by the TIL-002 bulk culture could be observed (Fig. 2). Some nonspecific killing activity can sometimes be detected (~10%), however, as observed with the L2G2 TIL clone (Fig. 2). Nevertheless, a considerable increase in the killing was observed when anti-CD3 mAbs were included in the assay (Fig. 2). No similar effect was observed when anti-CEACAM Kat4c mAb or the control anti-CD99 mAb were used (Fig. 2). Remarkably, a significant reduction in the killing activity was observed when both anti-CD3 mAb and anti-CEACAM mAb were included in the assay (Fig. 2). The anti-CEACAM Kat4c mAb is agonistic when bound either to a solid phase (32) or to Fc receptors of P815 cells (29). Thus, activation of TIL killing activity is reduced if CEACAM1 is engaged concomitantly to CD3. Importantly, this effect was specific and due to the coengagement of CEACAM1, because no similar reduction could be observed when the anti-CD3 mAb and the control anti-CD99 mAb were used (Fig. 2).

Homophilic CEACAM1 interactions inhibit various effector TIL functions

To test the role of the CEACAM1 protein in the interactions between TIL and melanoma cells, primary melanoma cells and melanoma cell lines were stained for the expression of the CEACAM1, the common melanoma Ags MART1 and gp100 as well as for the HLA-A2 protein (Fig. 3). Effector functions of bulk TIL cultures and clones against the melanomas, such as cytotoxic activity and IFN-γ secretion, were assayed. Several TIL clones, directed against different melanoma Ags, such as the JKF6 clone (directed against a MART1-derived peptide 27–35 in complex with HLA-A2) and the L2D8 clone (directed against a gp100-derived peptide 209–217 in complex with HLA-A2) were used. The irrelevant TIL001 cells were used as control. IFN-γ secretion assays and killing assays were performed as described in *Materials and Methods*. Specific secretion of IFN-γ by the different TIL clones in response to coincubation with various melanoma cells was evident only with the HLA-A2-positive melanoma cells, including MEL009, 526mel, or 624mel cells (Fig. 4, *A* and *B*, ▨). Accordingly, concomitant specific lysis of the same target cells could be observed (Fig. 4, *D* and *E*, ▨). These effects were the result of specific Ag recognition and TIL activation, because no

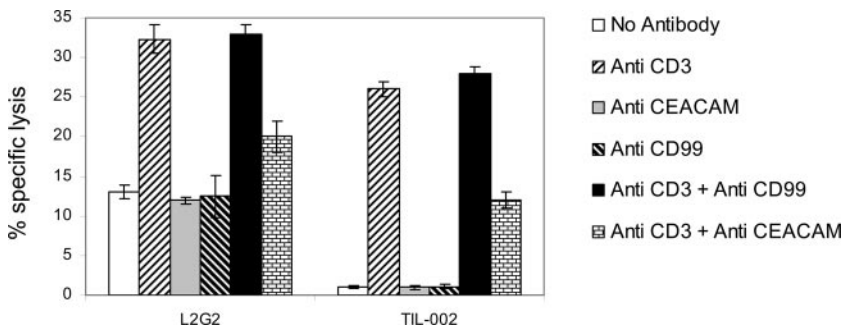

FIGURE 2. Engagement of CEACAM1 inhibits the redirected killing activity of TIL. The redirected killing experiments were performed by preincubation of the murine Fc-expressing P815 cells with various combinations of anti-human mAbs as indicated in the figure, followed by addition of two different human TIL cells (indicated in the figure). The mAbs cross-linked their target proteins expressed by the TIL cells and engaged them. The lysis of P815 target cells by TIL cultures was induced by the presence of anti-CD3 mAb and was measured by standard 5-h [^{35}S]methionine release. Figure shows the mean ± SD of three independent experiments.

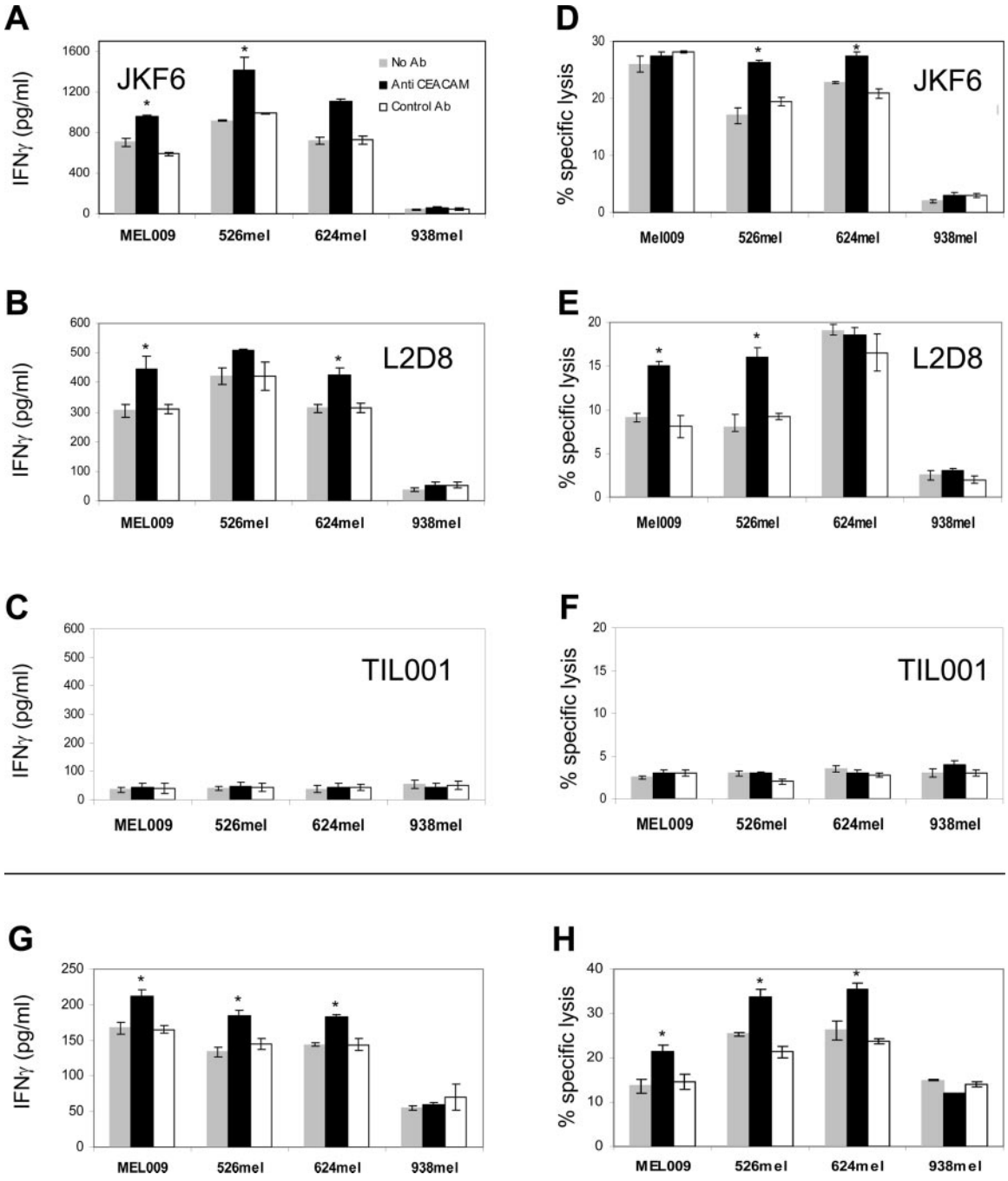

FIGURE 4. The presence of CEACAM1 on melanoma inhibits specific effector TIL functions. Melanoma cell lines 526mel, 624mel, and 938mel, and primary melanoma cells MEL009 were independently incubated either with the HLA-A2-restricted JKF6 or L2D8 TIL clones or with the inert TIL001 control cells, as indicated in each graph. The specific TIL effector functions tested were IFN-γ release to the cultures supernatants (*A–C*, *G*) and specific killing (*D–F*, *H*). Different treatments were tested: no Ab (▨), anti-CEACAM Abs (■), and control anti-CD99 Abs (□). In the *upper panel* (*A–F*, *above horizontal line*) the TIL cells were treated, and in the *lower panel* (*G* and *H*, *below horizontal line*) the target cells were treated. The data presented are the mean ± SD results of triplicates in one representative experiment of five performed. ∗, Represent statistical significance of p value <0.05.

IFN-γ secretion or TIL killing activity could be observed when the HLA-A2-negative 938mel cells were used (Fig. 4) or when the irrelevant TIL001 cells were used (Fig. 4, *C* and *F*). Importantly, the effector capabilities of the TIL clones were significantly augmented when cultured in the presence of blocking anti-CEACAM Abs, including both IFN-γ production (Fig. 4, *A* and *B*, ■) and cytotoxic activity (Fig. 4, *D* and *E*, ■). Similar results were obtained when anti-CEACAM Abs were cultured with target cells prior to coincubation (Fig. 4, *G* and *H*). Thus, abrogation of CEACAM1 function either on TIL cells or on target cells augments TIL effector functions, thereby demonstrating that homophilic CEACAM1 interactions are required. These effects were specific, because the control Abs had little or no effect (Fig. 4, □). In addition, the anti-CEACAM Abs did not enhance the TIL effector functions tested in the absence of initial reactivity against the HLA-A2-negative 938mel cells (Fig. 4), or when the irrelevant TIL001 cells were used (Fig. 4, *C* and *F*), thus implying that CEACAM1 inhibits ongoing activation.

Surprisingly, however, a discrepancy between the killing activity and the secretion of IFN-γ in response to the presence of

blocking anti-CEACAM Abs was observed in some of the cases. For example, the addition of blocking anti-CEACAM Abs resulted in augmentation of the IFN-γ secretion activity by JKF6 cells that were incubated with MEL009 cells (Fig. 4*A*), but not of the killing activity (Fig. *4D*). A similar phenomenon was observed when 624mel cells were used as target cells with L2D8 cells as effectors (Fig. 4, *B* and *E*). Noteworthy, when no Abs were included in the assay, MEL009 and 624mel cells were most efficiently killed by JKF6 and L2D8, respectively, as compared with the other melanomas (Fig. 4, *D* and *E*). It is possible, therefore, that in these cases the TIL clones already function in their maximal killing capacity under the in vitro experimental settings. Indeed, the augmentation of the killing activity observed with the other melanomas reached similar levels to the basal killing of MEL009 by JKF6 and of 624mel by L2D8 cells (Fig. 4, *D* and *E*). It is not clear why these cells are more efficiently killed. Alternatively, additional interactions between activating receptors and ligands are involved, which overcome the CEACAM1-mediated inhibition. Another possibility is that the lack of concordance between IFN-γ secretion and cytotoxicity reflects the oddities of the assay conditions.

721.221 cells expressing the CEACAM1 protein are protected from lysis by TIL clones

To directly test the role of the CEACAM1 protein in inhibition of TIL cytotoxicity, the MHC class I-deficient and CEACAM1-deficient human 721.221 target cells were transfected either with the HLA-A2 cDNA alone (.221/A2) or along with the CEACAM1 cDNA (.221/A2/CCM1). Transfection was performed as described in *Materials and Methods* to generate stable transfectants. The expression of HLA-A2 and CEACAM1 was monitored with the anti-HLA-A2 and the Kat4c mAb, respectively, as shown in Fig. 5*A*. Importantly, the expression level of the HLA-A2 was similar among the .221/A2 and .221/A2/CCM1 transfectants (Fig. 5*A*). Thus, it is expected that the recognition efficiency of the HLA-A2 with specific peptide complexes by TIL cells would be similar. The expression level of the CEACAM1 by the .221/A2/CCM1 was similar to that of the previously described .221/CEACAM1 cells (29).

We further demonstrated that the CEACAM1 protein of the .221/A2/CCM1 is intact and functional with the BW cell system. Mouse BW cells were stably transfected with a chimeric molecule composed of the extracellular portion of CEACAM1 fused to mouse ζ-chain, as described previously (30). Engagement of CEACAM1 leads to the secretion of mouse IL-2, mediated by the ζ-chain, in a dose-dependent manner (32). The amounts of IL-2 in the culture supernatants were evaluated with standard sandwich ELISA. The presence of IL-2 could be detected only in the supernatants of BW/CCM1-ζ cells when incubated with .221/A2/CEACAM1 cells, but not with .221 cells or with .221/A2 cells (Fig. 5*B*). IL-2 was not detected when the parental BW cells were used (Fig. 5*B*). These experiments demonstrate that the CEACAM1 protein is functionally intact on the .221/A2/CEACAM1 cells.

The effect of CEACAM1 on TIL killing activity was evaluated with the .221 transfectants system. The parental .221 cells are

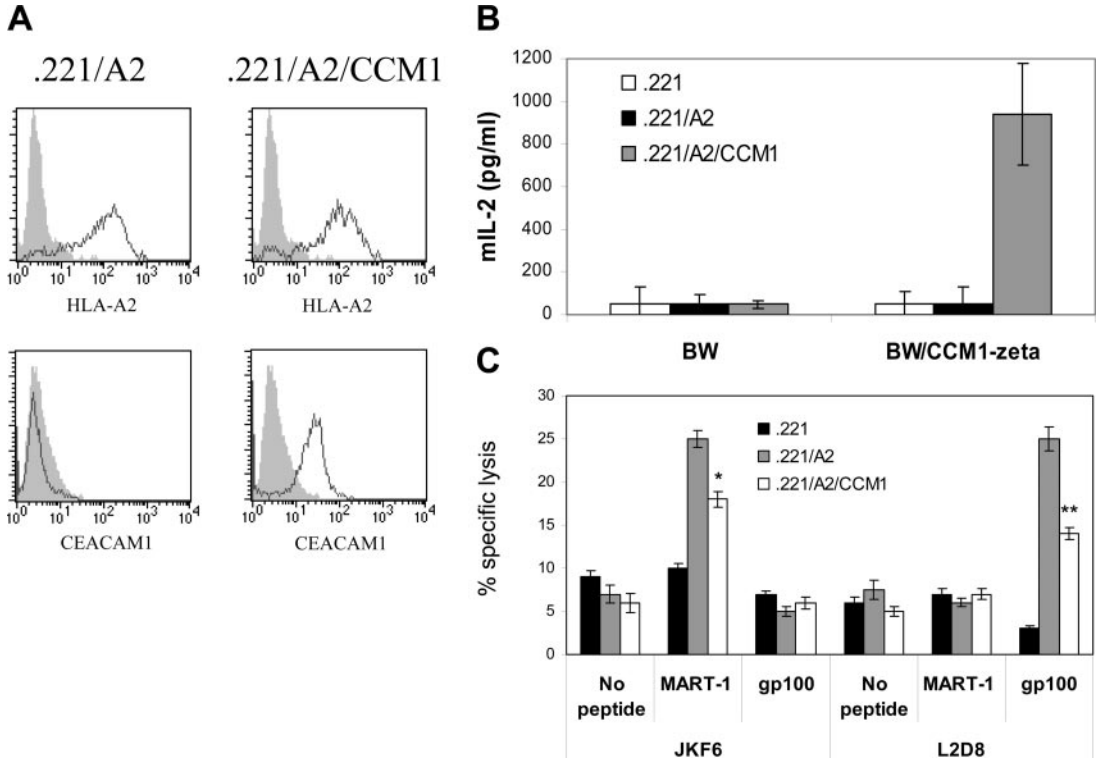

FIGURE 5. CEACAM1 protects 721.221 transfectants from specific killing by TIL clones. *A*, 721.221/A2 and .221/A2/CCM1 cells were stained for HLA-A2 or CEACAM1 expression with appropriate mAb (empty histograms). Staining results are overlaid on the background staining, which is the respective staining on the parental .221 cells (shaded histograms). Figure shows the results of a representative experiment of five performed. *B*, BW and BW/CCM1-ζ cells were independently incubated with the .221 cells or with the .221 transfectants, as indicated in the graph for 24 h. The amount of mouse IL-2 was measured in the supernatants with standardized ELISA. Figure shows the average of three independent experiments. *C*, Reactive TIL clones JKF6 or L2D8 were tested for specific killing of .221 transfectants. Target cells were separately preincubated with 1 mM of each peptide for 2 h in a humidified incubator to induce specific lysis. Statistical significance of decreased killing of .221/A2/CCM1 compared with .221/A2 by JKF6 is expressed by a p value of 0.001 (*), and similarly decreased killing by L2D8 is expressed by a p value of 0.0002 (**). Data presented are the mean ± SD of quadruplicates in one representative experiment of four performed.

MHC class I-deficient and are thus resistant to TIL-mediated killing. Specific TIL-mediated killing of .221 cells is enabled only upon introduction of both the appropriate MHC class I protein and an appropriate antigenic peptide. Two different TIL clones with defined reactivity against different melanoma Ags were used: JKF6, which recognizes the MART-1 (27–35) peptide; and L2D8 that recognizes the gp100 (209–217) peptide. Importantly, both TIL clones recognize their target peptides only in the context of the HLA-A2 protein. As expected, neither JKF6 nor L2D8 cells displayed any killing activity against the .221 parental cells, or against any of the transfectants when no peptides were included (Fig. 5*C*). Specific killing of .221/A2 cells was observed by the TIL clones only when the appropriate peptide was included because no cross-reactivity between the different peptides was evident (Fig. 5*C*). Remarkably, the .221/A2/CEACAM1 cells were more resistant to specific killing by both JKF6 and L2D8 cells, as compared with the .221/A2 cells. These results directly show that the presence of the CEACAM1 protein on tumor cells provides protection from the specific killing activity of TIL cells.

Coincubation of reactive TIL cells with melanoma cells results in the up-regulation of CEACAM1 on surviving melanoma cells

Treatment of melanoma patients with TIL ACT results in the migration of TIL cells in vivo into the tumors, subsequent lysis of the tumor cells, and eventually reduction of gross tumor mass (5, 18). However, it was recently demonstrated that the persistence of transferred TIL in the host is one of the most important predictors of tumor regression (37). It is thus logical that TIL cells and the tumor cells interact for many days in vivo. We therefore hypothesized that CEACAM1 expression by the surviving tumor cells might alter during the attack by reactive TIL cells.

To test this hypothesis, 526mel melanoma cells were cultured for 7 days alone or, alternatively, coincubated either with 526mel-reactive JKF6 cells or with 526mel-inert TIL001 cells. Every day, floating cells and adherent cells were collected and double-stained for CEACAM1 and MCSP as described in *Materials and Methods*. Basically, adherent cells were comprised mainly by PI-negative melanoma cells and are therefore considered as "surviving cells." The floating cells comprised a mixture of TIL cells with floating melanoma cells. Most floating melanoma cells (>75%) were stained by PI (data not shown). The TIL reactivity was monitored by concomitant daily measurements of the IFN-γ amounts in the culture supernatants and of the percentage of PI-positive melanoma cells. Assays were performed as described in *Materials and Methods*. The 526mel cells basally express CEACAM1 (median fluorescence intensity (MFI) = 60) and MCSP (MFI = 150) (data not shown). However, because the protein expression intensity on the melanoma cells could be influenced by other unrecognized parameters, the daily staining results of melanoma with TIL cells were normalized according to the staining results of melanoma without TIL cells. Calculation of expression ratio was performed independently for each day: (staining of melanoma incubated with TIL − background)/(staining of melanoma without TIL − background). Remarkably, when JKF6 cells were included in the assay, an up-regulation in CEACAM1 expression on surviving melanoma cells was observed after 24 h (ratio = 1.6) and increased to ratio of 2 on day 2 and 2.6 on day 3, followed by a decline to 1.34 on day 4 (Fig. 6*A*). This decline was probably observed due to decreased function of the TIL in vitro over time. Accordingly, the amount of IFN-γ detected in the culture supernatants, which reflects TIL response, decreased as well over time (Fig. 6*D*). The ratio of MCSP has not changed significantly during the entire week, arguing for the specificity of this up-regulation (Fig. 6*A*). Importantly, no significant change in any of the proteins tested could be observed when the nonreactive TIL001 cells were used (Fig. 6*B*). The expression of CEACAM1 was also daily monitored on the surface of TIL cells by double staining the floating cells for CEACAM1 and MCSP. The CEACAM1 expression ratio on $MCSP^-$ cells was normalized every day: (staining of TIL incubated with melanoma − background)/(staining of TIL without melanoma − background). Interestingly, no change in the expression of CEACAM1 could be observed either on the reactive JKF6 cells or on the nonreactive TIL001 cells (Fig. 6*C*). The reactivity of the JKF6 and TIL001 cells was demonstrated by the presence or absence, respectively, of IFN-γ in the culture supernatants (Fig. 6*D*). In addition, the percentage of PI-positive melanoma cells also reflects TIL reactivity, because more PI-positive 526mel cells were observed when incubated with JKF6 cells, when compared with 526mel cells only or 526mel cells with TIL001 cells (Fig. 6*E*). At day 4, the percentage of PI-positive melanoma cells evened between the different cultures, suggesting that from this point the main reason for PI-positive melanoma cells was not due to TIL function, but rather may be due to metabolic stress in overloaded cultures (Fig. 6*E*). Indeed, from this point on, the percentage of PI-positive melanoma cells similarly and continuously increased in all different cultures (data not shown). Similar results were also obtained when other reactive TIL cultures were used (data not shown). These results imply that a specific recognition of the tumor cells by the TIL triggers the expression of the CEACAM1 protein by the surviving melanoma cells. Supporting that notion, when the HLA-A2-negative 938mel cells were used, no change in the ratio of any of the above proteins could be observed (data not shown). The up-regulated CEACAM1 might contribute to protection of the remaining tumor cells from an ongoing TIL-mediated killing via the homophilic CEACAM1 interactions. In contrast, reactive TIL cells do not up-regulate CEACAM1 following recognition and killing of cognate melanoma cells, and are therefore not, probably, susceptible to an even more drastic inhibition by homophilic CEACAM1 interactions.

Up-regulated CEACAM1 on surviving melanoma cells is functional

To test whether the up-regulated CEACAM1 is of physiological relevance, we have isolated and analyzed the surviving melanoma cells following long-term coculture with reactive TIL cells. 526mel cells were cocultured either with the JKF6 TIL clone or with irrelevant TIL001 cells for 4 days. Up-regulation of CEACAM1 on surviving melanoma cells was monitored as described above. Every day, the activity or inactivity of JKF6 or TIL001, respectively, was confirmed by measuring secretion of IFN-γ and killing of 526mel cells (data not shown). The surviving 526mel cells were concomitantly stained with the CEACAM1-Ig fusion protein. Because CEACAM1 interacts in a homophilic manner (21, 22, 29), binding of CEACAM1-Ig to 526mel cells reflects the capability of these cells to functionally interact with CEACAM1 *in trans*. 526mel melanoma cells are basally stained with the CEACAM1-Ig fusion protein (MFI = 15; data not shown). The ratio of CEACAM1-Ig binding to surviving 526mel cells was calculated similarly as above. Remarkably, the ratio of CEACAM1-Ig binding was significantly increased on the surviving 526mel cells coincubated with the reactive JKF6 cells (Fig. 7*A*). Noteworthy, the increase in CEACAM1-Ig binding ratio correlated with the increase in CEACAM1 expression ratio as measured by the Kat4c mAb (Fig. 7*A*). This shows that surviving melanoma cells have enhanced capability to functionally interact with CEACAM1. Importantly, no similar augmentation in CEACAM1-Ig binding ratio could be observed when 526mel cells were cocultured with the irrelevant TIL001 cells (Fig. 7*B*).

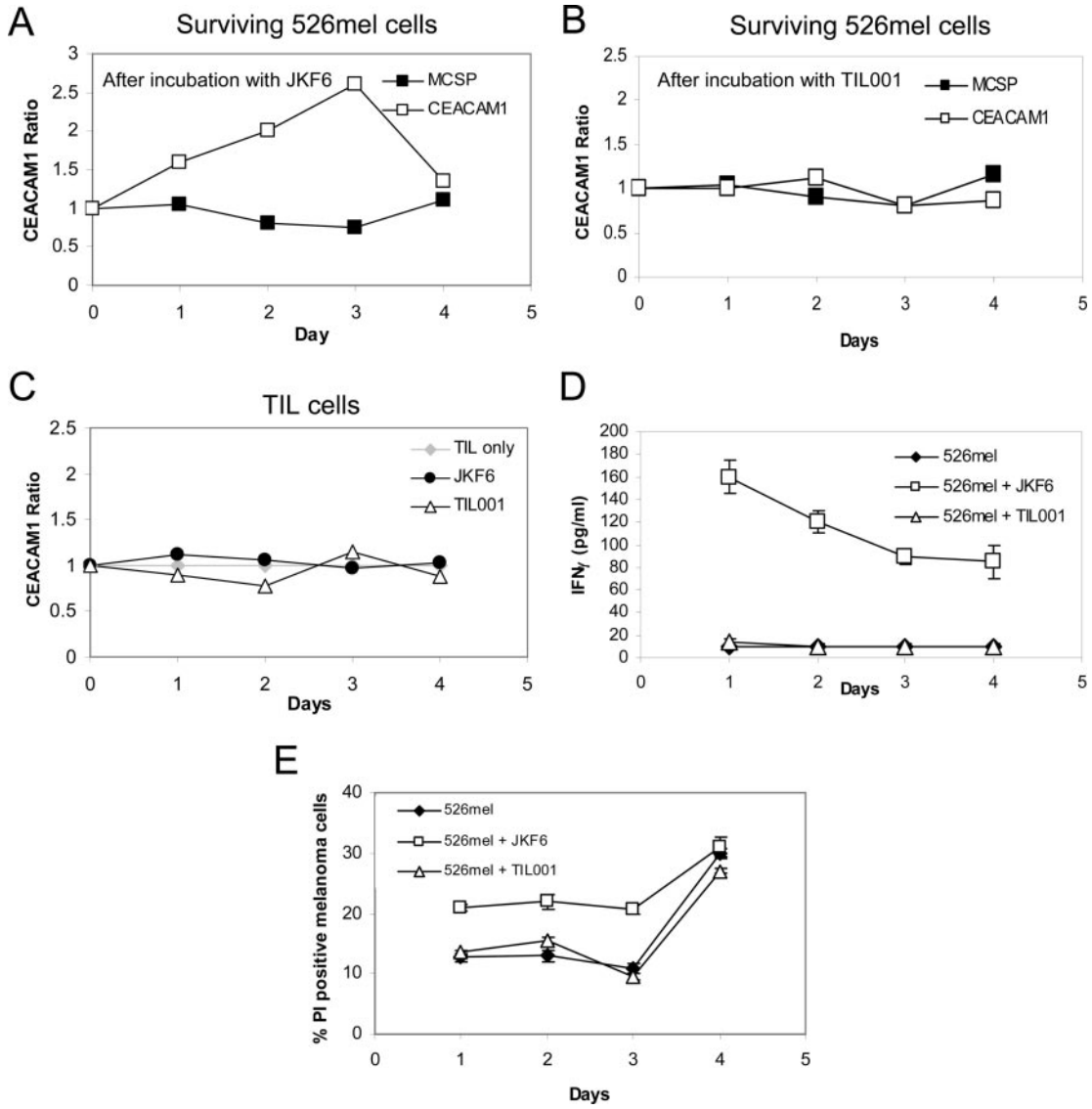

FIGURE 6. Specific recognition of melanoma by TIL leads to up-regulation of CEACAM1 on the surviving tumor cells. 526mel cells were coincubated for 7 days in 48-well plates either with 526mel-reactive (JKF6) or with the 526mel-inert (TIL001) TIL cells. Surviving 526mel cells and TIL cells were isolated and analyzed daily. Expression ratios of CEACAM1 and MCSP on surviving 526mel cells were monitored by dividing the staining MFI of the surviving 526mel cells following coculture with the JKF6 cells (*A*) or with the TIL001 cells (*B*) by the respective background MFI of 526mel cells cultured without the presence of TIL. CEACAM1 ratio was monitored daily on the TIL cells by dividing the staining MFI of the TIL cells incubated with 526mel cells, including the JKF6 cells or the TIL001 cells, by the respective background MFI of appropriate TIL cells incubated in the absence of 526mel cells (*C*). Expression of MCSP discriminated 526mel cells ($MCSP^{(+)}$) from TIL cells ($MCSP^{(-)}$). The specific reactivity of the different TIL cells was tested daily by the amount of IFN-γ in culture supernatants (*D*) and concomitantly via the percentage of PI-positive melanoma cells in the culture (*E*). These parameters were compared with 526mel cultures in the absence of any TIL cells (*D* and *E*). Figure shows a representative experiment of five performed.

Expression of CEACAM1 varies during TIL culture

The use of reactive TIL as a therapeutic modality in malignant melanoma requires massive expansion and activation (35) that eventually results in the up-regulation of the CEACAM1 protein (Fig. 1). However, before the massive expansion process, the TIL bulk cultures are slowly expanded with IL-2 until sufficient amounts are achieved. It is therefore interesting to evaluate the expression of CEACAM1 during the slow expansion phase. TIL cells obtained from a primary melanoma lesion of patient 001 (TIL001) were cultured in medium containing IL-2, exactly as described in previous references (35). Because only minute quantities of cells were available, the TIL culture was sequentially analyzed by flow cytometry at days 15, 20, 24, and 26 postoperation. After day 26, enough cells accumulated to initiate massive expansion. The cells were stained for several surface proteins, including CD3, CD4, CD8, CD16, CD56, and CEACAM1. At day 15 (the first analytic point), >95% of the cells were T cells ($CD3^+$ $CD56^-$), with <5% of the cells bearing the NK cell phenotype of $CD3^-CD56^+$ (data not shown). At this time point, the CD4:CD8 ratio was almost 1 (Fig. 8*A*). Remarkably, this ratio has skewed significantly toward CD8 during further culturing, until virtually all cells were $CD8^+$ and only negligible proportion expressed the CD4 protein (Fig. 8*A*). Importantly, however, the expression of CEACAM1, monitored with the 5F4 mAb, gradually decreased (Fig. 8*B*). On day 15, the MFI of CEACAM1 expression was highest (~14), decreased on day 20 to MFI~12 and reached the lowest MFI~6.1 on day 24, which remained similar to the last day of culture before massive expansion ensued (Fig. 8*B*). The expression level of CEACAM1 was similar among $CD4^+$ and $CD8^+$ cells

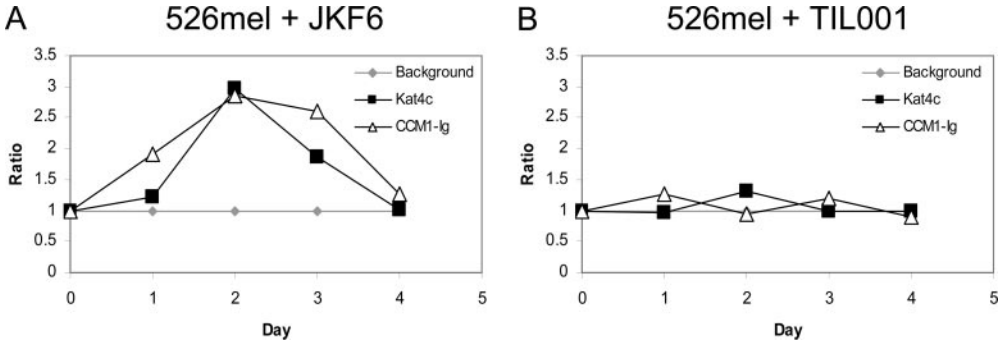

FIGURE 7. Up-regulated CEACAM1 on surviving melanoma cells is functional. 526mel cells were cocultured either with (*A*) JKF6 TIL clone or with (*B*) inert TIL001 cells for 4 days. Function of CEACAM1 was evaluated by monitoring the homophilic binding of CEACAM1-Ig fusion protein (CCM1-Ig) to the surviving cells divided by the binding of CEACAM1-Ig to the 526mel cells cultured without the presence of TIL cells (empty triangles). Binding of CEACAM1-Ig was detected in flow cytometry using FITC-conjugated goat anti-human $F(ab')_2$. The CEACAM1 expression ratio was concomitantly monitored on the surviving 526mel cells using the Kat4c mAb (black boxes). Background ratio is 1. Figure shows one representative experiment of four performed.

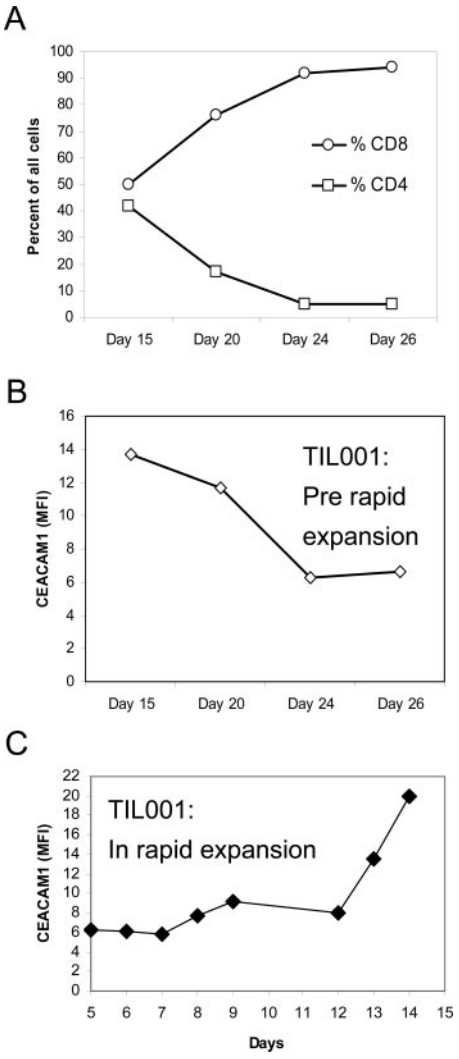

FIGURE 8. Kinetics of CEACAM1 expression on TIL during culture. Obtaining TIL cells from tumor specimen ex vivo might last >3 wk (slow expansion phase) before the rapid expansion protocol. Figure shows the variations exhibited by TIL001 population at the indicated time points during the slow expansion phase in (*A*) percentage of CD4 and CD8 cells and in (*B*) MFI of CEACAM1 expression on the entire TIL001 population. *C*, TIL001 cells during rapid expansion were analyzed for CEACAM1 expression using the 5F4 mAb (MFI) at indicated time points. Figure shows a representative bulk TIL culture.

(data not shown). The expression of CEACAM1 protein on T cells was reported to be activation-dependent in peripheral blood T cells (38) and decidual T cells (30). In this study, we suggest that changes in CEACAM1 expression might reflect the activation status of differentiated effector TIL cells.

As explained above, reactive TIL cells are massively expanded before ACT. Therefore, the kinetics of CEACAM1 expression during massive expansion was monitored with the 5F4 mAb almost daily. However, because the massive expansion protocol requires only minute amounts of TIL cells that are mixed with >3 orders of magnitude of irradiated PBMC (*Materials and Methods*), valid analysis of the TIL cells could be performed only from day 5. The MFI of CEACAM1 expression was ~6 until day 7, increased to 9 at day 9, 13 at day 12, and finally up to 20 at day 14 (Fig. 8*C*). In conclusion, expression of CEACAM1 increases through the course of the massive expansion. This further substantiates the correlation between CEACAM1 expression and the activation status of TIL cells. These temporal differences might prove important in the optimization of these protocols for clinical ACT therapy.

Discussion

The cellular immune response to tumors depends on a complex spatiotemporal interplay between various components. Yet, it is exerted by the final differentiation of immune cells into effector cells with the capacity to migrate and penetrate into the tumor. Delineation of the interactions between tumor cells and the effector immune cells stands in the focus of investigational efforts, particularly the identification of the various factors that control immune infiltration, immunologic cross-talk, antitumor immune functions, and tumor escape mechanisms. Therefore, TIL ACT technology provides an exciting platform for the treatment of resistant malignancies. For example, in malignant melanoma refractory to conventional immunotherapeutic modalities such as IFNα or IL-2, TIL ACT treatment has proven beneficial (5, 6). The mechanisms of melanoma refractoriness to these modalities are not completely understood and probably integrate multiple components. For example, in metastatic melanoma patients receiving high-dose IL-2, it has been shown that the frequency and number of $CD4^+$ $CD25^{high}$ T regulatory cells in the peripheral blood increased in patients with progressive disease and returned to normal in patients with objective clinical response (39). In another example, it has been demonstrated that IFN-α activates STAT5, which counteracts IFN-α's antiproliferative action by diminishing STAT1 activation, in melanoma cells and that in IFN-α-resistant melanoma cells the

STAT5 is already overexpressed (40). Other immunological approaches for priming the immune system through immunization strategies have been generally unsuccessful so far in causing regression of established vascularized tumors, although rare regressions have been reported (41). Multiple obstacles impair immunization strategies, such as inability of the sensitized lymphocytes to home and infiltrate the tumors (42), loss of Ag expression, and general or local immune suppressive states (43). TIL ACT is based on the ability to sensitize and expand ex vivo lymphocytes that have already acquired the ability to recognize, reach, and eliminate cancer cells. In current TIL ACT protocols, elimination of host immune suppressive state is achieved by prior nonmyeloablative chemotherapy that eliminates host T regulatory cells and biological competition over survival and expansion factors, such as IL-2 and IL-15 (44). Indeed, significant results were observed with modified TIL ACT in a recent study, where up to 50% of the melanoma patients that failed to respond to other modalities exhibited significant clinical response with some of the patients remarkably achieving complete response (18). These encouraging results further merit the continuous efforts placed on the identification of the biological parameters affecting the efficacy of this modality or predicting its success. Indeed, several approaches based on the underlying biology have already been taken to improve TIL ACT, including concurrent cytokine administration (15), pre-ACT nonmyeloablative chemotherapy (18), and genetic TIL manipulations (17). Additional studies have focused on prediction of treatment success and on selection of the appropriate patients suitable for this treatment (45, 46).

We have previously identified the MHC class I-independent inhibitory mechanism of human NK killing activity, which is mediated by homophilic CEACAM1 interactions (29–32). In this study, we show for the first time the presence of the inhibitory molecule CEACAM1 on virtually all activated human TIL cultures, which represent the cells used in TIL ACT. Remarkably, homophilic CEACAM1 interactions inhibited both TIL-specific killing activity and IFN-γ secretion in most of the melanoma cultures (Fig. 4). We have further shown the inhibitory effect of CEACAM1 on TIL function in a clean cell system, the 721.221 transfectants of CEACAM1, and HLA-A2 (Fig. 5). Inhibition of effector T cell functions by CEACAM1 might depend on cell differentiation, because TIL represent a differentiated effector subset of memory lymphocytes. These in vitro results suggest that CEACAM1 homophilic interactions may impair TIL-mediated antitumor response in vivo. This is emphasized by the common expression of CEACAM1 by different tumors, including melanoma (27, 29) and lung (47, 48) tumors. To our knowledge, the effect of CEACAM1 on the in vivo function of TIL or on the efficacy of TIL ACT has never been investigated before.

The continuous interactions of the immune system with the attacked tumor cells most probably lead to mutual reciprocal effects. These alterations in characteristics of both sides during the course of time eventually result in the in vivo coevolution of both tumor and immune response. For example, Yamshchikov et al. (49) followed and demonstrated "immune editing" by the tumor and the consequent adaptation of the dominant immune response in vivo in a long-term survivor of melanoma. We have observed an increased expression of CEACAM1 on melanoma cells that have survived specific recognition by reactive TIL cells in a 4-day in vitro coincubation assay (Fig. 6). Furthermore, we show that this is a functional phenomenon as evident by increased CEACAM1-Ig binding to the surviving melanoma cells (Fig. 7). This observation could be explained by selection for melanoma cells with higher CEACAM1 expression, which are more resistant to elimination. However, several points argue against this possibility: 1) in these experiments the melanoma killing rates were not phenomenal, which diminishes the possibility of efficient selection in such a short time; 2) there is a decrease in CEACAM1 expression after the initial increase over a course of only 4 days—this is more suggestive of alterations in protein expression rather than simple immune selection; and 3) all surviving tumor cells homogeneously increased CEACAM1 expression, which implies that all surviving cells were exposed to the same soluble or cell-bound factor. Alternatively, CEACAM1 is up-regulated on the surviving tumor cells, which represents a responsive escape mechanism from ongoing tumor lysis. The emergence of melanoma cells with higher CEACAM1 expression only following coincubation with reactive TIL emphasizes the significance of CEACAM1-mediated immune evasion mechanism, regardless of the underlying reason. In conclusion, these combined observations imply that CEACAM1 homophilic interactions may be involved in the local immunosuppressive tumor microenvironment. Importantly, the reactive TIL cells did not exhibit similar up-regulation in CEACAM1 expression following specific tumor recognition and killing. This enables the launched immune response to avoid an even stronger inhibition by CEACAM1 and maybe retain some antitumor capacity. It is still unclear why surviving cells up-regulate CEACAM1, as opposed to the reactive TIL cells.

Naturally occurring TIL cells exert the antitumor immune response in vivo and comprise a crucial component in ACT immunotherapy for malignant melanoma. Thus, our findings have not only scientific significance, but may also impact current clinical TIL ACT approach. Daily analysis of CEACAM1 expression during the preinjection expansion of reactive TIL cells suggests a correlation between CEACAM1 expression and the activation state (Fig. 8, *B* and *C*). We further show that CEACAM1 expression intensity on TIL cells increases through the time course of massive expansion process (Fig. 8*C*). We have previously shown that the strength of CEACAM1-mediated inhibition correlates directly with the intensity of CEACAM1 expression (29). Therefore, shortening of the expansion protocols might yield TIL cells with relatively reduced CEACAM1 expression and thus augmented antitumor capacity. However, the penalty for such alteration will probably be lesser quantities of reactive TIL cells. The optimization of TIL expansion protocols, considering numeral expansion efficiency, activation status, and CEACAM1 expression are to be investigated.

Finally, the identification of CEACAM1-mediated inhibition in TIL-melanoma interactions provides both a molecular basis and clinical platform for the development of novel immunomodulatory strategies. For example, administration of TIL ACT concomitantly with an agent that blocks the CEACAM1 homophilic interactions might boost the in vivo response, similar to the augmentation of TIL effector functions observed in vitro (Fig. 4). In addition, various methods for selective down-regulation or inhibition of CEACAM1 expression may be used to deliver CEACAM1^{dim} TIL cells in ACT treatments. Finally, these methods might eventually develop into adjuvant or even independent modalities for cancer immunotherapy.

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Disclosures

The authors have no financial conflict of interest.

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