

Immunological and Antitumor Effects of IL-23 as a Cancer Vaccine Adjuvant¹

Willem W. Overwijk,^{2*} Karin E. de Visser,^{3*} Felicia H. Tirion,* Laurina A. de Jong,* Thijs W. H. Pols,* Yme U. van der Velden,* Jasper G. van den Boorn,* Anna M. Keller,* Wim A. Buurman,[†] Marc R. Theoret,[‡] Bianca Blom,^{4*} Nicholas P. Restifo,[‡] Ada M. Kruisbeek,^{5*} Robert A. Kastelein,[§] and John B. A. G. Haanen*

The promising, but modest, clinical results of many human cancer vaccines indicate a need for vaccine adjuvants that can increase both the quantity and the quality of vaccine-induced, tumor-specific T cells. In this study we tested the immunological and antitumor effects of the proinflammatory cytokine, IL-23, in gp100 peptide vaccine therapy of established murine melanoma. Neither systemic nor local IL-23 alone had any impact on tumor growth or tumor-specific T cell numbers. Upon specific vaccination, however, systemic IL-23 greatly increased the relative and absolute numbers of vaccine-induced CD8⁺ T cells and enhanced their effector function at the tumor site. Although IL-23 specifically increased IFN- γ production by tumor-specific T cells, IFN- γ itself was not a primary mediator of the vaccine adjuvant effect. The IL-23-induced antitumor effect and accompanying reversible weight loss were both partially mediated by TNF- α . In contrast, local expression of IL-23 at the tumor site maintained antitumor activity in the absence of weight loss. Under these conditions, it was also clear that enhanced effector function of vaccine-induced CD8⁺ T cells, rather than increased T cell number, is a primary mechanism underlying the antitumor effect of IL-23. Collectively, these results suggest that IL-23 is a potent vaccine adjuvant for the induction of therapeutic, tumor-specific CD8⁺ T cell responses. *The Journal of Immunology*, 2006, 176: 5213–5222.

Cytokines are powerful mediators of innate and adaptive immunity and attractive candidate vaccine adjuvants (1, 2). Beneficial effects of cytokines as adjuvants include stimulation of T cell-mediated immunity at the level of Ag presentation and T cell proliferation, effector function, and memory formation (3, 4). Several cancer vaccines administered with cytokine adjuvants, particularly GM-CSF and IL-2, have had modest clinical success (5–10). It is therefore important to identify and understand the mechanism of action of new cytokine adjuvants that can augment the immunogenicity and therapeutic efficacy of cancer vaccines (4, 11).

IL-23 is a heterodimeric cytokine consisting of the IL-12 p40 subunit and a more recently discovered p19 subunit (12). It is

secreted by activated dendritic cells (DC)⁶ and enhances in vitro proliferation and IFN- γ production of memory CD4⁺ T cells, with a more modest effect on naive T cells (12). The IL-23R, composed of the IL-12R β 1 and the IL-23R subunit, is expressed on DC, macrophages, and T cells (13). Transgenic overexpression of the IL-23 p19 subunit in mice induced multiorgan inflammation (14), whereas IL-23-deficient mice were protected against autoimmune diseases, such as vaccination-induced T cell-mediated rheumatoid arthritis and experimental allergic encephalitis (11, 15, 16). This indicates that IL-23 can promote immunity to self Ags which bear immunological resemblance to the self Ags that are often the target of endogenous antitumor immunity (17–19). Indeed, immunogenic mouse colon carcinoma cells transduced with a p40-p19 IL-23 fusion protein grew into palpable tumors, which then spontaneously regressed in a CD8⁺ T cell-dependent fashion (20, 21). Furthermore, insertion of the *IL-23* gene into a DNA vaccine encoding hepatitis C virus envelope protein 2 increased the number of hepatitis C virus Ag-specific memory T cells in the spleens of DNA-vaccinated mice (22). Despite these powerful in vivo effects, little is known about the mechanism of action of IL-23. In addition, it is unclear whether IL-23 can act as an effective adjuvant for active immunization against cancer. To study this, we have targeted the melanocyte differentiation Ag, gp100, that is naturally expressed by melanocytes and melanoma and is recognized by specific CD8⁺ T cells in mice and humans. As an experimental system, we have used the pmel-1 gp100-specific TCR transgenic mouse model, which closely reflects many aspects of gp100-specific vaccine therapy of human melanoma (4). In this study we examined the immunological and antitumor effects of IL-23 on vaccine-induced, gp100-specific T cell responses.

*Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands; [†]Department of General Surgery, University Maastricht, Maastricht, The Netherlands; [‡]National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; and [§]DNAX Research Institute, Palo Alto, CA 93404

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² Address correspondence and reprint requests to Dr. Willem W. Overwijk, Department of Melanoma Medical Oncology, M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0904, Houston, TX 77030. E-mail address: woverwijk@mdanderson.org

³ Current address: Department of Molecular Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.

⁴ Current address: Department of Cell Biology and Histology, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, The Netherlands.

⁵ Current address: Free University Medical Center, Cancer Center Amsterdam, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.

⁶ Abbreviations used in this paper: DC, dendritic cell; CM, culture medium; HGT, hydrodynamic gene transfer; iIFN- γ , intracellular IFN- γ ; IRES, internal ribosome entry site; YFP, yellow fluorescent protein; WT, wild type; mgp100, mouse gp100.

Materials and Methods

Mice and tumor cells

C57BL/6 and pmel-1 TCR transgenic mice (4) were bred and housed at the National Institutes of Health and The Netherlands Cancer Institute animal facilities and were used at 5–12 wk of age. All animal experiments were reviewed and approved by the institutes' internal review boards. B16, B16.yellow fluorescent protein (YFP), and B16.IL-23 tumor cell lines were maintained in culture medium (CM) comprised of RPMI 1640 with 10% heat-inactivated FBS (Biofluids), 0.03% L-glutamine, 100 μ g/ml streptomycin, 100 μ g/ml penicillin, and 50 μ g/ml gentamicin sulfate (National Institutes of Health Media Center). B16 is an H-2^{b+}gp100⁺ spontaneous murine melanoma obtained from the National Cancer Institute tumor repository. To create B16.internal ribosome entry site (IRES)-YFP and B16.IL-23-IRES-YFP tumor cell lines, the N-terminally FLAG-tagged IL-23 fusion gene (12) was cloned into the retroviral LZRS-IRES-YFP vector, which is based on LZRS-IRES-GFP (23), to obtain LZRS-IL-23-IRES-YFP. Phoenix A retroviral packaging cells were transfected with a mixture of 400 μ l of serum-free IMDM, 12.5 μ l of FuGene 6 (Roche), 7.5 μ g of pCLeco (24), and 5 μ g of LZRS-IRES-YFP or LZRS-IL-23-IRES-YFP plasmid DNA and incubated at 37°C for 48 h; medium was replaced with fresh CM after 24 h. Phoenix A retroviral supernatant was supplemented with 8 μ g/ml Lipofectamine, added to 5×10^5 B16 cells (Invitrogen Life Technologies) in a 6-well plate (Corning Costar). The plate was spun for 90 min at 2000 rpm (Hettich Rotana/p centrifuge), and cells were incubated for 2 days at 37°C. The transduced cell lines were FACS sorted three times for high YFP signal to obtain the >98% YFP-positive B16.IRES-YFP and B16.IL-23-IRES-YFP (hereafter, B16.YFP and B16.IL-23) cell lines.

Peptides

All synthetic peptides were synthesized using regular F-moc chemistry. The synthetic, H-2D^b-restricted peptides, hgp100_{25–33} (KVPRNQDWL) and mouse (mgp100_{25–33} (EGSRNQDWL)), were synthesized by HPLC in-house to a purity >95%.

In vitro activation of pmel-1 T cells

pmel-1 splenocytes were depleted of erythrocytes by hypotonic lysis, activated with 1 μ M mgp100_{25–33} peptide, and cultured in CM with 30 IU/ml human rIL-2 (Chiron) until use on days 5–10 after the start of the culture.

In vitro T cell proliferation assays

pmel-1 splenocytes were activated with 1 μ M mgp100_{25–33} peptide for 1 h (25), washed twice with PBS, labeled with 0.5 μ M CFSE in 1 ml of serum-free medium for 10 min at 37°C, and washed twice before plating in CM or CM plus 1 μ g/ml murine rIL-23 (11). CM and CM plus rIL-23 were replaced daily until cell analysis.

Flow cytometry and intracellular IFN- γ assay

To obtain appropriate lymphocyte samples, mice were either tail-bled on the indicated days or sacrificed before organ isolation. Splenocytes were cultured as previously described (25). Erythrocytes were removed by hypotonic lysis or Ficoll gradient separation, and cells were stained with the indicated dilutions of mAbs against CD8 α (1/200; clone 53-6.7) and V β 13 (1/200; clone MR12-3). All Abs were purchased from BD Pharmingen. Propidium iodide-staining cells were excluded from analysis. Intracellular IFN- γ assay was performed with the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's recommendations after 4 h of stimulation with 1 μ M mgp100_{25–33} peptide and using a 1/800 dilution anti-IFN- γ mAb (clone XMG1.2). Samples were analyzed using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences).

In vitro tumor cell recognition

B16, B16.YFP, and B16.IL-23 cells were cultured for 48 h in the presence or the absence of 100 IU/ml mIFN- γ . Cells were harvested and incubated for 4 h with pmel-1 T cells that had been in vitro activated for 7 days. CD8⁺ pmel-1 T cells were then tested for intracellular IFN- γ release as described.

IL-23 ELISA and Western blot

High affinity binding plate type I (Corning/Costar; no. 2592) 96-well plates were coated with 0.5 μ g/ml anti-p40 capture mAb (clone C15.6; BD Pharmingen) in 0.1 M Na₂HPO₄ buffer (pH 9.5) for 3 h at 4°C and blocked for 30 min with 10% FCS in PBS at room temperature. Samples were applied and incubated for 2 h at room temperature, and plates were washed

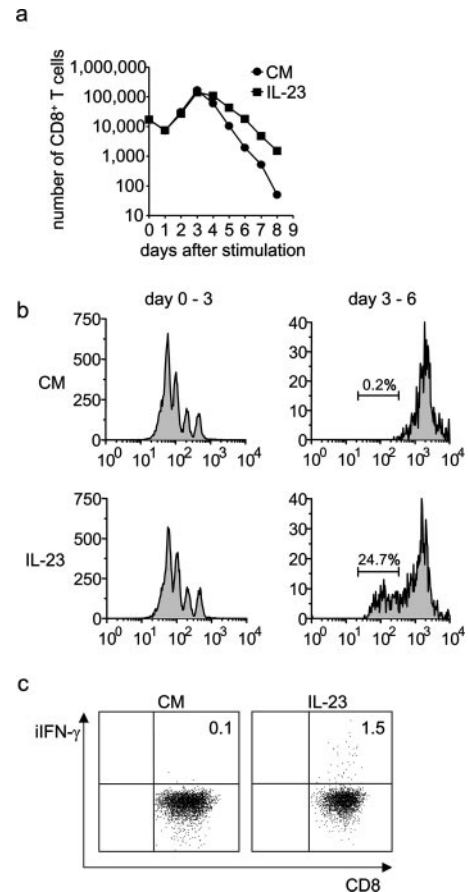


FIGURE 1. IL-23 sustains the proliferation of activated CD8⁺ T cells. *a*, pmel-1 splenocytes were activated with 1 μ M mgp100_{25–33} peptide and cultured at 100,000 cells/well in CM or CM plus 1 μ g/ml rIL-23; medium was replaced daily. Daily samples were counted and stained with anti-CD8 mAb and gp100 tetramer before FACS analysis. Plotted are the absolute number of gp100-specific CD8⁺ T cells per well through time. *b*, The pmel-1 splenocytes cultured in CM with or without rIL-23 as described in *a* were labeled with CFSE; CD8⁺ and tetramer-positive T cells were FACS analyzed 3 days later. *Left panels*, Labeling directly before T cell activation (day 0) and analysis on day 3; *right panels*, labeling on day 3 and analysis on day 6. The data shown are representative of six independent experiments. *c*, The pmel-1 splenocytes cultured in CM with or without rIL-23, as described in *a*, for 7 days were tested in a 4-h intracellular IFN- γ assay without peptide stimulation and were FACS analyzed after staining for CD8 and intracellular IFN- γ .

with wash buffer (0.05% Tween 20 (Merck) in PBS). Biotin-conjugated detection mAbs were added (anti-p40 clone (C17.8, 0.5 μ g/ml) or anti-FLAG M2 (10 μ g/ml)) and incubated for 1 h at room temperature. Plates were washed, and avidin-peroxidase (1/500; Sigma-Aldrich) was added and incubated for 30 min at room temperature. Plates were washed and developed with 100 μ l/well ABTS (diammonium salt) solution (150 mg of ABTS and 500 ml of 0.1 M citric acid (pH 4.35)) with 1% perhydrol (Darmstadt; Merck) and measured photospectrometrically at 405 nm. For Western blot, 20 μ l of 24-h B16.YFP and B16.IL-23 culture supernatants were run on a polyacrylamide gel and stained overnight with a 1/240 dilution of anti-FLAG M1-biotin mAb, followed by a 1/2000 dilution of streptavidin-HRP and developed using the Pierce ECL Far-West substrate (no. 34080) according to the manufacturer's instructions.

Adoptive transfer and tumor treatment

Mice ($n = 5$ /group unless indicated otherwise) were injected s.c. with 2×10^5 B16, B16.YFP, or B16.IL-23 melanoma cells and treated with i.v. adoptive transfer of 2×10^7 fresh TCR-Tg pmel-1 splenocytes ($\sim 4\text{--}5 \times 10^6$ CD8⁺V β 13⁺ T cells) (4). Mice were vaccinated by two separate s.c. injections with 100 μ l of water/IFA emulsion, each containing 100 μ g of hgp100_{25–33} peptide, followed by two daily i.p. injections of 100 μ g of anti-CD40 mAb

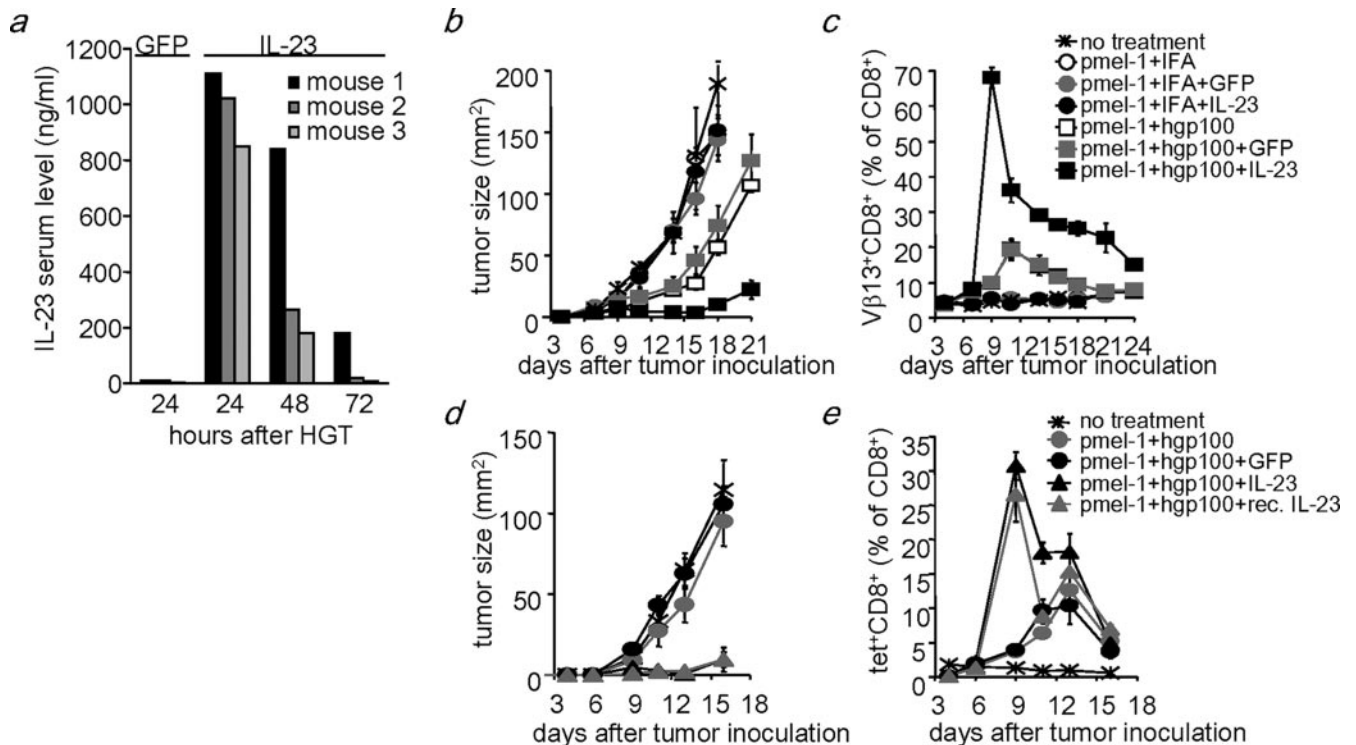


FIGURE 2. Systemic IL-23 as a cancer vaccine adjuvant. *a*, Anti-FLAG sandwich ELISA detection of IL-23 fusion protein in serum from three mice that received HGT with 5 μ g of IL-23 plasmid at time 0. As a control, three mice received HGT with 5 μ g of GFP plasmid. *b* and *c*, Mice ($n = 5$) bearing 3-day B16 tumors received 2×10^7 pmel-1 splenocytes and PBS/IFA or gp100/IFA vaccination, followed by HGT with 5 μ g of GFP or IL-23 plasmid on days 0, 3, and 6 after vaccination. Tumor growth (*b*) was followed, and pmel-1 T cell responses in peripheral blood (*c*) were FACS analyzed after staining for CD8 and transgenic V β 13 chain. The data shown are representative of four independent experiments. *d* and *e*, Mice ($n = 5$) bearing 3-day B16.WT tumors received 2×10^7 pmel-1 splenocytes, gp100 vaccination, and HGT with 10 μ g of GFP or IL-23 plasmid on days 0, 3, and 6 after vaccination or 10 μ g of rIL-23 (i.p.) twice daily from days 0–7 after vaccination. Mice were followed for tumor growth (*d*) and CD8⁺ gp100 tetramer-positive T cell response (*e*). The data shown are representative of two independent experiments.

purified from FGK45 hybridoma culture supernatant. Hydrodynamic gene transfer (HGT) consisted of i.v. injection of 5 μ g of plasmid DNA in 2 ml of saline on days 0, 3, and 6 after vaccination as previously described (26–28). Anti-TNF- α mAb (TN3) was given i.p. (200 μ g/injection) every 3 days. Tumors were measured with calipers, and the products of perpendicular diameters were recorded. Mice were killed once tumors reached 200 mm². All experiments were performed at least twice, with similar results.

Histology

Tumor samples were analyzed as described previously (29). Briefly, 4- μ m cryostat sections were air-dried overnight, fixed in acetone for 10 min at room temperature, preincubated in 5% (v/v) normal goat serum (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service), and stained with 0.5 μ g/ml anti-mouse v β 13 mAb-FITC or mIgG1-FITC control Ab (BD Pharmingen) in PBS/1% BSA, followed by rabbit anti-FITC (1/40,000; DakoCytomation), biotinylated goat anti-rabbit Ab (1/400; DakoCytomation), and streptavidin/biotin-conjugated alkaline phosphatase complex (1/100; ABC-protocol, DakoCytomation). Color was developed using permanent red chromogen substrate (PRC kit; Cell Marque), and sections were counterstained with hematoxylin.

Statistics

Statistical analyses to compare tumor growth rate between treatment and control groups was performed by ANOVA-repeated measures test and Wilcoxon's rank-sum test. Statistical analyses to compare tumor sizes and cell numbers between treatment and control groups were determined by non-parametric Kruskal-Wallis test.

Results

IL-23 enhances late, but not early, numbers and IFN- γ production of Ag-stimulated, tumor-specific CD8⁺ T cells in vitro

To assess whether and how IL-23 could function as a cancer vaccine adjuvant, we first studied its effects on cultured CD8⁺ T cells

derived from TCR transgenic pmel-1 mice. These T cells specifically recognize the mouse melanocyte differentiation Ag gp100-derived peptide, gp100_{25–33}; are readily detected by FACS staining with gp100_{25–33} tetramers or mAb against transgenic TCR V β 13 chain; and can have potent antimelanoma activity in vivo (4). Addition of IL-23 to cultures of mouse gp100_{25–33} peptide-stimulated pmel-1 splenocytes reproducibly increased T cell numbers 10- to 50-fold after 6–8 days. (Fig. 1*a*). Simultaneous CFSE dilution analysis did not reveal a significant impact of IL-23 on T cell number or the rate of T cell proliferation early after antigenic stimulation (days 0–3; Fig. 1*b*, left panels). However, when labeled with CFSE after 3 days in culture and cultured for an additional 3 days, Ag-stimulated T cell cultures supplemented with IL-23 showed an increased fraction of divided, CFSE^{low} T cells (Fig. 1*b*, right panels). Cells grown without IL-23 showed negligible CFSE dilution, suggesting that IL-23 sustained the prolonged proliferation of activated CD8⁺ T cells or protected proliferating cells from death. Finally, there was a modest, but reproducible, enhancement of spontaneous IFN- γ production by CD8⁺ pmel-1 T cells upon culture in IL-23 (Fig. 1*c*). Taken together, these results suggested that IL-23 could enhance both the number and the activity of tumor Ag-activated CD8⁺ T cells.

IL-23 augments vaccine-induced, but not spontaneous, antitumor immunity

The previously reported proinflammatory in vivo effects of IL-23 (11, 14–16) together with our in vitro data suggested that IL-23 could function as an effective cancer vaccine adjuvant. Therefore,

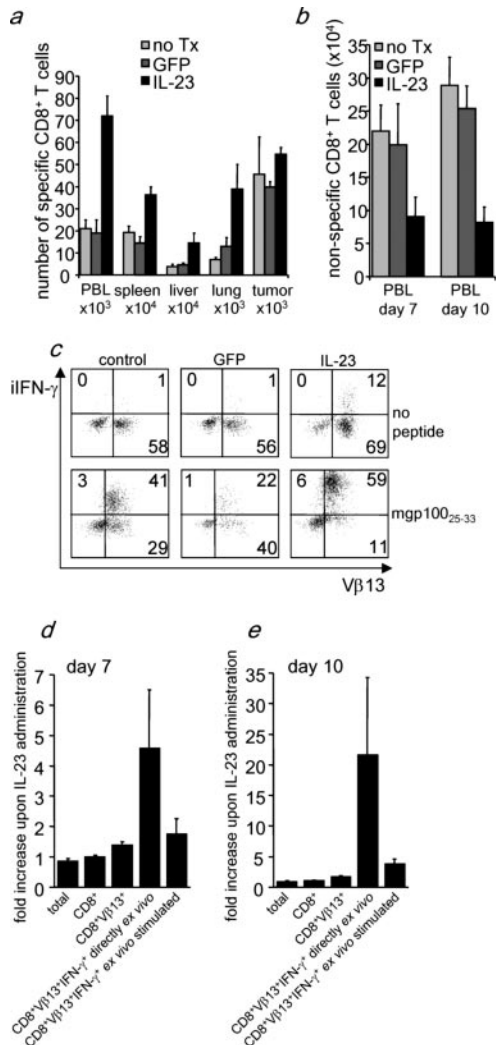


FIGURE 3. IL-23 induces systemic vaccine-induced CD8⁺ T cell accumulation and enhanced effector function while depleting bystander T cells. *a–d*, Mice bearing 10-day B16 tumors received 2×10^7 pmel-1 splenocytes and gp100 vaccination, followed by HGT with 5 μ g of GFP or IL-23 plasmid on days 10, 13, and 16 after tumor inoculation. Mice ($n = 3$) were individually analyzed on day 7 (*a*, *b*, and *d*) or 10 (*b*, *c*, and *e*) after vaccination. *a*, Total lymphocytes were isolated from peripheral blood, tumor, and solid organs on day 7 after gp100 vaccination, counted, and FACS analyzed after staining with anti-CD8 mAb and gp100_{25–33} tetramer. The total numbers of tetramer-positive cells are plotted per complete organ (spleen, liver, and lung) or per milliliter of blood (PBL) or 300 mg of tumor (the average weight of excised tumors on day 17 after tumor inoculation). *b*, PBL from mice treated as described in *a*, but FACS analyzed for absolute number of tetramer-negative CD8⁺ cells per milliliter of peripheral blood on days 7 and 10 after gp100 vaccination. *c*, Ex vivo intracellular IFN- γ production by total tumor-derived CD8⁺ T cells from mice receiving vaccination and HGT as described in *b*. Cells were cultured for 4 h with or without 1 μ M mgp100_{25–33} peptide and were FACS analyzed after staining for transgenic V β 13 chain, CD8, and iIFN- γ and gating on CD8⁺ cells. Individual representative samples (of $n = 3$) are shown. *d* and *e*, Tumor-derived total lymphocytes from treated mice were counted and FACS analyzed for V β 13, CD8, and spontaneous vs mgp100_{25–33} peptide-induced iIFN- γ production on days 7 (*d*) and 10 (*e*) after adoptive transfer and gp100 vaccination. Displayed as ratios are the total cell numbers in the indicated lymphocyte subpopulations isolated from IL-23-treated mice divided by the corresponding cell numbers from GFP-treated mice. The data shown are representative of three independent experiments.

we examined the impact of IL-23 on spontaneous and vaccine-induced immunity to the aggressive, poorly immunogenic B16 melanoma. Like human melanoma, B16 naturally expresses the

melanocyte differentiation Ag, gp100, yet grows uninhibited in the face of large numbers of gp100-specific, CD8⁺ T cells (4, 17, 30). We induced continuous systemic levels of IL-23 using the approach of in vivo transient IL-23 HGT (26, 27, 31), resulting in detectable serum IL-23 levels for 2–3 days (Fig. 2*a*). When we treated mice bearing B16 tumors with adoptive pmel-1 T cell transfer, control vaccination, and three rounds of IL-23 HGT, we observed no effect of IL-23 on T cell number or tumor growth. Adoptive T cell transfer along with gp100 vaccination induced strong T cell responses that were unaffected by control GFP HGT and had a minor impact on tumor growth (0–30% smaller size in multiple experiments). In contrast, adoptive transfer and gp100 vaccination combined with IL-23 induced slightly more rapid and markedly greater T cell accumulation and strongly suppressed tumor growth. (Fig. 2, *b* and *c*). To ensure that the observed results were not related to the HGT method, we compared the administration of either 10 μ g of IL-23 or GFP cDNA on days 0, 3, and 6 after vaccination with 8-day treatment (0–7 days after vaccination) of twice daily i.p. injections of 10 μ g of mouse rIL-23. The effects of IL-23 HGT and rIL-23 on vaccine-activated CD8⁺ T cell response and tumor growth were potent and comparable (Fig. 2, *d* and *e*).

IL-23 induces selective, systemic accumulation of Ag-specific and depletion of bystander CD8⁺ T cells

When measuring specific T cell responses in vaccinated mice receiving IL-23, we observed a striking depletion of Ag nonspecific (bystander) CD8⁺ T cells in PBL. To ensure that this phenomenon did not confound our measurements of relative specific CD8⁺ T cell levels, we used gp100 tetramers to count the absolute numbers of specific and nonspecific T cells in blood, spleen, liver, lung, and tumor. We found that IL-23 increased the number of Ag-specific CD8⁺ T cells in all organs examined (Fig. 3*a*). Simultaneously, bystander CD8⁺ T cells were depleted in those organs, as in peripheral blood (Fig. 3*b*), as were CD4⁺ T cells (not shown). In contrast to these changes in normal organs, the absolute number of Ag-specific CD8⁺ T cells infiltrating tumors was unaffected by IL-23 (Fig. 3, *a*, *d*, and *e*). Ex vivo stimulation of tumor-derived lymphocytes with gp100 peptide revealed that IFN- γ was exclusively produced by gp100-specific, V β 13⁺CD8⁺ T cells, and that IL-23 treatment increased the mean Ag-induced IFN- γ production by these T cells \sim 10-fold on a per cell basis (Fig. 3*c*, lower panels). In addition, spontaneous ex vivo IFN- γ production was dramatically increased (Fig. 3*c*, upper panels), resulting in up to 20-fold higher numbers of spontaneously IFN- γ -secreting, gp100-specific CD8⁺ T cells/g tumor (Fig. 3, *d* and *e*). Taken together, these data suggest that IL-23 strongly enhances in situ IFN- γ production by vaccine-activated, gp100-specific, CD8⁺ T cells.

IFN- γ is not a major mediator of IL-23-induced tumor destruction

The observation that IL-23 caused enhanced production of IFN- γ by tumor-infiltrating, gp100-specific, CD8⁺ T cells sparked the question of whether IFN- γ itself mediated the adjuvant effect of IL-23 or was a more general marker for overall increased effector function of vaccine-activated pmel-1 T cells. To examine this, we adoptively transferred suboptimal numbers of wild-type pmel-1 (pmel-1.WT) or IFN- γ -deficient pmel-1 (pmel-1.IFN- γ ^{-/-}) T cells before vaccination and IL-23 treatment. Similar to results obtained in another system by Badinovac et al. (32), IFN- γ -deficient pmel-1 T cells accumulated to higher numbers upon activation than their WT counterparts. Yet despite their higher numbers, the antitumor effect of pmel-1.IFN- γ ^{-/-} T cells was similar, if not inferior, to that of the much less abundant pmel-1.WT cells (Fig. 4, *a* and *b*).

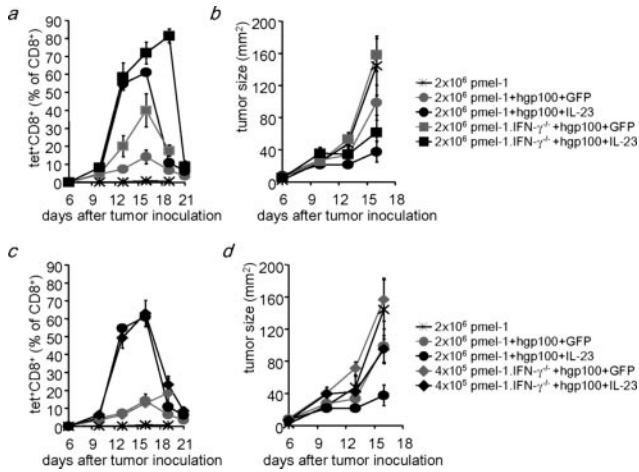


FIGURE 4. Contribution of IFN- γ to the antitumor effect of adjuvant IL-23. Mice ($n = 5$) bearing 3-day B16.WT tumors received suboptimal numbers of pmel-1 or pmel-1.IFN- $\gamma^{-/-}$ splenocytes, equivalent of either 2×10^6 pmel-1 vs 2×10^6 pmel-1.IFN- $\gamma^{-/-}$ CD8⁺tetramer⁺ T cells (*a* and *b*) or 2×10^6 pmel-1 vs 4×10^5 pmel-1.IFN- $\gamma^{-/-}$ CD8⁺tetramer⁺ T cells (*c* and *d*), followed by gp100 vaccination and HGT with 5 μ g of GFP or IL-23 plasmid on days 3, 6, and 9 after tumor inoculation. Mice were followed for gp100-specific T cell responses (*a* and *c*) and tumor growth (*b* and *d*). The data shown are representative of three independent experiments.

When the number of transferred pmel-1.IFN- $\gamma^{-/-}$ T cells was reduced 5-fold to result in matching T cell responses, this inferior antitumor effect of IFN- γ -deficient pmel-1 T cells was even more

striking (Fig. 4, *c* and *d*). However, at all cell doses tested in multiple experiments, the reduction in tumor growth that could be attributed to IL-23 treatment was similar in mice receiving pmel-1.WT vs that in mice receiving pmel-1.IFN- $\gamma^{-/-}$ T cells. This suggests that although IFN- γ production by vaccine-induced T cells is important for antitumor immunity, its observed increase after IL-23 administration does not explain the concurrent increase in tumor destruction.

IL-23 induces weight loss

The vaccine adjuvant effect of IL-23 on T cell responses and tumor growth was accompanied by weight loss (Fig. 5*a*). Weight loss was not an indirect effect of the HGT method, because it could be similarly induced by rIL-23 protein injection (data not shown). Weight loss was not dependent on a vaccine-induced T cell response, because mice receiving IL-23 HGT without adoptive transfer or vaccination displayed similar weight loss (Fig. 5*b*). It was also not mediated by IFN- γ , because IFN- γ -deficient mice were not protected against weight loss (data not shown). Histological examination of C57BL/6 mice 10 days after initiation of HGT with IL-23 revealed inflammation characterized by dense infiltration with mostly neutrophilic granulocytes in the gastrointestinal tract, including duodenum, large intestine, cecum, and, in particular, small intestine, but not stomach (Fig. 5*c*). There were no histological or macroscopic signs of severe villus atrophy or diarrhea, but the amount of fecal matter in the intestinal tract of IL-23-treated mice was visibly reduced, suggesting that weight loss was primarily due to decreased food intake. To determine whether effective therapy can be realized with less toxicity, we treated vaccinated,

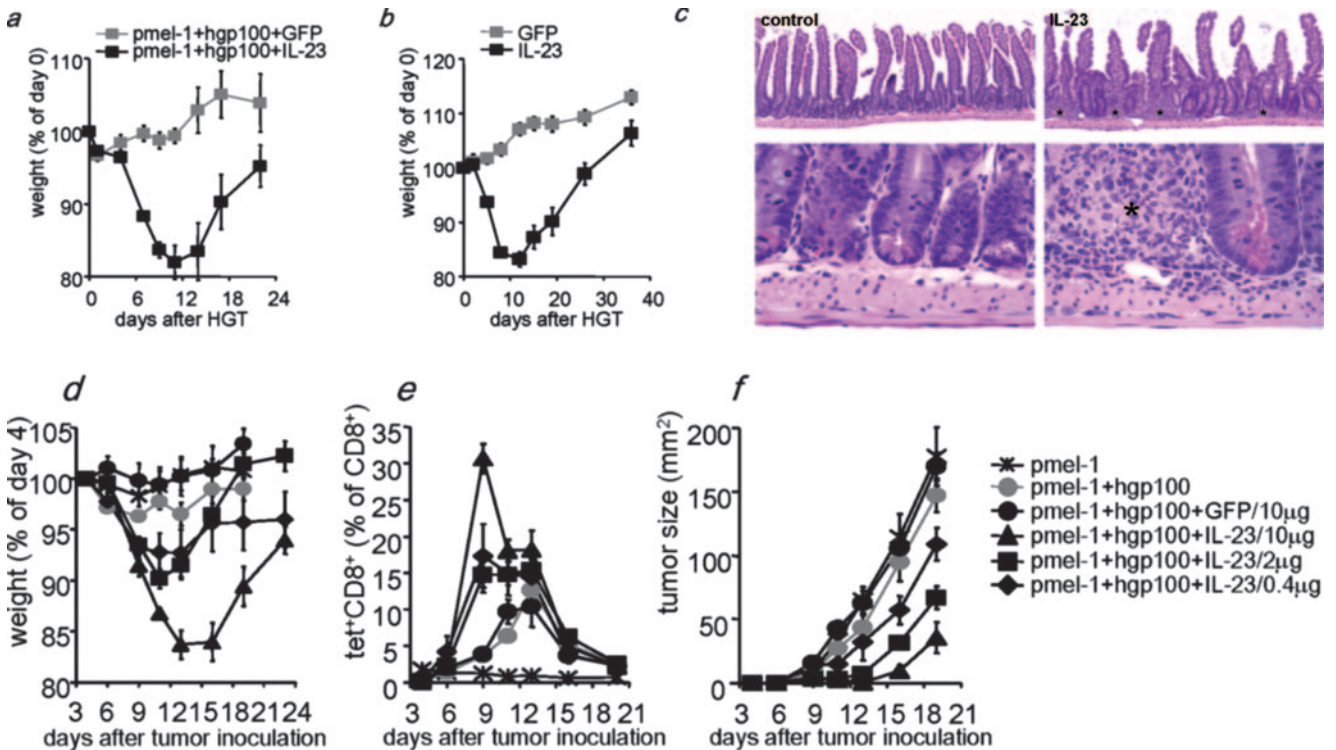


FIGURE 5. Effect of IL-23 on body weight. *a* and *b*, HGT with 5 μ g of GFP or IL-23 plasmid on days 3, 6, and 9 after tumor inoculation was given to mice ($n = 5$) bearing 3-day B16 tumors that had received 2×10^7 splenocytes and gp100 vaccination (*a*) or to naive mice ($n = 5$) on days 0, 3, and 6 (*b*). Individual mouse body weight was monitored and plotted as a percentage of body weight on the first day of HGT. *c*, Mice ($n = 2$) receiving HGT with 10 μ g of GFP (control) or IL-23 plasmid on days 0, 3, and 6 were killed on day 10, and paraffin sections of the small intestine were stained with H&E and photographed at $\times 25$ (*top panels*) and $\times 400$ (*bottom panels*) the original magnification. Asterisks indicate mononuclear cell infiltration. *d-f*, Mice ($n = 5$) bearing 3-day B16 tumors received 2×10^7 pmel-1 splenocytes, gp100 vaccination, and HGT with 10 μ g of GFP plasmid or 10, 2, or 0.4 μ g of IL-23 plasmid on days 3, 6, and 9 after tumor inoculation. Mice were followed for body weight (*d*), CD8⁺tetramer⁺ T cell response (*e*), and tumor growth (*f*). The data shown are representative of two independent experiments.

tumor-bearing mice with HGT with limiting doses of IL-23 plasmid (Fig. 5, *d–f*). We found a direct dose-response relationship and no dose at which the effects of IL-23 on the vaccine-induced CD8⁺ T cell response, antitumor effect, and toxicity were separated.

Antitumor effect and weight loss after IL-23 administration are partially mediated by TNF- α

TNF- α is a known inducer of weight loss in animals and humans (33). Because macrophages express functional IL-23Rs (13) and are prime producers of TNF- α in vivo, we examined the hypothesis that TNF- α mediates IL-23-induced weight loss. Mice that received anti-TNF- α mAb at the time of IL-23 administration were partially protected from IL-23-induced weight loss (Fig. 6*a*). TNF- α blockade did not alter the vaccine-induced T cell response, but the antitumor effect was greatly reduced (Fig. 6, *b* and *c*), suggesting that both the antitumor effect and the weight loss after IL-23 treatment are at least partially mediated by TNF- α .

Local IL-23 augments vaccine-induced antitumor immunity without weight loss

Systemic IL-23 levels induced powerful T cell responses, antitumor effects, and weight loss. Because we found that IL-23 enhanced the effector function, but not the number, of specific T cells at the effector (tumor) site (Fig. 3), we hypothesized that this locally enhanced effector function was a dominant component of the antitumor effect of IL-23. This suggested that IL-23 at the tumor site might enhance T cell effector function and antitumor effects without a need for the high systemic IL-23 levels that led to weight loss. To test this, wild-type (WT) B16.WT cells were retrovirally transduced and FACS sorted to produce either B16.YFP control cells or B16.IL-23 cells, which secrete IL-23 as a fusion protein of its two subunits (11, 12), as detected by p40- or FLAG-specific ELISA and Western blot (Fig. 7, *a* and *b*). Specific, short-term in vitro recognition of B16.WT and that of the transduced progeny by activated gp100-specific pmel-1 CD8⁺ T cells was comparable (Fig. 7*c*). After injection of IL-23 into normal C57BL/6 mice, the

growth of B16.WT, B16.YFP, and B16.IL-23 was nearly identical, indicating that no productive spontaneous immunity was induced to growing B16 tumors even when they secreted IL-23 (Fig. 7*d*). IL-23 was undetectable by ELISA in the serum of mice bearing B16.IL-23 tumors of up to 150 mm² (data not shown). When mice carrying B16.IL-23 tumors were injected with large numbers (up to 1×10^7 tetramer-positive cells) of gp100-specific pmel-1 CD8⁺ T cells, these T cells did not inhibit the growth of B16.IL-23 (Fig. 7*d*). Adoptive transfer of pmel-1 T cells, followed by gp100 vaccination, induced a marginal growth reduction of B16.WT and B16.YFP tumors. The growth of B16.IL-23 tumors, however, was greatly suppressed, demonstrating a specific augmentation of vaccine-induced antitumor immunity by locally produced IL-23 (Fig. 7*d*). These results were reminiscent of the observations after treatment with IL-23 HGT or rIL-23, with the important difference that the antitumor effect of locally produced IL-23 was not accompanied by systemically increased levels of gp100-specific T cells (Fig. 7*e*). In addition, in multiple experiments there was no significant weight loss or other clinical symptoms in mice bearing B16.IL-23 tumors that received adoptive transfer and vaccination (Fig. 7, *f* and *g*).

Local IL-23 increases levels and effector function of tumor Ag-specific CD8⁺ T cells

It appeared that the production of IL-23 at the tumor site resulted in a locally enhanced antitumor immune response without having an impact on systemic Ag-specific T cell levels (Fig. 7*e*). We also found that gp100-specific CD8⁺ T cells accumulated in B16.WT, B16.YFP, and B16.IL-23 tumors to comparable high levels (Fig. 8*a*), reminiscent of observations after IL-23 HGT (Fig. 3*a*). In addition, histological analysis of tumors from these mice at multiple time points after vaccination showed no difference in specific pmel-1 T cell infiltration between groups, even though tissue destruction was more extensive in B16.IL-23 tumors (Fig. 8*c*). Yet, as observed after IL-23 HGT, the ability to produce IFN- γ directly upon isolation without ex vivo antigenic stimulation was greatly increased in CD8⁺ T cells derived from B16.IL-23 tumors compared with T cells from B16.WT or B16.YFP tumors (Fig. 8*b*). This difference was evidenced as increases in both the percentage of T cells that produced IFN- γ and the level of IFN- γ production on a per cell basis. Thus, the majority of tumor-derived CD8⁺ T cells, although inherently proficient to produce IFN- γ , did not display this effector function unless exposed to IL-23 in vivo. Taken together, these data show that local IL-23 can augment vaccine-induced, CD8⁺ T cell-mediated, effector function and tumor destruction without increasing systemic T cell levels or limiting toxicity.

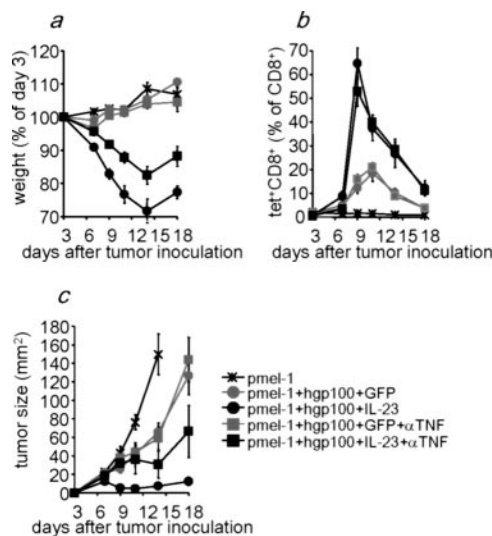


FIGURE 6. TNF- α is a mediator of IL-23 vaccine adjuvant effects. *a–c*, Mice ($n = 5$) bearing 3-day B16 tumors received 2×10^7 pmel-1 splenocytes and gp100 vaccination, followed by HGT with 5 μ g of GFP or IL-23 plasmid on days 3, 6, and 9 after tumor inoculation and 200 μ g of anti-TNF- α mAb i.p. on days 3, 6, 9, 12, and 15 after tumor inoculation. Mice were followed for body weight (*a*), CD8⁺tetramer⁺ T cell response (*b*), and tumor growth (*c*). The data shown are representative of two independent experiments.

Discussion

The modest, although promising, results of most clinical cancer vaccine trials have prompted a search for adjuvants that can increase the quantity and quality of vaccine-induced, antitumor immune responses. Destroying the growth and spread of cancer will probably require strong immune responses comparable to those observed during successfully controlled viral infections. However, most clinically tested cancer vaccines induce CD8⁺ T cell responses that are 1–2 orders of magnitude smaller (0.05–5% of circulating CD8⁺ T cells) (34–37) than those observed during successfully controlled acute viral infections, such as with influenza and EBV (up to 20% of circulating CD8⁺ T cells) (38–40). One possible reason is the relatively low number, avidity, and effector function of tumor-specific T cells due to central and peripheral tolerance to tumor Ags (6, 19, 41–44). Another reason may be the predominant use of defined “clean” vaccination approaches using

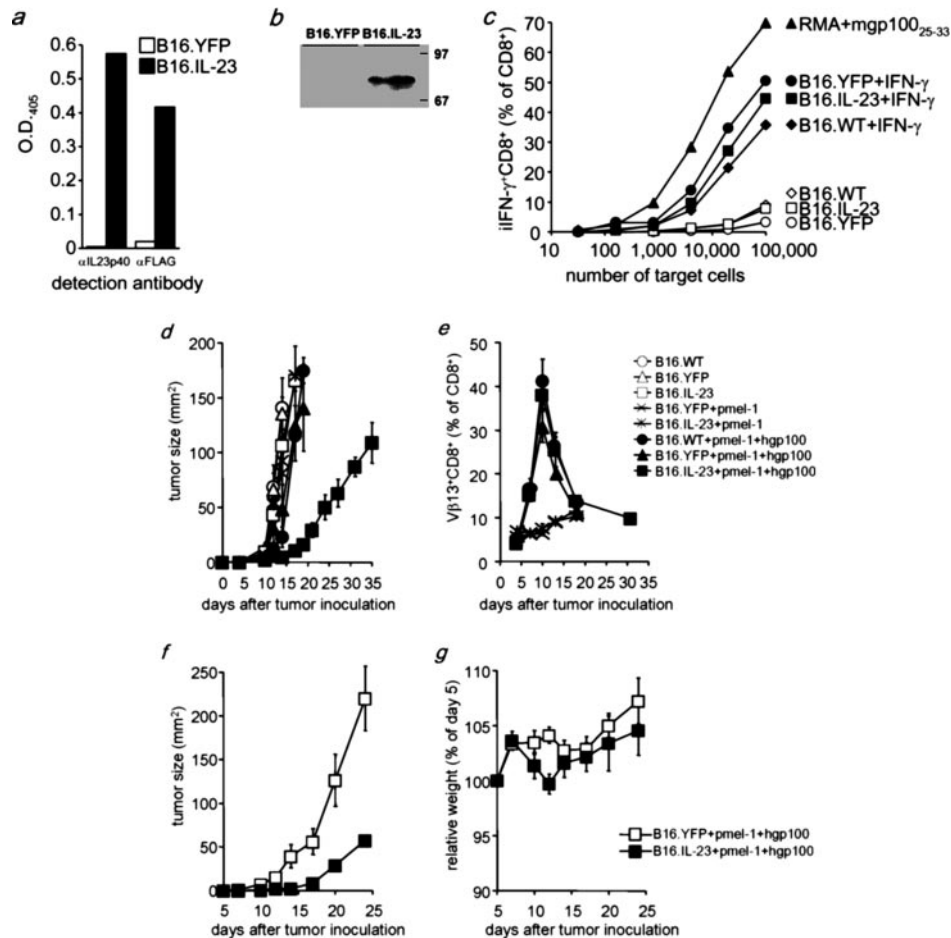


FIGURE 7. Tumor-derived IL-23 enhances CD8⁺ T cell-mediated tumor immunity. *a*, Detection of IL-23 fusion protein in 24-h B16.YFP and B16.IL-23 culture supernatants, as measured by IL-23p40 subunit-specific and FLAG-specific sandwich ELISA. *b*, Western blot detection of IL-23 fusion protein by anti-FLAG mAb staining of 24-h B16.YFP and B16.IL-23 culture supernatants. *c*, Short-term recognition of 48-h IFN- γ -treated or untreated B16.WT, B16.YFP, and B16.IL-23 tumor cells and mpg100₂₅₋₃₃ peptide-pulsed RMA cells by in vitro-activated CD8⁺ pmel-1 T cells. *d* and *e*, Intratumoral IL-23 enhances the antitumor effect of vaccine-activated CD8⁺ T cells. Mice ($n = 5$) bearing 3-day B16.WT, B16.YFP, or B16.IL-23 tumors received 2×10^7 pmel-1 T splenocytes with or without gp100 vaccination and were followed for tumor growth (*d*) and pmel-1 T cell response by FACS analysis after staining of PBL for CD8 and transgenic V β 13 chain (*e*). The data shown are representative of two independent experiments. *f* and *g*, Mice ($n = 5$) bearing 3-day B16.YFP or B16.IL-23 tumors received 2×10^7 pmel-1 T splenocytes with gp100 vaccination and were followed for tumor growth (*f*) and body weight (*g*).

peptide, DNA, or purified proteins that lack some of the inflammatory and costimulatory properties of successfully controlled pathogens. For example, inflammatory signals, such as ligation of CD40 and TLRs on DC, lead to the expression of costimulatory molecules and cytokines that control T cell expansion, effector function, persistence, and memory formation (45, 46). In addition, these inflammatory signals dictate specific T cell localization and effector phenotype (4, 47–49). It is becoming increasingly clear that these effector functions of tumor-specific T cells are often impaired during both endogenous and vaccine-induced antitumor immunity (4, 6, 47, 50, 51). With the availability of clinical cancer vaccines that induce measurable tumor-specific T cell priming (17, 18, 30), there is a growing interest in adjuvants that will further increase T cell levels and/or effector function (41).

IL-23 is a cytokine with potent effects on T cells, DC, and macrophages (11, 12, 52). Endogenous IL-23 plays a central proinflammatory role in diverse animal models of self Ag-mediated human disease, such as experimental allergic encephalitis, inflammatory bowel disease, and rheumatoid arthritis (11, 15, 16, 53, 54). We found that IL-23 enhanced the in vitro proliferation and effector function of tumor self-Ag-specific, CD8⁺ pmel-1 T

cells and set out to examine the immunological and anti-tumor effects of IL-23 as an adjuvant for self-specific cancer vaccination.

Our initial in vivo experiments showed that systemic IL-23 levels alone did not induce effective de novo tumor immunity or measurable activation of adoptively transferred, tumor-Ag specific T cells. However, upon gp100 peptide immunization, IL-23 specifically enhanced tumor destruction. Surprisingly, IL-23 increased the number of tumor-specific CD8⁺ T cells in all organs examined, but not in the tumor itself. Most vaccine-activated CD8⁺ T cells that were isolated from tumors after vaccination alone did not spontaneously secrete IFN- γ , even though they could be induced to do so by only 4 h of ex vivo antigenic stimulation. In contrast, freshly isolated, tumor-derived T cells from vaccinated mice receiving IL-23 spontaneously produced large amounts of IFN- γ both with and without ex vivo stimulation, indicating that IL-23 could unlock the effector potential of vaccine-activated T cells at the tumor site.

Our results indicated that systemic IL-23 1) suppressed tumor growth by vaccine-induced T cells; 2) enhanced tumor-specific T cell levels in all tested organs, but not in tumor; and 3) enhanced the effector function of intratumoral T cells. This suggested that

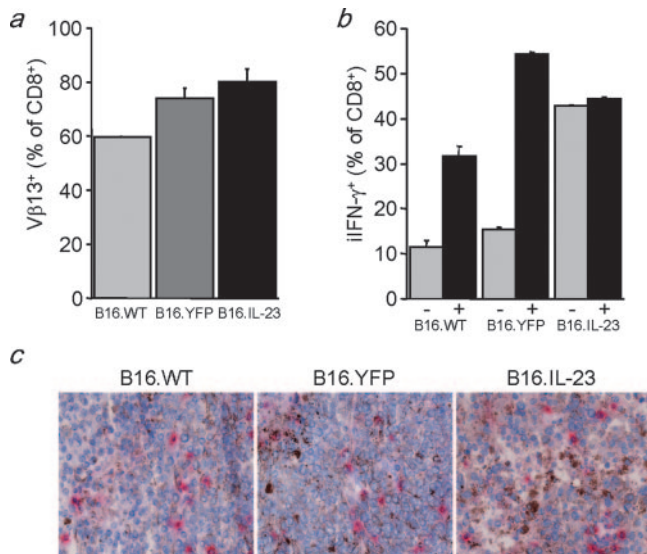


FIGURE 8. Tumor-derived IL-23 increases gp100-specific CD8⁺ T cell numbers and effector function. *a–c*, Mice ($n = 5$) bearing 10-day B16.WT, B16.YFP, or B16.IL-23 tumors received 2×10^7 pml-1 splenocytes and gp100 vaccination. Lymphocytes were isolated from tumor at the peak of the response (day 7 after vaccination) and were FACS analyzed for gp100-specific CD8⁺ pml-1 T cells (*a*) or iIFN- γ production after a 4 h-culture with (+) or without (-) 1 μ M mgp100_{25–33} peptide (*b*). The data shown are representative of two independent experiments. *c*, Immunohistochemical detection of pml-1 T cells and tissue integrity in tumors from mice from the same experiment (*a* and *b*) on day 11 after vaccination. Staining of pml-1 T cells with V β 13 (red) and hematoxylin counterstain shown at $\times 20$ the original magnification. Control mAb and IgG stainings were negative (data not shown). The data shown are representative of three independent experiments.

the enhanced T cell effector function, rather than the increased T cell number, was the most important factor for tumor suppression. When we tested this hypothesis using B16 tumor cells locally secreting IL-23, we found that enhanced tumor suppression was again accompanied by increased IFN- γ production by intratumoral T cells, but without higher systemic or intratumoral T cell levels. This suggests that the stimulatory effect of IL-23 on effector function was more important for tumor destruction than a systemic or local increase in vaccine-activated T cells. It also extends avenues of research into the use of cancer vaccines combined with tumor-targeted IL-23, for example through the use of fusion proteins of IL-23 with tumor-specific Ab (55–57); IL-23-encoding viruses that specifically home to, replicate within, or express their transgene within tumors (58–60); liposome-directed delivery of IL-23 to tumors (61, 62); or linking IL-23 to peptides that specifically bind to tumor endothelium (63, 64).

It is important to note that the increased IFN- γ production by vaccine-activated CD8⁺ T cells upon IL-23 treatment was not a main contributing factor to the antitumor effect of IL-23. IFN- γ , which was produced exclusively by vaccine-activated, tumor-infiltrating, CD8⁺ T cells, was important for B16 tumor destruction, as previously reported (65, 66). However, the vaccine adjuvant effect of IL-23 did not depend on the intrinsic ability of vaccine-activated T cells to produce IFN- γ . Thus, it appears that the enhanced IFN- γ production induced by local or systemic IL-23 is a marker of enhanced T cell effector function, rather than a direct mediator of the observed increase in tumor destruction. The partial abrogation of tumor suppression after anti-TNF- α treatment indicates that TNF- α mediates IL-23-induced tumor destruction. We found TNF- α production in tumor-derived macrophages from IL-

23-treated mice (data not shown), but whether these macrophages are relevant to tumor destruction remains to be determined.

An unexpected effect of IL-23 was the rapid, systemic, vaccination-independent depletion of Ag-nonspecific CD8⁺ and CD4⁺ T cells. This was also observed in nontumor-bearing, unvaccinated mice receiving IL-23 and is reminiscent of previous reports of bystander T cell depletion during acute infection with pathogens such as *Listeria* and lymphocytic choriomeningitis virus (67–69). It seems likely that IL-23 induces this bystander depletion via other cells, because naive murine T cells are reported to lack a functional IL-23R complex (12, 13). The significance, if any, of bystander T cell depletion for the vaccine adjuvant effect of IL-23 remains to be determined.

The utility of a vaccine adjuvant is determined by its desired therapeutic effects and its undesired side effects. We found that the dose-limiting toxicity of IL-23 was weight loss, apparently due to a loss of appetite, that was reversed upon IL-23 discontinuation. The therapeutic and side effects of IL-23 were linked across the entire dose-response range. Weight loss was partially mediated by TNF- α , which can, like other proinflammatory cytokines, such as IL-1, IL-6, and IFN- γ , directly suppress appetite (33). In addition, TNF- α can induce intestinal inflammation, a phenomenon that was also observed in mice transgenically overexpressing the IL-23 p19 subunit (14) and in our IL-23-treated mice. It is possible that IL-23 exacerbates local immune reactivity to intestinal commensals, because no other organs, including the stomach, appeared inflamed upon histological examination (54). The resulting inflammation could explain the malaise and loss of appetite. For now, the exact mechanism underlying weight loss after IL-23 treatment is unclear; however, TNF- α does not appear to be a suitable target for intervention, because its neutralization not only reduced weight loss, but also markedly impaired tumor suppression.

The effect of IL-23 on T cell effector function is reminiscent of previous results with IL-2 in this model and others, reinforcing the idea that impaired T cell effector function at the tumor site is an important bottleneck in both spontaneous and vaccine-induced T cell immunity to cancer (2, 4, 44, 70, 71). It is possible that cytokine adjuvants, such as IL-23 and IL-2, act through direct stimulation of Ag-specific T cells or the APC, such as DC and Langerhans cells, that prime them (72). Alternatively, cytokine adjuvants could stimulate immune or matrix cells at the effector (tumor) site, enhancing tumor cell recognition by or susceptibility to effector T cells, as recently demonstrated for IFN- α (48).

Our results using IL-23-producing B16 tumor cells are at odds with those obtained using a retrovirally transduced CT26.IL-23 tumor cell line in both the pulmonary lung nodule and s.c. setting and an independent B16.IL-23 cell line in the pulmonary setting (20, 21). In these studies, tumors initially grew and then spontaneously regressed. These studies did not examine the effect of IL-23 on active, Ag-specific immunization or on Ag-specific T cell responses. The difference between these results and our data may be due to the fact that CT26 tumors are significantly more immunogenic than B16 tumors (6, 73) (our unpublished observations) and the commonly observed greater resistance against immune-mediated destruction of established s.c. tumors compared with pulmonary nodules.

In conclusion, systemic IL-23 accelerates the accumulation and enhanced effector function of increased numbers of vaccine-induced T cells, leading to tumor destruction and growth suppression. It also induces depletion of bystander T cells and dose-dependent weight loss that is partially mediated by TNF- α . Local delivery of IL-23 prevents weight loss while preserving antitumor effects that appear to be mediated by the enhanced local T cell effector function rather than by increased T cell numbers. These

results suggest that IL-23 is a potential cancer vaccine adjuvant that deserves additional study.

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Disclosures

The authors have no financial conflict of interest.

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