Melanoma immunotherapy by targeted IL-2 depends on CD4+ T-cell help mediated by CD40/CD40L interaction

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The induction of tumor-protective immunity against malignancies remains a major challenge in cancer immunotherapy. A novel, humanized anti-ganglioside-GD_2–IL-2 immunocytokine (hu14.18–IL-2) induced $CD8^+$ T cells to eradicate established pulmonary metastases of B78-D14 murine melanoma, in a process that required help by $CD4^+$ T cells and was mediated by the CD40/CD40 ligand (CD40L) interaction. The anti-tumor effect was diminished in mice deficient in $CD4^+$ T-cells. Three lines of evidence show that $CD4^+$ T-cell help was mediated by CD40/CD40L interaction but not by endogenous IL-2 production. First, the hu14.18–IL-2–induced anti-tumor response is partially abrogated in C57BL/6J CD40L knockout (KO) mice in contrast to C57BL/6J IL-2 KO animals, in which the immunocytokine was completely effective. Second, partial abrogation of the anti-tumor effect is induced with anti-CD40L antibodies to the same extent as with $CD4^+$ T-cell depletion. Third, a complete anti-tumor response induced by hu14.18–IL-2 can be reconstituted in C57BL/6J CD40L KO mice by simultaneous stimulation with an anti-CD40 mAb. These results suggest that help provided by $CD4^+$ T cells via CD40/CD40L interactions in our tumor model is crucial for effective immunotherapy with an IL-2 immunocytokine.

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Introduction

A critical step in induction of T cell–mediated, tumor-protective immunity against syngeneic malignancies is the effective initial activation and subsequent maintenance of a tumor-specific $CD8^+$ T cell–mediated immune response.

One strategy for achieving this goal is to increase cytokine concentration in the local tumor microenvironment by introduction of cytokine genes into autologous tumor cells, commonly referred to as cytokine gene therapy (1). We demonstrated the feasibility of an alternative non–patient-specific therapy with tumor-specific Ab-cytokine fusion proteins called immunocytokines, which direct immunomodulatory substances into the tumor microenvironment (2, 3). Tumor-specific targeting of human recombinant IL-2 using a human/mouse chimeric anti-ganglioside-GD_2–IL-2 immunocytokine (ch14.18–IL-2) targeted IL-2 to the tumor and induced a $CD8^+$ T cell–mediated, long-lived, transferable tumor-protective immunity against murine GD_2-positive B78-D14 melanoma cells in syngeneic C57BL/6J mice. In contrast, a nonspecific IL-2 immunocytokine proved ineffective (4–6). These results are consistent with the successful induction of a vaccination effect against syngeneic melanoma. Three major findings support this conclusion. First, depletion of $CD8^+$ T cells abolished the treatment effect mediated by ch14.18–IL-2. Second, this therapy failed in T cell–deficient C57BL/6J *scid/scid* mice, but was fully operational in T cell–competent, natural killer cell–deficient (NK cell–deficient) C57BL/6J *beige/beige* mice. Third, adoptive transfer of $CD8^+$ T cells from animals successfully treated with immunocytokine protected C57BL/6J *scid/scid* mice from lethal tumor cell challenge.

Three major events must occur to induce $CD8^+$ T cell–mediated, tumor-protective immunity against syngeneic melanoma. First, the T-cell receptor must be triggered by a (or multiple) self antigen–derived peptide MHC class I complex (7–13). Therefore, this event depends entirely on appropriate antigen presentation, which is most efficiently provided by mature dendritic cells (14). Peripherally tolerant or "ignorant" self-reactive T-cell clones, once properly activated, may serve as tumor-specific effector T cells (15, 16). Second, simultaneously with T-cell receptor triggering, a distinct second costimulatory signal must be delivered, mediated by IL-2, B7-1, or B7-2, which engage IL-2 receptors and CD28 on the surface of the T cell, respectively (17). A source of these cofactors for effective $CD8^+$ T-cell stimulation can be provided by $CD4^+$ T cells that release critical amounts of IL-2, or by mature dendritic cells that display an increased level of B7-1/B7-2 costimulatory molecules on their cell surfaces. Third, inflamma-

tory cytokines, including IL-1, IL-6, IL-12, and IFN-γ provide a third signal that acts directly on T cells (18), referred to as the "danger signal" (19, 20). This signal was found to optimally activate T_H1 differentiation and lead to clonal expansion of T cells (18).

In the context of optimal stimulation of $CD8^+$ T cells, $CD4^+$ T cells were reported to play a crucial role not only in the release of soluble immunomodulatory factors such as IL-2 (21) into the tumor microenvironment, but also in activation of antigen presentation. This effect is also mediated by ligation of CD40 on the surface of antigen presenting cells (APC) (21), and is induced by CD40L (CD154) expressed on activated $CD4^+$ T cells, leading to maturation of dendritic cells (22) and upregulation of antigen presentation in functions of the MHC and costimulatory molecules.

Here, we extend prior findings on the induction of a tumor-protective immunity against syngeneic melanoma by tumor-targeted IL-2 using humanized versions of anti-GD_2 immunocytokine hu14.18–IL-2. In our model, most optimal immunotherapy with IL-2 immunocytokines depended on help from $CD4^+$ T cells. This was mediated by interactions between CD40 and CD40L, but was independent of endogenous production of IL-2.

Methods

Mice. Syngeneic female C57BL/6J mice, C57BL/6J *scid/scid* mice, C57BL/6J *beige/beige* mice, and CD40L knockout (KO) mice (B6/129-T*nfsf5*^{tm1Imx}) were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Syngeneic C57BL/6Tac-[KO]Abb N5 $CD4^+$ helper T cell–deficient MHC class II KO mice and C57BL/6Gph-Tac-[KO]B2m N5 $CD8^+$ T cell–deficient MHC class I KO mice were purchased from Taconic Farms (Germantown, New York, USA). All mice were housed in groups of four each in the pathogen-free mouse colony at our institution, and were used at 6–8 weeks of age for each experiment. Mice were fed ad libitum on standard mouse laboratory chow. Animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Immunocytokines. The mouse-human chimeric Ab ch225–IL-2 control immunocytokine directed against the human EGF receptor has been described previously (23). The humanized anti-disialoganglioside GD_2 antibody hu14.18, and its fusion protein with hIL-2 were generated and provided by Lexigen Pharmaceuticals Corp. (Lexington, Massachusetts, USA). An equimolar amount of 3,000 IU rhIL-2 (specific activity 16×10^6 IU/mg; Chiron Corp., Emeryville, California, USA) contained in 1 μg hu14.18–IL-2 was used in Ab-cytokine mixture controls (24).

Cells and animal model. Generation and propagation of the GD_2-positive murine B78-D14 melanoma cell line have been described previously (4, 25). Briefly, the murine melanoma cell line B78 was transfected with genes encoding β-1,4-*N*-acetylgalactosaminyltransferase and α-2,8-sialyltransferase, inducing constitutive expression of the gangliosides GD_2 and GD_3 on a subclone called B78-D14. These cells were grown as monolayers in the presence of 400 μg/mL G418 and 50 μg/mL hygromycin B in RPMI containing 10% FCS and 2 mM L-glutamine. The level of pulmonary metastases was increased by two in vivo passages of 5×10^6 B78-D14 cells. This was followed by in vitro culture of single pulmonary metastases 5 weeks after initial tumor cell injection and the isolation of an amelanotic subline, B78-D14 2.34.

Experimental lung metastases were induced by injections of a single-cell suspension of 1.25×10^6 B78-D14 2.34 cells into the lateral tail vein. To prevent pulmonary embolism caused by the injection of tumor cells, they were administered in a total volume of 500 μL PBS containing 0.1% BSA over a period of 60 seconds. Four days later, established micrometastases were present throughout the lungs; after 28 days, grossly visible disseminated metastases were observed on the surface of these organs. Therefore, treatment of established pul-

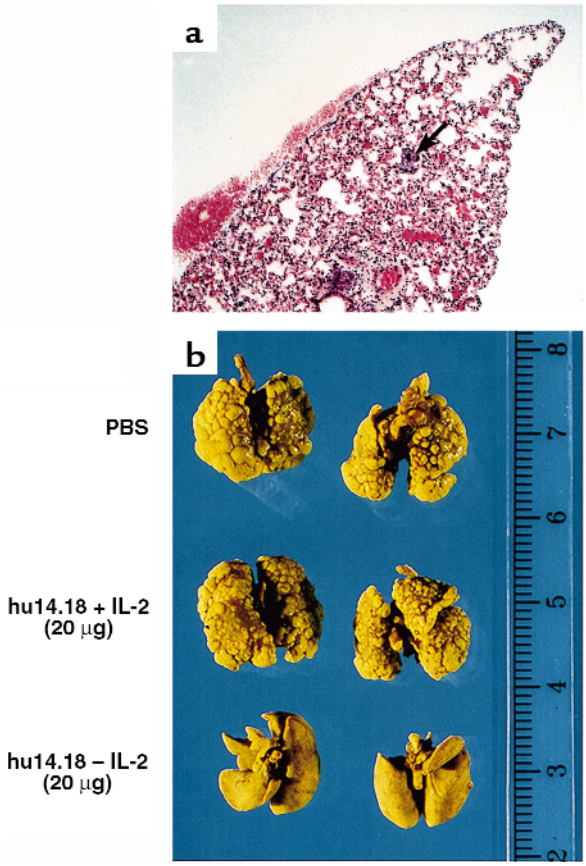

Figure 1
Treatment of established micrometastases with hu14.18–IL-2 immunocytokine. (**a**) The presence of micrometastases (arrow) was determined in lungs of C57BL/6J mice injected intravenously with 1.25×10^6 B78-D14 2.34 cells, 4 days after tumor cell injection. (**b**) Treatment of established melanoma metastases was initiated at that time with daily intravenous applications (for 5 days) of either 100 μL PBS (top row), an equivalent mixture of hu14.18 Ab (20 μg) and rhIL-2 (60,000 IU) (second row), or 20 μg hu14.18–IL-2 immunocytokine (third row). Two representative specimens of each treatment group of six mice were selected for photography.

monary metastases was initiated 4 days after tumor-cell inoculation by daily intravenous injections (for 5 days) with 20 μg each of tumor-specific hu14.18–IL-2. Results of this treatment were compared with results of treatment with an equivalent mixture of either 20 μg hu14.18 Ab and 60,000 IU rhIL-2, or 20 μg of a nonspecific ch225–IL-2 immunocytokine. The metastatic load in the lungs of individual mice was assessed 28 days after tumor-cell inoculation by determining lung weights of freshly prepared specimens.

Depletion of $CD4^+$ and $CD8^+$ T-cell subsets was accomplished by weekly intraperitoneal injections for 4 weeks (on days –2, 5, 12, and 19) of 500 μg rat anti-mouse anti-CD4 mAb (GK1.5) and anti-CD8 mAb (53-6.7), respectively. The greater than 95% absence of $CD4^+$ and $CD8^+$ T cells was confirmed by FACS® analysis of splenocytes from depleted animals, as described previously (ref. 4 and data not shown). The generation, characterization, and production of anti-CD40L (MR-1) and anti-CD40 (FGKK5) mAb's has been described elsewhere (21, 26–28). Experiments blocking CD40/CD40L interaction with anti-CD40L Ab were performed by daily intraperitoneal injections (for 3 days) of 200 μg anti-CD40L, given simultaneously with hu14.18–IL-2 treatments. Surrogate CD40 stimulation was accomplished by one intraperitoneal injection of 200 μg anti-CD40 Ab 4 days after tumor-cell inoculation.

Flow cytometry. Activation of $CD8^+$ T cells and CD11c-positive APCs was measured by multiple color flow cytometry analysis. For this purpose, animals were inoculated with 1.25×10^6 B78-D14 2.34 melanoma cells, treated as outlined in the legends to Figures 5 and 6, and sacrificed on day 12. $CD8^+$ T-cell activation was determined by staining of freshly isolated splenocytes with anti-CD8 FITC (53-6.7), anti-CD25 PE (H129.19), or anti-CD69 PE (H1.2F3). Activation of APCs was measured using anti-CD11c FITC (HL-3), anti-CD86 PE (GL1), biotinylated anti-IA^b (KH74), and streptavidin-allophycocyanin. All flow experiments were performed in the presence of 0.1 μg/mL propidium iodide and CD16/CD32 (Fcγ III/II receptor) Fc Block™ in order to exclude dead cells and block nonspecific Fc-receptor binding, respectively. All reagents were obtained from PharMingen (La Jolla, California, USA). Cells were incubated in PBS (pH 7.4) containing 0.05% NaN_3 and 1% FCS in the presence of labeled antibodies at 1:200 dilution, for 30 minutes at 4°C. After being washed in the same buffer, cells were analyzed by FACScan (Becton Dickinson, Bedford, Massachusetts, USA). A minimum of 10,000 labeled cells per sample were analyzed.

Statistics. The statistical significance of differential findings between experimental groups of animals was determined by two-tailed Student's *t* test. Findings were regarded as significant if two-tailed *P* values were below 0.05.

Results

Effect and characterization of the immune response induced by the hu14.18–IL-2 immunocytokine. We previously demonstrated a T cell–mediated eradication of established pulmonary metastases after tumor-targeted IL-2 therapy using a mouse-human chimeric anti-ganglioside GD_2 antibody–IL-2 immunocytokine, ch14.18–IL-2 (4). Here, we extend these findings, showing that daily injections for 5 days with 20 μg of a humanized version, hu14.18–IL-2, also elicit effective eradication of established pulmonary metastases in the majority of animals (Figures 1 and 2; Table 1). This was demonstrated by a complete absence of both micrometastases (data not shown) and macroscopic metastatic foci on the lung surfaces of 4 of 6 mice (Figure 1, Table 1), determined by histologic examination. A correlation between the number of metastatic foci and organ weight was established for this model (Table 1), and proved to be the most sensitive and quantitative parameter for metastatic disease. Therefore, lung

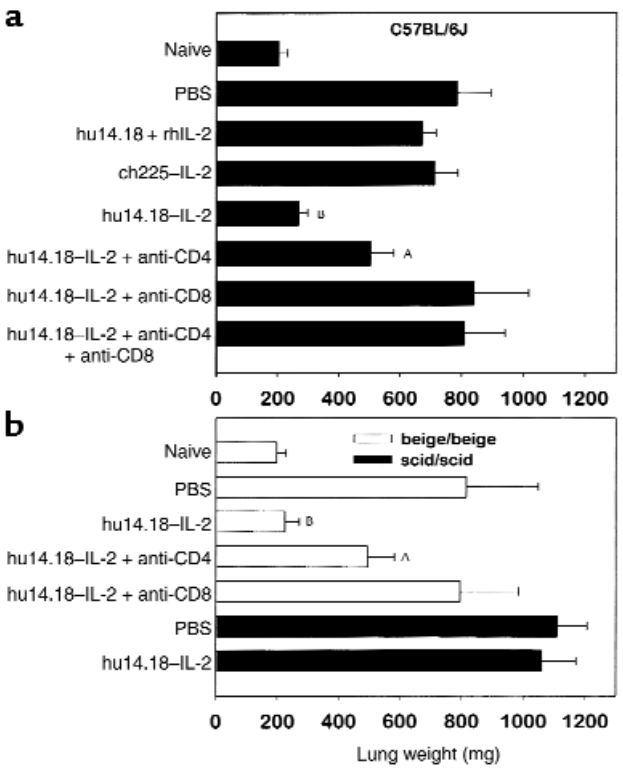

Figure 2
Effect of in vivo depletion of $CD4^+$ and $CD8^+$ T cells on hu14.18–IL-2 therapy of established pulmonary melanoma metastases. The therapeutic effect of hu14.18–IL-2 was determined in immunocompetent C57BL/6J (**a**) and NK cell–deficient C57BL/6J *beige/beige* mice depleted of $CD4^+$ and/or $CD8^+$ T cells and compared with C57BL/6J *scid/scid* controls (**b**). Experimental metastasis was induced by intravenous injection of 1.25×10^6 B78-D14 2.34 cells, followed by treatment beginning 4 days thereafter with daily intravenous applications (for 5 days) of either 20 μg hu14.18–IL-2, an equivalent mixture of hu14.18 Ab and rhIL-2, 20 μg nonspecific ch225–IL-2, or PBS. A group of six mice were not injected with B78-D14 2.34 cells (naive control). Depletion of $CD4^+$ and $CD8^+$ T cells was accomplished as described in Methods. Twenty-eight days after tumor-cell injection, mice were euthanized before determination of total fresh lung weights. Bars represent mean and SD of six mice per group ($^AP < 0.02$, $^BP < 0.01$ compared with all control groups).

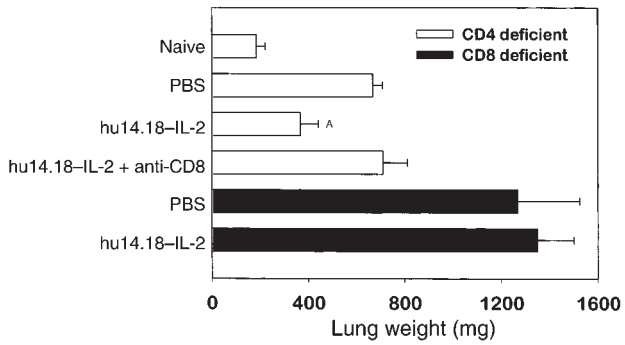

Figure 3
Anti-tumor immune response in CD8+ and CD4+ T cell–deficient C57BL/6J MHC class I and class II KO mice induced by hu14.18–IL-2. The therapeutic effect of hu14.18–IL-2 was determined in C57BL6/J MHC class I and II KO mice characterized by CD8+ and CD4+ T-cell subpopulation deficiencies, respectively. Treatment was initiated 4 days after tumor-cell inoculation by daily intravenous injections (for 5 days) with 20 μg hu14.18–IL-2 or PBS. Depletion of CD8+ T cells in CD4+ T cell–deficient mice was accomplished as described in Methods. One group of six mice was not injected with B78-D14 2.34 cells (naive control). Twenty-eight days after tumor-cell injection, mice were euthanized before determination of total fresh lung weights. Bars represent mean and SD of six mice per group ($^{A}P < 0.01$ compared with all control groups).

weights were used for further evaluation of anti-melanoma responses. The anti-tumor response observed with hu14.18–IL-2 was primarily mediated by CD8+ T cells, as demonstrated in three independent experimental settings. First, in vivo depletion of CD8+ T cells from immunocompetent C57BL/6J mice decreased the therapeutic effect of hu14.18–IL-2 back to the control levels that were obtained with injections of either PBS, an equivalent mixture of hu14.18 Ab and IL-2, or a nonspecific ch225–IL-2 immunocytokine (Figure 2). Second, treatment of melanoma metastases with hu14.18–IL-2 was ineffective in T cell–deficient C57BL/6J *scid/scid* mice, in contrast to NK cell–deficient, T cell–competent C57BL/6J *beige/beige* mice. This indicates a T cell–mediated but not an NK cell–mediated immune response. The effect of treatment with hu14.18–IL-2 was also reversed in this mouse strain by depletion of CD8+ effector T cells (Figure 2). Third, pulmonary metastases remained disseminated throughout the lung after treatment with hu14.18–IL-2 immunocytokine in CD8+ T cell–deficient MHC class I KO mice (Figure 3).

The induction of an optimally effective CD8+ T cell–mediated anti-tumor response by hu14.18–IL-2 clearly depends on the presence of CD4+ T cells. This was demonstrated by partial abrogation of the anti-tumor effect mediated by hu14.18–IL-2 in three different experimental settings that were characterized by a deficient CD4+ T-cell compartment, but presence of CD8+ T cells in vivo. Immunocompetent C57BL/6J mice and NK cell–deficient, T cell–competent C57BL/6J *beige/beige* mice depleted of CD4+ T cells revealed a partial abrogation (to about 50%) of the treatment effect mediated by hu14.18–IL-2 (Figure 2). A similar result was obtained in CD4+ T cell–deficient MHC class II KO mice, in which the treatment with hu14.18–IL-2 was also only partially effective (Figure 3). These findings contrast sharply with those obtained with CD4+ and CD8+ T cell–competent C57BL/6J and C57BL/6J *beige/beige* mice, respectively, in which the hu14.18–IL-2 immunocytokine treatment was completely effective (Figure 2). In summary, these data demonstrate a helper function for CD4+ T cells in CD8+ T cell–mediated anti-melanoma immune responses induced by hu14.18–IL-2.

Determination of the help mechanism provided by CD4+ T cells. There are currently two models for help provided by CD4+ T cells, including production of T-cell growth factor IL-2 and activation of APCs for most efficient antigen presentation via CD40/CD40L interaction. We determined which model applied for help provided by CD4+ T cells in CD8+ T cell–mediated anti-melanoma immune responses induced by hu14.18–IL-2 by using mouse strains deficient in endogenous IL-2

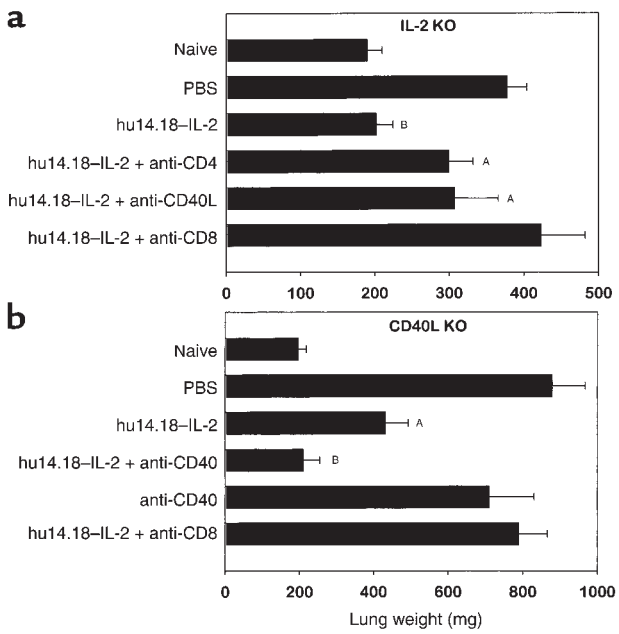

Figure 4
Role of endogenous IL-2 and CD40L in anti-tumor effects on established pulmonary melanoma metastases mediated by hu14.18–IL-2. The anti-tumor immune response mediated by hu14.18–IL-2 was determined in C57BL/6J IL-2 KO (**a**) and CD40L KO mice (**b**). All mice were inoculated with 1.25×10^6 B78-D14 2.34 cells, and treatment was initiated 4 days thereafter with daily intravenous administration of 20 μg hu14.18–IL-2, for 5 days. This treatment was combined with daily intraperitoneal injections of 250 μg anti-CD40L Ab for three days (**a**), or one intraperitoneal injection with 200 μg anti-CD40 mAb (**b**), also starting at day 4. Depletion of CD4+ and CD8+ T cells was performed as described in Methods. One group of six mice was not injected with B78-D14 2.34 cells (naive control). Twenty-eight days after tumor-cell injection, all mice were euthanized, followed by determination of total lung weights on fresh specimins. Bars represent mean and SD of six mice per group ($^{A}P < 0.05$, $^{B}P < 0.01$ compared with all control groups).

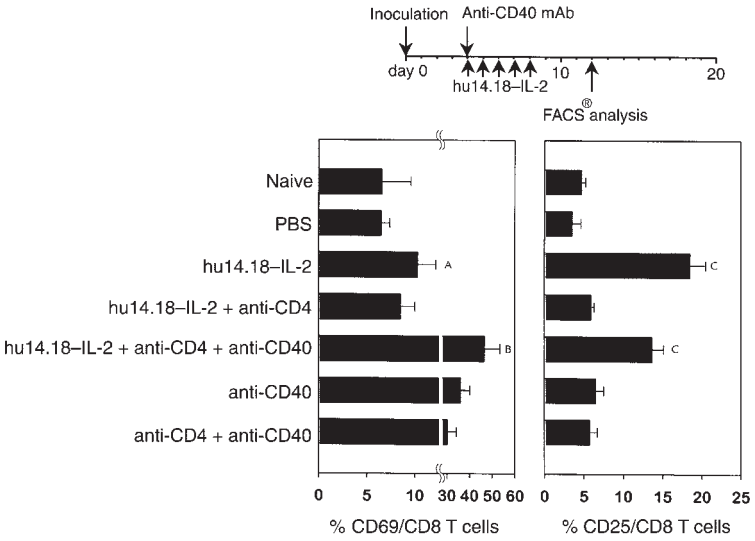

Figure 5
T-cell activation after hu14.18–IL-2 and anti-CD40 therapy of established pulmonary melanoma metastases. Increase in the proportion of $CD8^+$ T cells characterized by elevated CD69 and CD25 expression was investigated in mice bearing established pulmonary metastases (resulting from 1.25×10^6 B78-D14 2.34 cells injected intravenously) after therapy with hu14.18–IL-2 and anti-CD40. Treatment was initiated 4 days after tumor-cell inoculation by daily intravenous administration of either 20 μg hu14.18–IL-2 or PBS for 5 days, or a single intraperitoneal injection with 200 μg anti-CD40 mAb. Depletion of $CD4^+$ T cells was accomplished as described in Methods. One group of six mice was not injected with B78-D14 2.34 cells (naive control). Four days after completion of the treatment, mice were euthanized, and splenocytes were subjected to FACS® analysis for CD69/CD8 and CD25/CD8 double-positive T cells. Bars represent mean and SD of five mice per group. Findings between experimental groups and all control groups were statistically significant ($^AP < 0.05$, $^BP < 0.01$, $^CP < 0.001$).

production and CD40L expression, respectively. Interestingly, the treatment with hu14.18–IL-2 was completely effective in C57BL6/J IL-2 KO mice, in contrast to C57BL6/J CD40L KO mice (Figure 4). In the latter mice, the therapeutic effect was partially abrogated, indicating a CD40/CD40L–mediated mechanism. Two additional lines of evidence supported this supposition. First, the efficacy of hu14.18–IL-2 treatment in the IL-2 KO mouse was partially decreased by anti-CD40L Ab blocking of CD40L interaction with CD40 (Figure 4). This inhibition occurred to a similar extent to that achieved by depletion of $CD4^+$ T cells in the same mouse strain. A control group depleted of $CD8^+$ T cells also revealed complete abrogation of the treatment effect mediated by hu14.18–IL-2, indicating that the anti-tumor response is also mediated by $CD8^+$ effector T cells in this mouse strain. Second, the partial efficacy of hu14.18–IL-2 in CD40L KO mice could be reestablished by in vivo stimulation with anti-CD40 mAb (Figure 4), a reagent that was reported to provide surrogate $CD4^+$ T-cell help by stimulating APCs expressing CD40 (21). In fact, we demonstrated that a combination of hu14.18–IL-2 with anti-CD40 induced a synergistic, elevated anti-melanoma immune response in CD40L KO mice, which was in contrast to controls treated with each agent used alone.

Effect of hu14.18–IL-2 treatment on the activation of T cells and APCs in the presence or absence of CD40 stimulation. The stimulation of T cells and APCs after depletion of $CD4^+$ T cells or depletion of $CD4^+$ T cells plus treatment with hu14.18–IL-2 in the presence or absence of anti-CD40 mAb was determined by analysis of T-cell activation markers CD69 and CD25, and parameters associated with activation of APCs, including B7-2 and MHC class II $I\text{-}A^b$ molecules (Figures 5 and 6). This analysis revealed a fourfold increase in $CD25^+/CD8^+$ T

Table 1
Effect of hu14.18–IL-2 immunocytokine on established pulmonary melanoma metastases

Treatment[A]	Number of foci[B]	Lung weight (mg)[C]
PBS	> 250, > 250, > 250, > 250, > 250, > 250	721 ± 89
hu14.18 (20μg) + rhIL-2 (60,000 IU)	>250, > 250, > 250, 181, 160, 123	527 ± 145
hu14.18–IL-2 (20 μg)	0, 0, 0, 0, 5, 13	197 ± 43[D]

All animals received an intravenous injection of 1.25×10^6 B78-D14 2.34 melanoma cells. [A]Treatment was initiated 4 days after tumor-cell inoculation consisting of daily intravenous injections with 20 μg hu14.18–IL-2 immunocytokine, an equivalent mixture of 20 μg hu14.18 Ab plus 60,000 IU rhIL-2, or PBS, for 5 days. [B]Pulmonary metastases were determined 1 week after fixation in Bouin's solution by counting metastatic foci under a low-magnification microscope. [C]Lung weights were determined on fresh specimens, before fixation in Bouin's solution. [D]The differences in numbers of metastatic foci and lung weights between this experimental group and all control groups was statistically significant ($P < 0.005$).

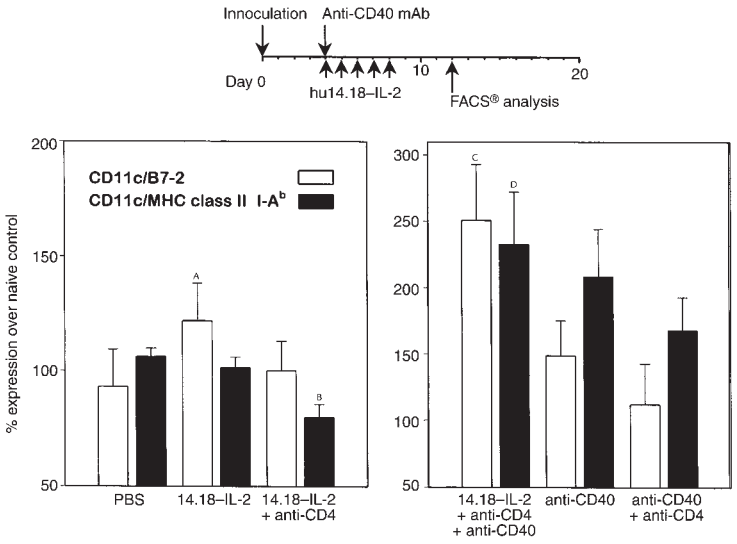

Figure 6
Activation of APCs by administration of hu14.18–IL-2 and anti-CD40 in melanoma metastases–bearing mice. APC activation as defined by upregulation of B7-2 and MHC class II molecules was determined in mice with established pulmonary metastases (resulting from 1.25×10^6 B78-D14 2.34 cells injected intravenously) after hu14.18–IL-2 and anti-CD40 therapy, compared with that of naive mice. Treatment was initiated 4 days after tumor-cell inoculation by daily intravenous administrations of 20 μg hu14.18–IL-2 for 5 days, or a single intraperitoneal injection with either 200 μg anti-CD40 mAb or PBS. Depletion of $CD4^+$ T cells was accomplished as described in Methods. Four days after completion of the treatment, mice were euthanized and splenocytes were subjected to FACS® analysis for CD11c/B7-2 (open bars) and CD11c/MHC class II I-A^b (closed bars) double-positive APCs. Values were expressed as percentage of values from naive controls. Bars represent mean and SD of four mice per group. Findings between experimental groups and all control groups were statistically significant ($^AP < 0.02$, $^BP < 0.01$, $^CP < 0.0005$, $^DP < 0.02$).

cells, and a 50% increase in the percentage of $CD69^+/CD8^+$ T cells after treatment with hu14.18–IL-2 (Figure 5). The increase indicates effective $CD8^+$ T-cell activation consistent with a $CD8^+$ effector T cell–mediated mechanism that has been described in previous experiments (4). This increase was reversed in groups of mice depleted of $CD4^+$ T cells, which was more pronounced for CD25 than CD69, highlighting the importance of the $CD4^+$ T-cell helper function observed in this system. However, the proportion of $CD25^+/CD8^+$ and $CD69^+/CD8^+$ T cells could be reestablished and amplified, respectively, in mice that were depleted of $CD4^+$ T cells but treated simultaneously with hu14.18–IL-2 and anti-CD40 (Figure 5). These results further support a crucial role for the CD40/CD40L interaction in optimally efficient $CD8^+$ T-cell activation in this mouse model. The contention that this effect is mediated by increased activation of CD11c-positive APCs is indirectly supported by an increase in the proportion of B7-2–positive and MHC class II I-A^b CD11c–positive APCs in mice that were depleted of $CD4^+$ T cells and were treated simultaneously with hu14.18–IL-2 and anti-CD40 (Figure 6). Direct evidence for activation of CD11c-positive APCs isolated from those mice was established by an increase in the fluorescence intensity of both markers, B7-2 and MHC class II I-A^b (data not shown). This finding contrasts with results from mice depleted of $CD4^+$ T cells that received hu14.18–IL-2 or anti-CD40 monotherapy. These animals revealed either no increase or a reduced increase in the proportion of B7-2– and I-A^b–positive CD11c APCs (Figure 6). Similarly, there was no increase or a reduced increase in the fluorescence intensity of B7-2 and I-A^b on CD11c-positive APCs compared with control animals injected with PBS (data not shown).

Discussion

Targeting of cytokines into the tumor microenvironment may emerge as a promising strategy for adjuvant immunotherapy of cancer, based on a tumor-specific increase in immunogenicity of the patient's autologous tumor cells. This can be accomplished by directing IL-2 into the tumor microenvironment with tumor-specific Ab-cytokine fusion proteins, called immunocytokines; this technique has been demonstrated in several xenograft and syngeneic preclinical animal models for colorectal carcinoma (29–31), prostate cancer (32), neuroblastoma (24, 33), and melanoma (4–6, 23). In two such model systems—murine colorectal carcinoma and melanoma—eradication of established metastases was mediated by $CD8^+$ effector T cells. This preceded the induction of long-lasting, tumor-protective memory immune responses in both systems, and was effective against tumor-cell challenges long after the initial therapy, consistent with a therapeutic T-cell vaccination effect. The later stages of the anti-tumor effects observed in these model systems, specifically the characterization of T-cell memory after IL-2 immunocytokine therapy, have already been described (6, 31). However, little is known about the mechanisms involved in the initial events necessary for most effective activation in the T-cell compartment, which may subsequently lead to the induction of T-cell memory. In this report we have defined some of the key players involved in optimally effective IL-2 immunocytokine-mediated induction of $CD8^+$ T cell–mediated eradication of established pulmonary melanoma metastases.

The induction of a $CD8^+$ T cell–mediated anti-tumor response after hu14.18–IL-2 therapy was found to depend on help provided by $CD4^+$ T cells, as demonstrated by the partial abrogation of the treatment effect in mice lacking $CD4^+$ T cells (Figures 2–4). A helper

function of $CD4^+$ T cells in adaptive immunity was first described for the B-cell compartment. Subsequent production of antibodies was followed by reports describing a role for $CD4^+$ T cells in the induction of specific $CD8^+$ T cell–mediated immune responses. The mechanism by which $CD4^+$ T cells provides help for the development of humoral and cellular adaptive immunity is a matter of debate essentially favoring two models, either the endogenous production of IL-2 from $CD4^+$ T cells or the interaction of $CD4^+$ T cells with APCs through CD40/CD40L interaction (34, 35). The latter interaction was reported to activate functional properties of APCs by upregulation of costimulatory molecules (36), MHC antigens (21), and production of the T_H1 cytokine IL-12 (37, 38), leading subsequently to effective priming, T_H1 differentiation, and maturation of naive $CD8^+$ T cells into $CD8^+$ effector T cells.

The results obtained in our syngeneic melanoma model indicate a requirement for $CD4^+$ T-cell help to optimize tumor-targeted, IL-2–induced $CD8^+$ T cell–mediated eradication of pulmonary metastases, and clearly indicate a CD40/CD40L–mediated mechanism rather than endogenous IL-2 production. This is supported by highly effective treatment of established pulmonary metastases with hu14.18–IL-2 in IL-2 KO mice (Figure 4), which are unable to produce IL-2. This finding contrasts with results obtained in CD40L KO mice, which are deficient in CD40L-dependent APC stimulation, but are competent in endogenous IL-2 production. In this mouse strain, the treatment effect of hu14.18–IL-2 is partially abrogated, but can be reconstituted by replacing the missing CD40/CD40L signal with a stimulatory anti-CD40 Ab (Figure 4).

Importantly, a simultaneous combination of hu14.18–IL-2 and anti-CD40 was synergistic in the induction of an anti-tumor immune response in CD40L KO mice (Figure 4) and in the upregulation of activation markers on $CD8^+$ T cells and APCs (figures 5 and 6). This synergy is most likely based on activation of anti-tumor immune responses that occurs by two different mechanisms, and is mediated by hu14.18–IL-2 and anti-CD40. IL-2 immunocytokines were shown to amplify inefficient tumor-specific T-cell responses (16, 31, 39), demonstrating that they act more directly to expand and proliferate T cells than does anti-CD40, which was clearly demonstrated to activate dendritic cells and subsequent antigen presentation (21, 35, 36, 40), leading to more efficient T-cell priming. A similar observation was reported after activation of CD40 with anti-CD40 Ab, which was demonstrated to overcome peripheral $CD8^+$ and $CD4^+$ T-cell tolerance, subsequently augmenting anti-tumor vaccine efficacy (40, 41). Because a vaccination effect was observed by targeted IL-2 therapy in our melanoma model, amplification by additional anti-CD40 therapy, as suggested in this report, may benefit clinical efforts to establish the use of targeted IL-2 for active cancer immunotherapy.

In summary, we have demonstrated that a humanized anti-GD_2 immunocytokine, hu14.18–IL-2, is effective in the induction of a $CD8^+$ T cell–mediated immune response against syngeneic melanoma, and that this effect depends on help provided by $CD4^+$ T cells. The mechanism of $CD4^+$ T cell–mediated help involves the interaction of CD40/CD40L, and does not require endogenous production of IL-2. This finding suggests a synergistic role for stimulatory anti-CD40 immunotherapy in combination with tumor-targeted IL-2 for the treatment of cancer patients with minimal residual disease.

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