Targeted Delivery of TNF Potentiates the Antibody-Dependent Cell-Mediated Cytotoxicity of an Anti-Melanoma Immunoglobulin

Patrizia Murri1, Jonathan D. Kiefer1, Louis Plüss1, Mattia Matasci2, Sandra L. Blümich3, Marco Stringhini2 and Dario Neri1

The recombinant murine IgG2a antibody TA99, directed against a melanoma antigen, was used to study combination modalities that potentiate antibody-dependent cell cytotoxicity. As previously reported, IgG2a(TA99) was extremely efficacious in preventing the growth of B16 lung metastases. However, the same antibody mediated only minimal tumor growth retardation when used to treat established neoplastic masses. The therapeutic activity of IgG2a(TA99) could be substantially enhanced by co-administration with an antibody-cytokine fusion (TA99-murine tumor necrosis factor [mTNF]), consisting of the TA99 antibody in single-chain variable fragment format fused to murine TNF. This fusion protein efficiently killed endothelial cells in vitro and displayed only minimal activity against B16 melanoma cells. In vivo, TA99-mTNF boosted the influx of natural killer cells and macrophages into B16 melanoma lesions. Therapy studies with two different administration schedules showed that the combination of TA99-mTNF and IgG2a(TA99) was superior to the individual products used as single agents. The combination treatment converted most of the tumor mass into a necrotic lesion, but a vital tumor rim eventually regrew, even when dacarbazine was included in the therapeutic regimen. The treatment modality described in this article may be applicable to the treatment of melanoma patients, given the specificity of the gp75 antigen and its conservation across species.

INTRODUCTION

Intact antibodies in IgG format can be efficacious for the selective depletion of certain lymphocyte populations and for the treatment of hematological malignancies (Carter, 2006; Maloney et al., 1994; Siwkowski and Mellman, 2013). However, antibody-dependent cell-mediated cytotoxicity (ADCC) is much less efficient against solid tumors (Slamon et al., 2001; Van Cutsem et al., 2009). An efficient implementation of ADCC would be highly desirable for pharmaceutical applications. In principle, if the target antigen were specifically expressed on tumor cells, IgGs capable of connecting tumor cells with the killing power of natural killer (NK) cells would come close to an embodiment of those magic bullets (Zauberkugeln) originally envisaged by Paul Ehrlich (Kaufmann, 2008).

The cell membrane gp75 antigen is selectively expressed in melanocytes and in melanoma cells, but it is otherwise undetectable in all other normal tissues (Takechi et al., 1996; Uhlen et al., 2015). This antigen, which is conserved from mouse to man (Vijayasaradhi et al., 1990), represents an ideal target for antibody-based pharmaceutical applications and an excellent model system to study ADCC. Nimmerjahn and Ravetch (2005) showed that the simultaneous intravenous administration of B16 melanoma cells and the gp75-specific TA99 antibody in murine IgG2a format could efficiently prevent formation of lung metastases in immunocompetent mice (Nimmerjahn and Ravetch, 2005). The antibody isotype was important, because murine IgG variants with lower affinity toward the cognate FcγRI and FcγRII did not display similar anticancer activity (Boross et al., 2014; Nimmerjahn et al., 2010; Nimmerjahn and Ravetch, 2005). Other reports, performed using IgG2a(TA99), showed that this agent could not eradicate melanoma lesions once the tumors had established as small subcutaneous solid nodules (Moyaihan et al., 2016; Sockolosky et al., 2016; Tzeng et al., 2015). We aimed to characterize the tumor-homing properties and therapeutic activity of murine IgG2a(TA99) and to investigate whether suitable combination modalities could potentiate ADCC.

Cytokine-based therapies can substantially boost ADCC activity in mouse models of cancer. There is a considerable biomedical interest in the use of engineered cytokines (Arenas-Ramirez et al., 2016; Charych et al., 2016; Hess et al., 2014; Lode et al., 1998; Neri and Sondel, 2016; Pasche and Neri, 2012). The recent transaction for a PEGylated version of IL-2 (NKTR-214) has been dubbed “the largest licensing deal in the pharmaceutical industry.”

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Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; mTNF, murine tumor necrosis factor; NK, natural killer; TNF, tumor necrosis factor

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(Taylor, 2018). Wittrup and colleagues recently reported that the ADCC activity of the intact immunoglobulin TA99 could be substantially enhanced by combination with an Fc fusion of murine IL-2 (Zheng et al., 2015). Similarly, we previously reported that the ADCC activity of IgG therapeutics can be boosted by the antibody-based delivery of IL-2 in mouse models of cancer (Borschel et al., 2015; Kaspar and Trachsel, 2012; Schliemann et al., 2009) and in patients (Gutbrodt et al., 2013; Schliemann et al., 2015).

In addition to IL-2, the antibody-based delivery of tumor necrosis factor (TNF) to the tumor environment holds promises for the implementation of an efficient anticancer therapy. TNF may display a direct anticancer activity against certain cancer cell types and against endothelial cells (Fajardo et al., 1992; Old, 1985; van Horssen et al., 2006; Watanabe et al., 1988). This strong proinflammatory cytokine may also favor the uptake of therapeutics into the solid tumor mass (Folli et al., 1993; Halin et al., 2003; Khawli et al., 1994). We recently showed that the antibody-based delivery of murine TNF to splice variants of fibronectin leads to the complete eradication of soft-tissue sarcoma in immunocompetent mouse models (Borsi et al., 2003; Hemmerle et al., 2013; Probst et al., 2017). Cured mice were able to reject subsequent challenges of heterologous tumor cells from the same mouse strain by a mechanism that depended on CD8^+ T cells recognizing an endogenous retroviral antigen (Probst et al., 2017). Encouraged by these results and by the tolerability of TNF fusions in patients (Papadia et al., 2013; Spitaleri et al., 2013), the L19-TNF fusion protein has recently progressed to phase III clinical trials for the treatment of patients with metastatic soft-tissue sarcoma (clinical trial number NCT02076620). Antibody-TNF fusions capable of selective localization at the tumor site promote hemorrhagic necrosis of neoplastic lesions and favor the influx of leukocytes (including NK cells and macrophages) into the tumor mass (De Luca et al., 2017; Khawli et al., 1994; van Horssen et al., 2006). Collectively, these findings suggest that the targeted delivery of TNF may be considered as a strategy for ADCC potentiation.

In this article, we characterize the tumor-homing properties and anticancer activity of murine IgG2a(TA99) in B16 melanoma, both as single agent and in combination with the recombinant TA99-murine TNF (mTNF) fusion protein. The combination treatment was extremely efficacious in converting the tumor mass into a necrotic lesion, but a few tumor cells survived the treatment and eventually regrew. These findings may open new clinical applications for the management of melanoma in patients, since the gp75 antigen is conserved from mouse to human.

RESULTS

Figure 1 shows the biochemical properties of the recombinant antibody-based therapeutics used for this study. Murine IgG2a(TA99) and the noncovalent homotrimeric TA99-mTNF fusion migrated as a single band in nonreducing SDS-PAGE analysis, eluted as a single peak in gel filtration and bound to B16F10 melanoma cells. TA99-mTNF efficiently killed fibroblasts and endothelial cells but did not display a potent biocidal activity against B16 melanoma in vitro (Figure 1c and d, and see Supplementary Figure S1 online).

We then assessed the tumor-homing properties of the TA99 antibody in immunocompetent C57/B16 mice bearing subcutaneously grafted B16 melanomas. An ex vivo immunofluorescence analysis, performed 24 hours after intravenous administration of IgG2a(TA99), showed a selective and homogenous homing of the antibody to the tumor cells and to melanocytes, consistently with the very specific pattern of gp75 antigen expression (see Supplementary Figure S2 online) (Takechi et al., 1996; Uhlen et al., 2015). By contrast, an IgG2a of irrelevant specificity (KSF, directed against hen egg lysozyme) (Pasche et al., 2011) did not exhibit any preferentially tumor homing (Figure 2).

In keeping with what had previously been reported by the Ravetch group (Nimmerjahn and Ravetch, 2005), IgG2a(TA99) efficiently prevented B16 lung metastasis formation when the antibody was administered 1 hour after intravenous injection of B16 melanoma cells (Figure 3). However, when the product was administered to mice bearing small tumors (i.e., 3 or 6 days after subcutaneous injection of B16 cells), only minimal tumor growth retardation was observed (Figure 4a). The IgG2a(TA99) was biologically active in this setting, as evidenced by the discoloration of the mouse coat (Figure 4b).

To address whether TA99-mTNF may display a direct melanoma killing activity in vivo, we repeated the experiment of Figure 3b, adding a treatment group with TA99-mTNF used as single agent. Although IgG2a(TA99) was potently active in inhibiting lung metastases, TA99-mTNF did not show any detectable antitumor activity (Figure 3c). By contrast, treatment of mice with established subcutaneous B16 melanoma using TA99-mTNF inhibited tumor growth, and this anticancer activity was potentiated by combination with IgG2a(TA99) (Figure 4c). These results suggest that the main role played by the TA99-mTNF fusion protein is at the level of the tumor endothelium, inducing a rapid hemorrhagic necrosis and an influx of leukocytes into the residual neoplastic mass.

At the dose used (7 µg), TA99-mTNF caused a transient body weight loss of 10%. In an attempt to improve activity and tolerability, a more frequent dosing of the product (5.5 µg) was tested. Although in a combination group (i.e., the one with simultaneous injection of the two biotherapeutics) tumor progression could be substantially delayed, neoplastic masses eventually regrew (Figure 4d). Selective depletion of NK cells led to a substantial loss of therapeutic activity.

Analysis of tumor sections in mice treated with saline or with the combination of TA99-mTNF and IgG2a(TA99) showed that the combination treatment rapidly transformed the neoplastic mass into a largely necrotic scab (Figure 5). However, a few areas with vital tumor cells, surrounding tumor blood vessels, could still be detected. The combination treatment mediated an increase in the density of macrophages and NK cells in the remaining vital portions of the tumor (Figure 5). These cells are crucially important for ADCC. The combined action of IgG2a(TA99) plus TA99-mTNF causes either a direct tumor cell death (evidenced by
caspase 3 staining) or the generation of strongly hypoxic regions, which exhibit an intense staining for carbonic anhydrase IX (Neri and Supuran, 2011) (see Supplementary Figure S3 online).

In an attempt to further improve therapeutic activity by killing the surviving tumor rim, a therapy experiment was performed featuring the use of IgG2a(TA99), TA99-mTNF, and/or dacarbazine (alone or in combination). Dacarbazine has long been used for the treatment of patients with metastatic melanoma (Ugurel et al., 2013) and was applied at a dose (70 mg/kg) previously reported in other studies (Jin et al., 2011). The triple combination regimen substantially delayed tumor progression but failed to induce cancer cures (Figure 6).
DISCUSSION

In keeping with previous reports, IgG2a(TA99) efficiently prevented B16 melanoma lung metastasis (Nimmerjahn and Ravetch, 2005) but could not eradicate solid tumor masses, even of small size (Moynihan et al., 2016; Sockolosky et al., 2016; Tzeng et al., 2015) (Figures 3b and 4). The antibody
Figure 4. TA99-mTNF is able to boost the therapeutic effect of IgG2a(TA99) on B16F10 tumors. (a) Therapy in B16F10 melanoma-bearing C57/BL6 mice with IgG2a(TA99) as single agent. Mice were injected three times intravenously every 48 hours with 300 μg IgG2a(TA99) or saline (arrows). Therapy was started at day 3 (red arrows) or at day 6 (black arrows) after tumor implantation. Data represent mean tumor volume ± standard error of the mean, n = 5 mice per group. *P < 0.05, **P < 0.01, by two-way analysis of variance followed by Bonferroni posttest. (b) Example of coat depigmentation in C57/BL6 mouse treated with IgG2a(TA99). Mice were shaved on day 1 over the right flank and on the back. (c) Left panel shows therapy in B16F10 melanoma-bearing C57/BL6 mice with IgG2a(TA99) and TA99-mTNF as single agents or in combination. Mice were injected three times intravenously every 72 hours with 300 μg IgG2a(TA99), 7 μg TA99-mTNF, or saline (red arrows). Data represent mean tumor volume ± standard error of the mean. ****P < 0.0001, ***P < 0.001, **P < 0.01, ns indicates P > 0.05 by two-way analysis of variance followed by Bonferroni posttest. Right panel shows toxicity monitoring by alterations in body weight change during therapy represented as mean percent weight change ± standard error of the mean. n = 5 mice per group for IgG2a(TA99) + TA99-mTNF combination and TA99-mTNF monotherapy; n = 4 mice per group for saline, IgG2a(TA99) monotherapy and TA99-mTNF monotherapy after day 13 (§). (d) Left panel shows therapy in B16F10 melanoma-bearing C57/BL6 mice with IgG2a(TA99) as TA99-mTNF as single agents or in combination. Mice were injected three times intravenously every 48 hours with 300 μg IgG2a(TA99), 5.5 μg TA99-mTNF, or saline (red arrows). For the combination treatment, TA99-mTNF was injected either 24 hours before or immediately after IgG2a(TA99) administration. Data represent mean tumor volume ± standard error of the mean. ****P < 0.0001, ***P < 0.001, **P < 0.01, ns indicates P > 0.05 by two-way analysis of variance followed by Bonferroni posttest. Right panel shows toxicity monitoring by alterations in body weight change during therapy represented as mean percent weight change ± standard error of the mean. n = 5 mice per group; n = 4 mice per group for saline and TA99-mTNF monotherapy after days 16 and 13, respectively (§). mTNF, murine tumor necrosis factor; ns, not significant; s.c., subcutaneous.
Figure 5. The combination of IgG2a(TA99) and TA99-mTNF kills most of the neoplastic mass and boosts infiltration of leukocytes, NK cells, and macrophages. B16F10 tumor section analyses of mice treated with saline or 300 μg IgG2a(TA99) + 7 μg TA99-mTNF combination therapy and killed 6 hours or 24 hours after injection. (a) From left: hematoxylin and eosin analysis (scale bar = 2.5 mm) and detection of activated caspase-3 and Asialo GM1 on NK cells, and F4/80 on macrophages by immunofluorescence (green, Alexa Fluor 488). Blood vessels stained with anti-murine CD31 (red, Alexa Fluor 594). Original magnification ×20. Scale bar = 100 μm. (b) Immunohistochemical analyses of CD45-positive leukocytes (red, aminoethyl carbazole) on B16F10 tumor sections. Entire tumor section (scale bars = 2.5 mm) and representative images of the infiltrate in the tumor center or periphery (scale bar = 100 μm). (c) Immunohistochemical analyses of CD68-positive macrophages (brown, 3,3′-diaminobenzidine) on B16F10 tumor sections. Entire tumor section (scale bar = 2.5 mm) and representative images of the infiltrate in the tumor center or periphery (scale bar = 100 μm). h, hour; mTNF, murine tumor necrosis factor; NK, natural killer.
could mediate a potent cytotoxic activity against melanocytes, as shown by discoloration of the black coat of C57/BL6 mice (Figure 2b). However, there are mechanisms that prevent an efficient ADCC within the solid tumor mass. In principle, antibody-mediated cellular phagocytosis (e.g., by macrophages) and complement-dependent cytotoxicity could also contribute to the observed antitumor effect (Derer et al., 2014; Gul and van Egmond, 2015; Johnson and Glennie, 2003; Klaus et al., 1979).

To achieve ADCC in vivo, a few prerequisites are needed: (i) the antibody needs to efficiently localize to tumor cells, (ii) a sufficiently high density of effector cells must be present, and (iii) the tumor cell must be sensitive to components of cytotoxic granules (e.g., perforin and granzymes). Ex vivo detection of tumor targeting clearly showed a selective and homogenous homing of the IgG2a antibody to B16 melanoma cells (Figure 2). The inhibition of lung metastasis formation confirms that the tumor cells could be killed by the in vivo action of IgG2a(TA99) (Figure 3b). By contrast, TA99-mTNF acts at the level of the tumor neovasculature (causing hemorrhagic necrosis and an increase in leukocyte infiltration), whereas the fusion protein is not capable of direct melanoma cell killing in vitro (Figure 1c) and in vivo (Figure 3c). Collectively, our results suggest that the main limitation for an efficient implementation of ADCC against solid B16 melanoma masses relates to the lack of active NK cells or macrophages in the neoplastic matrix (Figure 5).

Antibody-cytokine fusions, capable of selective localization in the tumor mass, can substantially increase the local density of NK cells and other leukocytes (Borsi et al., 2003; Carnemolla et al., 2002; De Luca et al., 2017; Halin et al., 2002; Lode et al., 1998; Moschetta et al., 2012; Penichet and Morrison, 2001). The antibody-based delivery of TNF to components of the tumor extracellular matrix can mediate a hemorrhagic necrosis of the tumor mass (van Horssen et al., 2006), followed by activation of the immune system against the (few) residual tumor cells (De Luca et al., 2017; Probst et al., 2017). This process may be boosted by chemotherapy (Hemmerle et al., 2013; Pretto et al., 2014) or by other combination modalities (e.g., other tumor-homing immunocytokines) (De Luca et al., 2017; Halin et al., 2003; Pretto et al., 2014).

There has been an intense industrial effort to potentiate the ADCC activity of antibodies by mutagenesis of the Fc portion (Presta, 2008; Sondermann and Szymkowski, 2016) or by glycoengineering (Umana et al., 1999). The use of antibody-cytokine fusions, capable of boosting the influx and activation of certain leukocytes into the tumor cell mass, may represent a valuable complementary approach. Two clinical trials feature the combination of L19-IL2 (a fusion protein directed against the alternatively spliced EDB domain of fibronectin) with rituximab (clinical trial number NCT02957019), or the combination of F16-IL2 with the anti-CD33 antibody BI 836858 (clinical trial number NCT03207191) (Gutbrodt et al., 2013; Schliemann et al., 2015). Although two TNF fusions are currently being investigated in advanced clinical trials (Danielli et al., 2015; Santoro et al., 2010), we are not aware of ongoing combination trials with IgG-based therapeutics.

In preclinical models of cancer, the main activity of targeted TNF products appears to be associated with a rapid induction of hemorrhagic necrosis (van Horssen et al., 2006). In patients, similar effects on superficial melanoma lesions have been reported in isolated limb perfusion procedures, both with recombinant TNF (Lejeune et al., 2001) and with the L19-TNF fusion (Papadia et al., 2013). When considering visceral metastases or internal solid tumor masses, advances in perfusion magnetic resonance imaging methodologies (Li and Padhani, 2012) may allow us to detect whether necrotic processes, similar to the ones observed in mice, can also be induced in patients with cancer.
ADCC remains an attractive and elegant avenue for exploiting the potential of the immune system against target antigens of choice. Biocidal events are manifested only when the IgG molecule engages in a binding interaction with a suitable marker on the surface of target cells. ADCC applications have not been very successful for the treatment of disseminated solid tumors, but the successful application of trastuzumab for the adjuvant treatment of patients with breast cancer is due, at least in part, to ADCC mechanisms (Slamon et al., 2001). ADCC may even play a role in the growing field of immune checkpoint inhibitors (Arce Vargas et al., 2018; Ingram et al., 2018; Simpson et al., 2013).

Gp75, the target of the TA99 antibody, is one of the cleanest tumor-associated antigens described so far, being expressed only in melanocytes and in melanoma lesions. The excellent quality of this target can be easily confirmed by inspection of the Protein Atlas database (Uhlen et al., 2015). We believe that molecular strategies directed against the gp75 antigen may be ideally suited for investigating whether ADCC approaches can be efficient for the management of disseminated solid tumors. Based on the results of our study, a fully human IgG1 product, specific to gp75, may deserve industrial and clinical development, especially if used in combination with antibody-cytokine products.

MATERIALS AND METHODS

Cell lines, animals, and tumor models

CHO cells and L-M fibroblasts were obtained from the ATCC between 2015 and 2017, expanded, and stored as cryopreserved aliquots in liquid nitrogen. The B16F10 melanoma cell line was kindly provided by the group of Michael Detmar (Swiss Federal Institute of Technology, Zurich, Switzerland), and mouse pancreatic islet endothelial cells (MS-1) cells were kindly provided by the group of Cornelia Halin (Swiss Federal Institute of Technology, Zurich, Switzerland). Cells were grown according to the manufacturer’s protocol. Authentication of the cell lines, including check of post-freeze viability, growth properties and morphology, testing for mycoplasma contamination, isoenzyme assay, and sterility testing, were performed by the cell bank before shipment.

Production, purification, and in vitro characterization of IgG2a(TA99) and TA99-mTNF

Sequences of the variable region of the light and heavy chains of the anti-gp75 antibody TA99 were taken from Boross et al. (2014). The genes encoding for the TA99 light and heavy chains and the constant regions of the IgG2a immunoglobulin were PCR amplified, PCR assembled, and cloned into the mammalian expression vector pMM137. The pMM137 vector was provided by Philochem AG and has been previously described (Putelli et al., 2014). The same cloning was performed with the light and heavy chains of the KSF antibody (binding the irrelevant hen egg lysozyme antigen) used as negative control (Pasche et al., 2011). The fusion proteins TA99-mTNF and KSF-mTNF contain the antibody (TA99 or KSF, respectively) as a single-chain variable fragment fused to mTNF at the C-terminus by a 15-amino acid linker (Hemmerle et al., 2013). The gene encoding for the TA99 or KSF single-chain variable fragments and the gene encoding mTNF were assembled through PCR and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Waltham, MA). IgG2a(TA99), IgG2a(KSF), TA99-mTNF, and KSF-mTNF were expressed using transient gene expression in CHO cells, as described previously (Pasche et al., 2011; Rajendra et al., 2011), and purified from the cell culture medium to homogeneity by protein L chromatography (Thermo Fisher Scientific, Waltham, MA). Quality control of the proteins was performed by SDS-PAGE, size exclusion chromatography (Superdex200 10/300GL, GE Healthcare, Little Chalfont, UK) and mass spectrometry. The full amino acid sequences for TA99-mTNF and IgG2a(TA99) can be found in Supplementary Figure S1.

The biological activity of murine TNF was determined on L-M fibroblasts, MS-1 endothelial cells, and B16F10 melanoma cells, as described previously (Hemmerle et al., 2013). Images of the cells incubated with 10−7 mol/L L-M fibroblasts and MS-1 cells or 10−9 mol/L TA99-mTNF for 24 hours were taken with the Axiovert 200 M microscope (Carl Zeiss, Oberkochen, Germany).

Ex vivo immunofluorescences

TA99-mTNF, IgG2a(TA99), and IgG2a(KSF) were labeled with FITC, as described in the manufacturer’s protocol (Sigma Aldrich, St Louis, MO). For ex vivo immunofluorescence analysis, C57BL6 mice bearing subcutaneous B16F10 tumors were injected with 300 µg FITC-labeled IgG2a(TA99) or IgG2a(KSF), respectively. Mice were killed 24 hours after injection. The organs were excised and embedded in cryoembedding medium (Thermo Fisher Scientific) and cryostat sections (8 mm) were stained using rabbit anti-FITC (Bio-Rad, Hercules, CA; 4510-780) and rat anti-CD31 (BD Biosciences, Franklin Lakes, NJ; 553370) as primary antibody and donkey anti-rabbit Alexa-488 (Invitrogen, A21206) and anti-rat Alexa594 (Invitrogen, A21209) as secondary antibodies. Slides were mounted with fluorescent mounting medium (Dako, Santa Clara, CA) and analyzed with Axiostop2 mot plus microscope (Carl Zeiss).

Immunofluorescence studies

The expression of the gp75 antigen in B16F10 tumors and the binding of IgG2a(TA99) and TA99-mTNF to their targets were assessed by immunofluorescence staining of iced-cold, acetone-fixed, 8-µm-thick B16F10 tumor sections. FITC-labeled IgG2a(TA99) or TA99-mTNF were used as primary reagents and detected by rabbit anti-FITC (Bio-Rad, 4510-780). Vital areas and hypoxic areas in the B16F10 melanoma masses were shown by staining with rabbit anti-caspase-3 (Sigma Aldrich, C8487) and goat anti-murine CAIX (R&D Systems, Minneapolis, MN; AF2344-SP). Infiltrations of NK cells and macrophages in the B16F10 tumor masses were assessed by immunofluorescence studies using rabbit anti-Asialo GM1 (Wako Chemicals, Richmond, VA) and rat F4/80 (BM8, ebBioscience, Waltham, MA). Blood vessels were stained with goat anti-CD31 (R&D Systems, AF3628). As detecting antibodies, anti-goat Alexa-Fluor594 (Invitrogen, A11058), anti-rabbit Alexa488 (Invitrogen, A21206) and anti-rat Alexa 488 (Invitrogen, A21208) were used. Slides were mounted with fluorescent mounting medium and analyzed with Axiostop2 mot plus microscope (Carl Zeiss).

Histology and immunohistochemistry studies

Tumor cryosections (7 µm) were fixed in acetone and stained with hematoxylin and eosin using routine methods. Tumor infiltrate was analyzed by immunostaining of mouse leukocytes (CD45; Abcam, Cambridge, UK; ab25386) and macrophages (CD68; Abcam, ab125212). The reaction was visualized with 3-amino-9-ethylcarbazole (CD45) or 3,3’-diaminobenzidine (CD68) as chromogens, followed by light counterstain with hematoxylin. The immunohistochemical staining was performed using an autostainer (Dako Autostainer Universal Staining System Model LV-1, Dako-
Agilent Technologies, Santa Clara, CA). All slides were scanned using a digital slide scanner (NanoZoomer-XR CX12000, Hamamatsu Photonics, Shizuoka, Japan), and images were obtained using the NDP.view2 software (Hamamatsu Photonics).

Flow cytometry
B16F10 cells cultured in a T-150 flask were detached with 2 mmol/L EDTA and stained with IgG2a(TA99) or FITC-labeled TA99-mTNF. IgG2a(TA99) was detected using anti-mouse AlexaFluor488 (Invitrogen, A21202). Rabbit anti-FITC (Bio-Rad, 4510-780) and anti-rabbit AlexaFluor488 (Invitrogen, A21206) were used to detect TA99-mTNF binding. All staining and washing steps were performed in 2 mmol/L EDTA 0.5% bovine serum albumin in phosphate buffered saline. Cells were sorted by FACS (CytoFLEX, Beckman Coulter, Brea, CA) and analyzed using FlowJo software (FlowJo, Ashland, OR).

Animal studies
We used 6–7-week-old C57BL/6 mice obtained from Janvier (Genest-St-Isle, France). B16F10 melanoma cells were injected intravenously (1 × 10^5 cells) through the tail vein or implanted subcutaneously in the flank (1 × 10^6 cells). Mice injected intravenously with B16F10 cells were perfused with 1% paraformaldehyde through the pulmonary artery and the aorta after they were killed. Lungs were excised and fixed for 1 hour.

For the in vivo depletion of NK cells, B16F10 tumor–bearing mice were injected intraperitoneally with 30 μL anti-Asialo GM1 (Wako Chemicals, 986-10001) on days 2, 5, and 8 after subcutaneous tumor implantation. Dacarbazine was purchased as lyophilized powder for infusion, Dacin (Lipomed, Switzerland).

SUPPLEMENTARY MATERIAL

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