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Dacarbazine-Mediated Upregulation of NKG2D Ligands on Tumor Cells Activates NK and CD8 T Cells and Restrains Melanoma Growth

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Dacarbazine (DTIC) is a cytotoxic drug widely used for melanoma treatment. However, the putative contribution of anticancer immune responses in the efficacy of DTIC has not been evaluated. By testing how DTIC affects host immune responses to cancer in a mouse model of melanoma, we unexpectedly found that both natural killer (NK) and CD8$^+$ T cells were indispensable for DTIC therapeutic effect. Although DTIC did not directly affect immune cells, it triggered the upregulation of NKG2D ligands on tumor cells, leading to NK cell activation and IFNγ secretion in mice and humans. NK cell–derived IFNγ subsequently favored upregulation of major histocompatibility complex class I molecules on tumor cells, rendering them sensitive to cytotoxic CD8$^+$ T cells. Accordingly, DTIC markedly enhanced cytotoxic T lymphocyte antigen 4 inhibition efficacy *in vivo* in an NK-dependent manner. These results underscore the immunogenic properties of DTIC and provide a rationale to combine DTIC with immunotherapeutic agents that relieve immunosuppression *in vivo*.

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INTRODUCTION

Beyond their cytotoxicity on tumor cells, some anticancer agents promote the activation of the host immune system, which can make a decisive contribution to therapeutic success (Apetoh *et al*., 2008; Zitvogel *et al*., 2008). Drugs such as cyclosphosphamide and 5-fluorouracil were indeed, respectively, shown to selectively deplete regulatory T cells (Tregs) (Ghiringhelli *et al*., 2004) and myeloid-derived suppressor cells (MDSCs) (Vincent *et al*., 2010; Apetoh *et al*., 2011) *in vivo*, resulting in enhanced antitumor responses. In addition, anthracyclines, oxaliplatin, and radiotherapy induce immunogenic cell death, leading to the activation of T cell–based anticancer immune responses (Apetoh *et al*., 2007; Obeid *et al*., 2007; Ghiringhelli *et al*., 2009). Platinum derivates were also shown to stimulate the expression of ligands for NKG2D, an natural killer (NK) cell–activating receptor, which determines the innate immune response against leukemic tumors (Gasser *et al*., 2005). NKG2D recognition by NK cells may be a preponderant mechanism in some cancers, including melanoma (Pende *et al*., 2001), and regulation of NKG2D ligand expression by tumor cells affects NK cell cytotoxicity (Pietra *et al*., 2012). Many different NKG2D ligands have been described to be differentially expressed between models, namely Rae-1, Mult-1, and H-60 in mice, and MICA, MICB and ULBPs in human cells. These ligands are activators of NK cell functions when membrane bound (Sutherland *et al*., 2006; Champsaur and Lanier, 2010), but may also inhibit immunity in their soluble form (Salih *et al*., 2008; Paschen *et al*., 2009).

Dacarbazine (DTIC) is an alkylating agent approved to treat metastatic melanoma in humans. This has been administered as a first-line treatment for metastatic melanoma since the 1970s (Gerner *et al*., 1975) and is still used as such when mutant BRaf-targeting therapy is not applicable. However, response rates to this treatment remain poor (Lui *et al*., 2007). Although the direct cytotoxic activity of DTIC has been known for long, little information is currently available about DTIC immunomodulatory effects. To address how DTIC affects anticancer immune responses, we evaluated the contribution of innate and adaptive immune cells in the anticancer effects of DTIC in a mouse melanoma model. We found that NK and CD8$^+$ T cells were both required for DTIC antitumor activity. DTIC induced upregulation of NKG2D ligands on B16 melanoma cells and human melanoma cells, leading to NK cell activation. Activated NK cells produced IFNγ, which enhanced major histocompatibility complex I (MHC-I) tumor

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Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; DAPI, 4′,6′-diamidino-2-phenylindole; DCs, dendritic cells; DTIC, dacarbazine; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; NK, natural killer; OVA, ovalbumin; PBS, phosphate-buffered saline; Tregs, regulatory T cells

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cell expression, resulting in the activation of antitumor-specific CD8$^+$ T cells *in vitro* and *in vivo*. Furthermore, a combination of DTIC with cytotoxic T lymphocyte antigen 4 (CTLA4) blockade also has an NK-dependent antitumor effect. These results underscore the immunogenic properties of DTIC that rely on its ability to induce NKG2D ligand expression on tumor cells.

RESULTS

DTIC exerts an antitumor effect in a CD8-, IFNγ-, and NK-dependent manner

We first treated wild-type or nude mice bearing a subcutaneous B16F10 melanoma tumor with DTIC. This treatment delayed tumor growth in wild-type mice but had no effect on tumor progression in nude mice, suggesting that the DTIC antitumor effect depends on T cells (Figure 1a).

To further delineate the contribution of the host immune response to the anticancer effect of DTIC, we treated tumor-bearing mice with DTIC along with isotype or depleting anti-CD8, CD4, IFNγ, or NK1.1 antibodies to eliminate, respectively, CD8$^+$ T cells (Figure 1b), CD4$^+$ T cells (Figure 1c), IFNγ (Figure 1d), or NK cells (Figure 1e). Although CD4$^+$ T cells were dispensable for DTIC therapeutic efficacy, we found that the DTIC antitumor effect relied on CD8$^+$ T cells, IFNγ, and NK cells. Interestingly, in NK- and IFNγ-depleted mice, the DTIC effect was totally abrogated, whereas in CD8$^+$ T cell–depleted mice DTIC only exerted an early and transient effect on tumor progression, suggesting that NK cells and IFNγ, which act as early effectors against tumor progression, might subsequently trigger CD8$^+$ T-cell activation. To further assess the immune-based effect of DTIC, we inoculated mice with 10 less B16F10 cells than usual and treated them according to the standard protocol. We observed that in mice in which the low-dose tumor was implanted, the DTIC was more effective, suggesting a nontumor-based phenomenon (Figure 1f).

Taken together, these data underscore the contribution of CD8$^+$ T cells, IFNγ, and NK cells in the anticancer activity of DTIC.

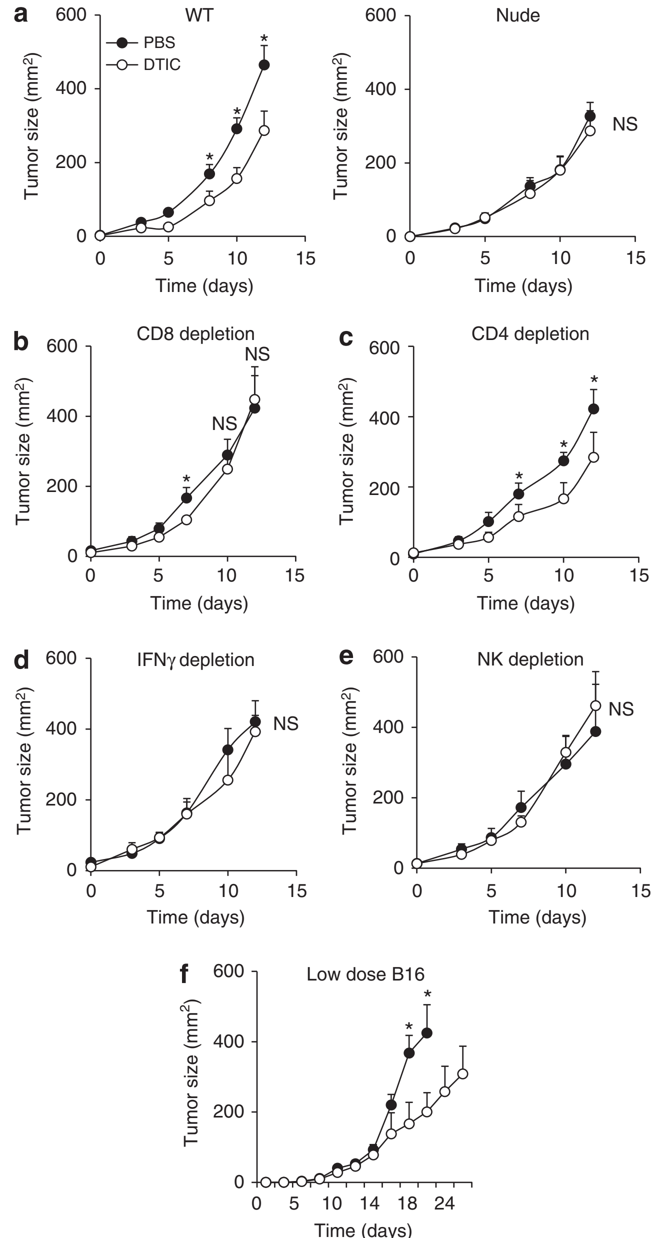

Figure 1. Dacarbazine (DTIC) exerts an antitumor effect in a CD8-, IFN-γ-, and natural killer (NK)–dependent manner. (**a**) Wild-type (WT) (left panel) or nude (right panel) mice were injected with 5×10^5 B16F10 cells and treated 7 days after injection with one intraperitoneal (IP) injection of DTIC (100 mg kg^{-1}). Day 0: day of treatment (10 mice per group). (**b–e**) Mice were injected with 5 × 105 B16F10 cells and treated 7 days after injection with one IP injection of DTIC (100 mg kg^{-1}). Mice received IP injection of (**b**) anti-CD8, (**c**) CD4, (**d**) IFNγ), (**e**) and anti-NK1.1 on day 5 after tumor cell injection and then twice a week (10 mice per group). (**f**) Same as **a** with injection of 5×10^4 B16F10 cells. The experiments were performed three times, yielding similar results. *$P<0.05$. NS, nonsignificant; PBS, phosphate-buffered saline.

DTIC does not directly affect immune effectors

Chemotherapy could harness the host immune system by eliminating immunosuppressive cells such as Tregs and MDSCs (Ghiringhelli *et al.*, 2004; Suzuki *et al.*, 2005; Vincent *et al.*, 2010), by inducing dendritic cell (DC) maturation (Tanaka *et al.*, 2009) or by eliciting ''immunogenic cell death'' of tumor cells, which has been shown to rely on the expression of cell surface calreticulin (Obeid *et al.*, 2007). To address these possibilities, we have first evaluated the frequency of Tregs and MDSCs in the spleen of DTIC-treated tumor-bearing mice and found that DTIC did not significantly alter the frequency of these immunosuppressive cells (Figure 2a and b).

We then assessed whether DTIC promoted the maturation of antigen-presenting cells by testing the effect of a single systemic injection of DTIC or lipopolysaccharide on the maturation pattern of splenic DCs. Although lipopolysaccharide induced a massive upregulation of CD86 and CD40, DTIC administration did not affect the expression of these markers, ruling out DTIC involvement in DC maturation (Figure 2c).

We next tested the ability of DTIC to trigger an immunogenic form of cell death by monitoring the expression of cell surface calreticulin on B16 cells. Unlike

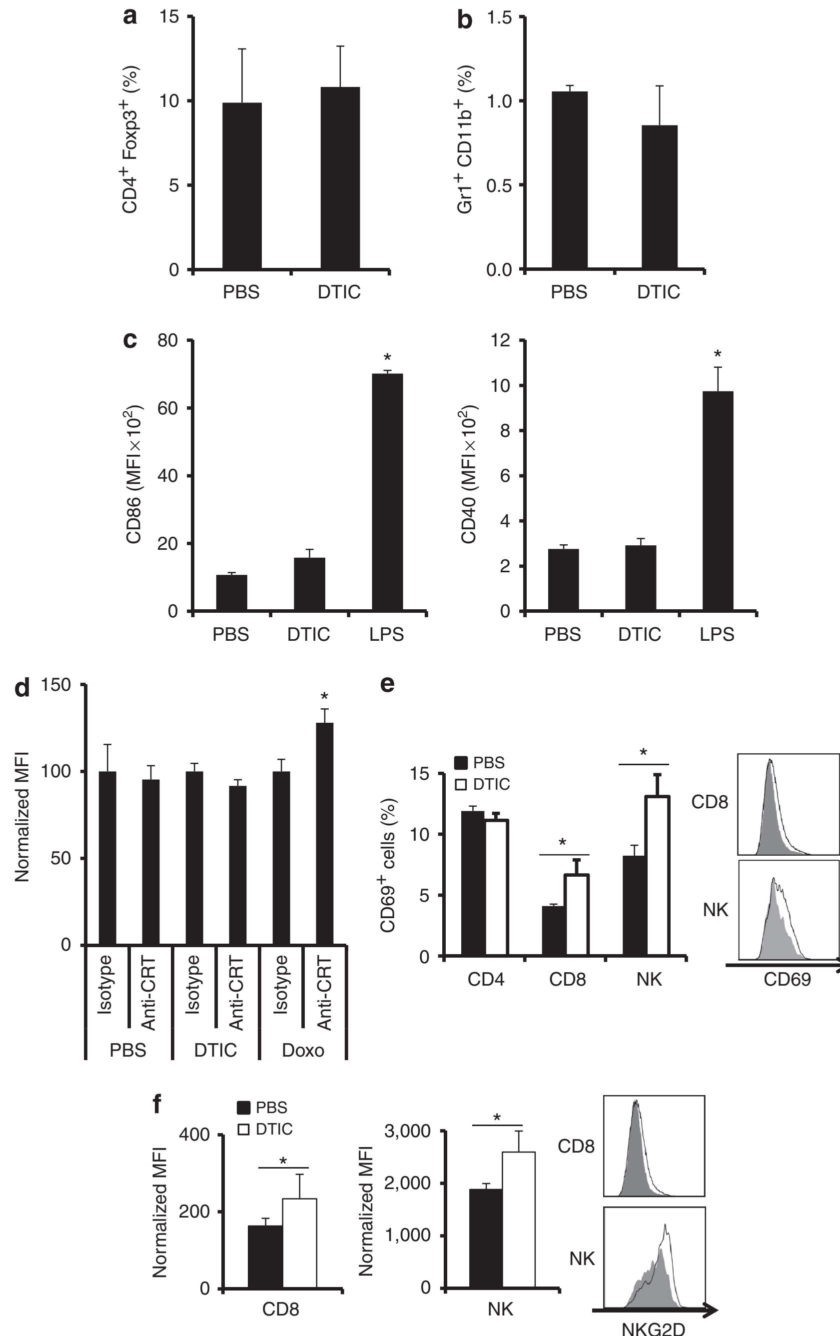

Figure 2. Dacarbazine (DTIC) activates natural killer (NK) and CD8$^+$ T cells, but does not induce immunogenic cell death or have an impact on suppressive cells. (**a**) Effect of DTIC on regulatory T cells (Tregs) (CD4$^+$ FoxP3$^+$). Mice bearing B16F10 tumors received either phosphate-buffered saline (PBS) or DTIC. The panel shows the percentage of splenic Treg 5 days after drug administration. The experiments were performed three times, yielding similar results. (**b**) Effect of DTIC on myeloid-derived suppressor cells (MDSCs) (Gr1$^+$ CD11b$^+$). Mice bearing B16F10 tumors either received PBS or DTIC. The panel shows the percentage of splenic MDSCs 5 days after drug administration. The experiments were performed three times, yielding similar results. (**c**) Effect of DTIC on splenic dendritic cell (DC) maturation. Tumor-free mice received an injection of PBS, lipopolysaccharide (LPS), or DTIC. Twenty-four hours after treatment, splenic CD11c^{hi} DCs were examined by flow cytometry for CD86 (left) or CD40 (right) expression. The experiments were performed twice, yielding similar results. (**d**) Effect of PBS, DTIC, and doxorubicin (Doxo) on calreticulin translocation. B16F10 tumor cells were cultured for 24 hours in complete medium alone (PBS) or supplemented with DTIC or Doxo as a positive control. Calreticulin expression on the cell surface was determined by flow cytometry, and MFI was standardized to the isotype control fluorescence. The experiments were performed twice. (**e**) Effect of DTIC on NK, CD8, and CD4 T-cell activation. Mice bearing B16F10 tumors either received no treatment (PBS) or one intraperitoneal administration of DTIC. Tumor-draining lymph nodes were harvested 2 days post treatment, and the proportion of CD69-positive NK cells, CD8$^+$ T cells, and CD4$^+$ T cells was determined by flow cytometry. Right panel shows representative FACS histograms for CD8$^+$ T and NK cells. (**f**) Same as **e** with NKG2D staining. The mean fluorescence intensity was normalized to isotype-matched cells. The experiments were performed twice, yielding similar results. *$P<0.05$. MFI, mean fluorescence index.

doxorubicin, which is known to trigger calreticulin cell surface expression (Obeid *et al*., 2007), DTIC treatment could not trigger calreticulin membrane expression on B16 cells (Figure 2d), suggesting that the antitumor effects of DTIC cannot be explained by its ability to trigger an immunogenic form of tumor cell death.

Finally, we found that DTIC injection did not modify the number of total CD4, CD8, CD19, CD11c, or NK cells in lymphoid organs of tumor-bearing mice (not shown). Nevertheless, we noted NK and $CD8^+$ T-cell activation assessed by CD69 (Figure 2e) and NKG2D (Figure 2f) labeling in tumor-draining lymph nodes after DTIC injection. *In vitro*, DTIC did not affect NK or CD8 T-cell phenotype (not shown).

Altogether, these data suggest that although the antitumor effect of DTIC is dependent on $CD8^+$ T and NK cells, DTIC did not affect Tregs, MDSC, and DC maturation, and failed to induce immunogenic death of tumor cells. In contrast, DTIC induced local activation of NK and $CD8^+$ T cells, suggesting that the DTIC-mediated NK and $CD8^+$ T-cell activation is indirect.

DTIC induces the expression of NKG2D ligands and induces NK cell activation

Previous reports have shown that chemotherapy-induced cell stress triggers the expression of NKG2D ligands on leukemic cell lines, leading to NK cell activation (Fine *et al*., 2010; Nice *et al*., 2010). As our results showed NK cell activation following DTIC treatment *in vivo*, we tested whether DTIC induced NKG2D ligand expression in melanoma cells. We found that DTIC induced *Rae1* and *Mult1* gene expression in B16 cells (Figure 3a). This increased NKG2D ligand expression resulted in detection of membrane Rae-1 and Mult-1 proteins, as well as total NKG2D ligands, on B16F10 tumor cells 24 hours following DTIC treatment (Figure 3b), whereas, according to the literature, we failed to detect any of them in phosphate-buffered saline (PBS)–treated B16F10 (Zhang and Sentman, 2011). Thereafter, we tested NK cell cytotoxicity against B16 cells after DTIC treatment. We observed that DTIC enhances NK cell toxicity (Figure 3c). In addition, we could demonstrate that coculture of NK cells with B16 cells treated with DTIC induced a production of IFNγ (Figure 3d). We further observed that the lysis of DTIC-treated B16 cells by NK cells was dependent on perforin/granzyme B but not TRAIL, as shown by the use of concanamycin A or blocking anti-TRAIL antibody (Figure 3e). Importantly, by using a blocking NKG2D-Fc, we could observe that NKG2D engagement was mandatory for both NK cell cytotoxic effects (Figure 3f) and IFNγ secretion (Figure 3g) when using DTIC-treated B16 cells. Finally, we tested the dependency of DTIC treatment efficacy *in vivo*. We treated melanoma-bearing mice with DTIC along with isotype or depleting anti-NKG2D. We observed that NKG2D blockade hinders the DTIC effect on tumor growth (Figure 3h).

To generalize our *in vitro* and animal results, we took advantage of a human melanoma cell line MelC (Fregni *et al*., 2011). To check whether DTIC treatment induces upregulation of NKG2D ligands in MelC cells, we analyzed transcripts for *MICA*, *MICB*, *ULBP1*, *ULPB2*, and *ULPB3* in MelC cells treated or not treated with DTIC (Figure 4a). MICA and MICB were significantly upregulated upon DTIC exposure, whereas the other NKG2D ligands showed a slight increase in expression. In accordance with these results, we also detected the presence of MICA and MICB proteins in MelC cells by flow cytometry (Figure 4b). To test the functional relevance of this observation, we performed coculture of human NK cells with PBS- or DTIC-treated MelC cells. DTIC treatment efficiently sensitizes the melanoma target to NK cell lysis, indicating the validity of our hypothesis in human settings (Figure 4c). We also noted that this increase in target mortality was prevented by a blocking anti-human NKG2D antibody (Figure 4d). We confirmed this observation in other human melanoma cell lines. We found that three out of five tested melanoma cell lines are prone to NKG2D ligand upregulation-dependent NK lysis induced by DTIC (data not shown).

Altogether, these data demonstrate that DTIC-induced NKG2D ligand upregulation on tumor cells leads to NK cell activation.

DTIC-driven production of IFNγ by NK cells triggers upregulation of MHC-I and favors tumor cell killing by CTL

B16 cells poorly express MHC-I molecules at steady state, and this phenomenon impedes their recognition by $CD8^+$ T cells (Zoller *et al*., 1988; Lugade *et al*., 2008). We observed that the supernatant of NK cells cocultured with DTIC-treated B16 cells induced the expression of MHC-I molecules on fresh B16 cells. This MHC-I upregulation was entirely due to IFNγ, as IFNγ blockade aborted the phenomenon (Figure 5a). By using B16 cells expressing ovalbumin (OVA) antigen (B16OVA), we showed that, although PBS-treated B16OVA express low levels of MHC-I as expected (Lugade *et al*., 2008), the supernatant of NK cells cocultured with DTIC-treated tumor cells induced the expression of OVA peptide (SIINFEKL)–MHC-I complex on tumor cell surface in an IFNγ-dependent manner, indicating enhanced endogenous tumor antigen presentation (Figure 5b). We hypothesized that this increased availability of peptide/MHC-I complexes may enhance B16OVA susceptibility to antigen-specific cytotoxic T cells. We took advantage of OT-I transgenic mice, in which $CD8^+$ T cells bear an OVA-specific TCR. *In vitro*, pretreatment of B16OVA cells with the supernatant of NK cells cocultured with DTIC-treated tumor cells enhances their killing by OT-I $CD8^+$ T cells (Figure 5c).

To test the *in vivo* relevance of this observation, we treated mice bearing B16OVA tumors with DTIC, and then injected them with carboxyfluorescein succinimidyl ester (CFSE)–labeled OT-I cells and monitored their outcome. We found that DTIC treatment induced higher OT-I proliferation in tumor-draining lymph nodes compared with control. This effect is blunted by IFNγ neutralization or NK cell depletion, suggesting that DTIC-driven OT-I *in vivo* activation was dependent on NK cell activation (Figure 5d).

Altogether, these data indicate that, *in vivo* and *in vitro*, IFNγ produced by NK cells after DTIC treatment enhances tumor cell capacity to present tumor antigens on MHC-I and favors $CD8^+$ T-cell activation.

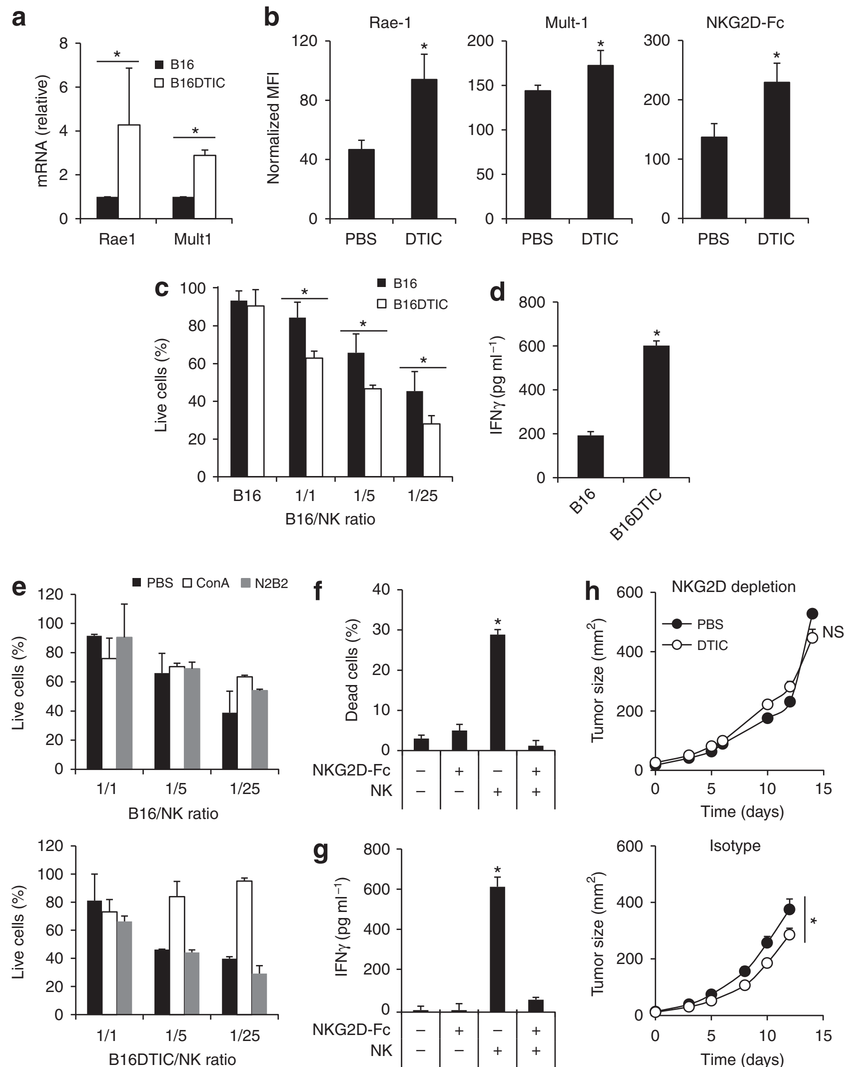

Figure 3. Dacarbazine (DTIC) induces expression of NKG2D ligands and natural killer (NK cell) activation. (**a**) Messenger RNA (mRNA) expression of NKG2D ligands in B16F10 cells before and after treatment with 24 hours DTIC (0.4 g l^{-1}). Relative expression normalized to β-actin level. The experiments were performed five times with similar results. (**b**) FACS expression of membranous Rae1 (left panel), Mult-1 (middle panel), and total NKG2D ligand (right panel) before and after treatment with 24 hours DTIC. The mean fluorescence intensity was normalized to isotype-matched cells. The experiments were performed three times, yielding similar results. (**c**) B16F10 cells treated or not treated with DTIC were incubated with the indicated number of NK cells. Cell death was assessed using 4′,6′-diamidino-2-phenylindole staining by flow cytometry after 48 hours. The experiments were performed twice, yielding similar results. (**d**) B16F10 cells treated or not treated with DTIC were incubated with NK cells at 25/1 E/T ratio. IFNγ production after 24 hours was determined by ELISA. The experiments were performed twice, yielding similar results. (**e**) Untreated B16F10 cells (upper panel) and B16F10 treated for 24 hours with 0.4 g l^{-1} DTIC (lower panel) were incubated with the indicated number of NK cells in the presence of phosphate-buffered saline (PBS), concanamycin A (ConA), or anti-TRAIL mAb. Cell viability was assessed using the crystal violet test after 48 hours. The experiments were performed twice. (**f**, **g**) B16F10 cells treated with DTIC were incubated with NK cells at 25/1 E/T ratio in the presence of NKG2D-Fc molecule or control Fc protein. (**f**) Cell viability was assessed using the crystal violet test. (**g**) IFNγ production after 24 hours was determined by ELISA. (**h**) Same as Figure 1, with injection of isotype (lower panel) or blocking anti-mouse NKG2D (upper panel). The experiments were performed twice. *$P<0.05$. NS, nonsignificant.

CTLA4 blockade + DTIC synergy relies on NK cells

Given that our previous experiments indicate the NK cell dependency of the DTIC treatment, we questioned whether NK cells are mandatory for efficacy of DTIC-based combined chemotherapeutic regimen. Recently, treatment inhibiting CTLA4, an inhibitor of T cell–based cytotoxicity (Walker and Sansom, 2011), has proven beneficial in combination with DTIC (Robert *et al.*, 2011). We decided to test CTLA4 + DTIC inhibition in our experimental model, with or without NK depletion. As expected from the literature (van Elsas *et al.*, 1999; Dranoff, 2011), the effect of anti-CTLA4 treatment alone on tumor growth is comparable to that of DTIC alone, and the anti-CTLA4 + DTIC combination gives better antitumor efficacy (Figure 6a). As already seen in Figure 1, DTIC efficacy is totally blunted in mice depleted of NK cells; furthermore, NK cell depletion abrogated not only the antitumor effect of anti-CTLA4 alone but also that of anti-CTLA4 + DTIC (Figure 6b). These data support the rationale that the *in vivo* antitumor effect of the combination of DTIC + anti-CTLA4 relies on NK cell action.

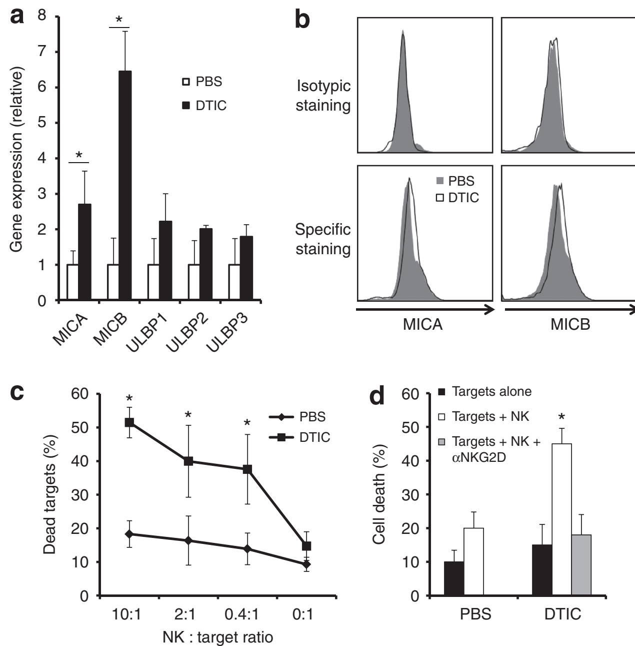

Figure 4. Dacarbazine (DTIC) induces the expression of NKG2D ligands and enhances tumor sensitivity to natural killer (NK) in a human model. (**a**) Messenger RNA (mRNA) expression of NKG2D ligands in MelC cells before and after 24 hours of DTIC ($0.4\,g\,l^{-1}$). Relative expression normalized to β-actin level. The figure shows mean and SEM from three independent experiments. (**b**) Representative FACS analysis of MICA and MICB expression by MelC cells treated with phosphate-buffered saline (PBS) (filled gray) or DTIC (open black). The bottom row shows representative specific staining, whereas the upper row shows the corresponding isotype control. (**c**) MelC cells treated for 24 hours with PBS or DTIC were incubated with indicated number of purified human NK cells. Death was assayed with 4′,6′-diamidino-2-phénylindole staining by flow cytometry. (**d**) MelC cells treated for 24 hours with PBS or DTIC were incubated with purified NK cells (10 NK for 1 target). In some conditions, anti-human NKG2D was added at a concentration of $10\,\mu g\,ml^{-1}$. Viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The figure shows mean and SEM from three independent experiments. $*P<0.05$.

DISCUSSION

This paper reports the immunological properties of DTIC in the context of growing melanoma. We unraveled the capacity of DTIC to trigger NKG2D ligand expression on both murine and human tumor cells, resulting in augmented NK cell cytotoxic properties and IFNγ production. This cytokine production is involved in the enhancement of MHC-I presentation of tumor-associated antigen and better CTL activity. In our *in vivo* model, the effect of DTIC on the immune system seems predominant, as its effect on tumor growth is lost in T cell–deficient *nude* mice, or in mice depleted of $CD8^+$ T cells, NK cells, or IFNγ. Thus, DTIC can be added to the expanding list of chemotherapeutic agents whose antitumor effect depends, at least in part, on their capability to enhance the anticancer immune response (Zitvogel *et al.*, 2008).

Much evidence demonstrates that mouse and human NKG2D ligands are often expressed poorly on normal cells, but their expression levels are upregulated on tumor cells or virus-infected cells (Mistry and O'Callaghan, 2007). NKG2D ligands are also expressed by melanoma cell lines and tumors (Casado *et al.*, 2009; Paschen *et al.*, 2009). A given tumor cell line or primary tumor typically expresses one or more NKG2D ligands, although not usually all of them, suggesting a complexity in their regulation. Radiation (Kim *et al.*, 2006; Gannage *et al.*, 2008) and DNA-damaging agents (Fine *et al.*, 2010) have demonstrated their capacity to upregulate NKG2D ligand expression. This regulation is mediated by the major DNA damage checkpoint pathway initiated by ATM (ataxia telangiectasia, mutated) or ATR (ATM- and Rad3-related) protein kinases (Soriani *et al.*, 2009). In this vein, we here report that DTIC is able to enhance expression of NKG2D ligand by mouse (Figure 3) and human (Figure 4) melanoma cell lines, favoring NK-driven killing and NK-derived IFNγ production. To our knowledge, this fact is previously unreported. The expression of NKG2D ligands in cells that do not express them endogenously renders the cells more prone to NK cell–mediated lysis *in vitro*, and reduces the tumorigenicity of tumor cell lines *in vivo* (Cerwenka *et al.*, 2000, 2001; Diefenbach *et al.*, 2000, 2001). Further research is, however, warranted to unravel the molecular mechanisms accounting for the enhanced NKG2D ligand expression on tumor cells following DTIC treatment.

The fact that DTIC treatment was ineffective in nude mice seems surprising, as DTIC is widely used as a cytotoxic drug for the treatment of melanoma. We propose that the NKG2D ligand modulation by the DTIC treatment may be sufficient to trigger an antitumor immune response, which strengthens this therapeutic effect. In previous studies, we also demonstrated that anthracyclines, oxaliplatine, and 5-fluorouracil antitumor effect was also dependent on an immune response (Apetoh *et al.*, 2007; Ghiringhelli *et al.*, 2009; Vincent *et al.*, 2010). It is conceivable that, in melanoma patients, NKG2D ligand upregulation triggers activation of immune effectors and enhances the efficacy of the treatment. Therefore, it could be relevant to check the NKG2D ligand expression in tumor cells before and after chemotherapy and perform a correlation analysis between NKG2D ligand expression and response to chemotherapy to corroborate this hypothesis.

DTIC effect was totally abrogated in NK- and IFNγ-depleted mice, yet featured an early and transient effect in $CD8^+$ T cell–depleted mice (Figure 1c), suggesting a sequential activation of these immune effectors following DTIC treatment. We propose that IFNγ secretion establishes a key link between the activation of innate and adaptive immune effectors. Indeed, enhanced MHC-I expression by IFNγ leads to higher accessibility of endogenous tumor antigens, processed and presented through peptide/MHC complexes on tumor cells, explaining the consequent vulnerability of melanoma cells to CTL. Furthermore, by augmenting the availability of antigens, DTIC enables the survival of low-avidity TCR clonotypes, easing epitope spreading (Palermo *et al.*, 2010), thereby favoring increased anticancer immunity and reduced tumor progression.

Specific stimulation of the host's immune system to induce melanoma tumor rejection has been attempted. Approaches to tumor-specific immunotherapy of melanoma have included chemoimmunotherapy combining IFN-α or IL-2 with

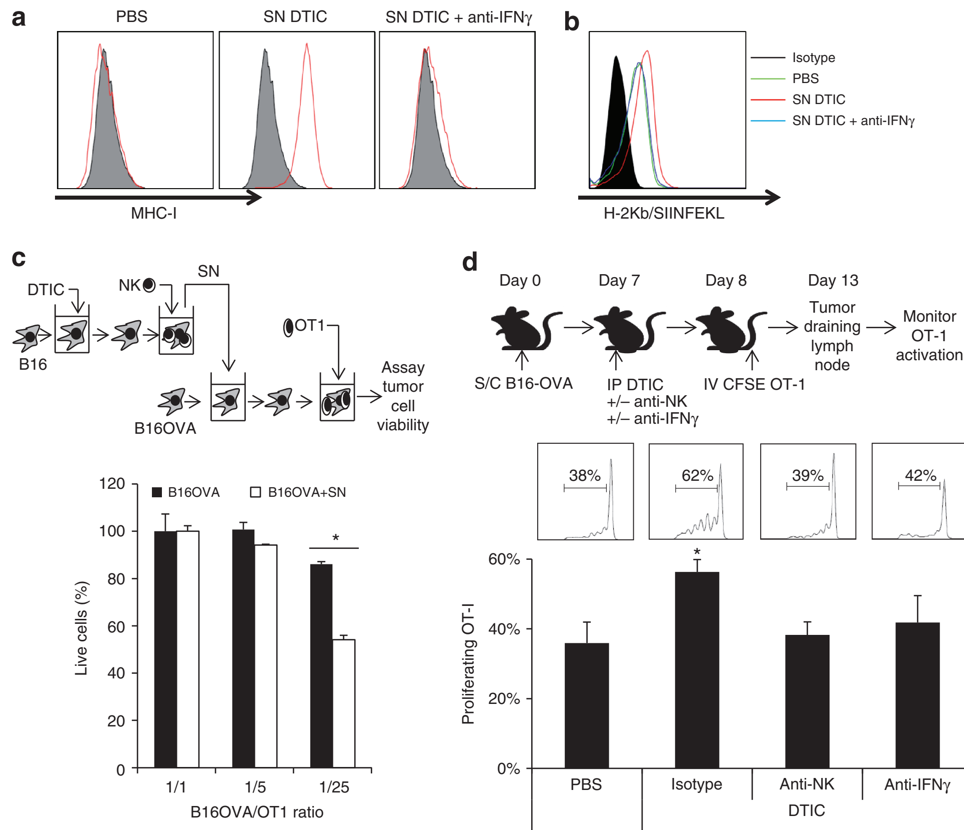

Figure 5. Dacarbazine (DTIC)-enhanced production of IFNγ by natural killer (NK) cells triggers upregulation of major histocompatibility complex-I (MHC-I) and favors tumor cell killing by cytotoxic T lymphocyte (CTL). (**a**) MHC-I (H-2K^b) expression was determined by cytometry on B16F10 cells treated for 24 hours with either phosphate-buffered saline (PBS) or supernatant of coculture of DTIC-treated B16F10 cells with NK cells, in the absence (SN DTIC) or presence (SN DTIC + anti-IFNγ) of an anti-IFNγ neutralizing antibody. The experiments were performed three times, yielding similar results. Filled black lines show isotype-stained cells; red line shows the H-2K^b staining. (**b**) MHC-I-SIINFEKL (H-2K^b/SIINFEKL) complex expression was determined by flow cytometry on B16OVA cells treated as in **a**. The experiments were performed twice, yielding similar results. (**c**) B16OVA cells were treated for 24 hours with either PBS (B16OVA) or the supernatant of coculture of DTIC-treated B16F10 cells with NK cells (B16OVA + SN). Then, tumor cells were cultured for 48 hours with OT-I cells. Cell viability was assessed using the crystal violet test. The experiments were performed twice, yielding similar results. (**d**) B16OVA cells (5×10^5) were subcutaneously (S/C) implanted in mice thigh. Seven days after injection, mice were treated or not treated (PBS) with DTIC and intravenously (IV) injected with carboxyfluorescein succinimidyl ester (CFSE)–labeled OT-I cells the day after. Some mice were depleted with anti-IFNγ or anti-NK1.1 mAb concomitantly to DTIC treatment. OT-I cell activation was assessed by CFSE dilution by flow cytometry. Inset shows representative FACS analysis of CFSE dilution in transferred OT-I. The experiments were performed twice. *$P<0.05$. IP, intraperitoneal.

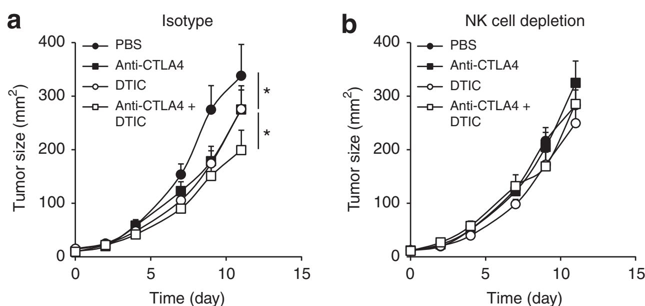

Figure 6. Dacarbazine (DTIC) + cytotoxic T lymphocyte antigen 4 (CTLA4) blockade therapeutic action is dependant of natural killer (NK) cells. Mice were injected with 5×10^5 B16F10 cells and 7 days after tumor cell inoculation mice were treated with phosphate-buffered saline (PBS), DTIC, anti-CTLA4, or a combination of DTIC plus anti-CTLA4 (DTIC + anti-CTLA4) (10 mice per group). (**a**) All mice were injected with isotype control. (**b**) All mice were injected with anti-NK1.1 antibody starting 5 days after tumor inoculation, and then twice a week. The experiments were performed twice. *$P<0.05$.

DTIC therapy, the use of tumor lysates and whole tumor vaccines, tumor-derived peptides, DC-based vaccination protocols, and adoptive transfer of tumor-specific T cells (Kadison and Morton, 2003). These approaches have so far obtained little success in the clinic, are complex and labor-intensive, thereby limiting their potential for widespread use. However, the implementation of a new immunotherapy was recently approved by Food and Drug Administration for the treatment of unresectable or metastatic melanoma. Targeting immunosuppression by the anti-CTLA-4 antibody ipilimumab revealed a clinical benefit after failure of first-line chemotherapy (Hodi *et al.*, 2010). Recently, a new chemoimmunotherapy protocol with the association of ipilimumab and DTIC demonstrated superiority in terms of overall survival compared with DTIC administered alone (Robert *et al.*, 2011). However, it should be pointed out that the clinical advantage is only observed in a small percentage of patients and should

therefore be balanced with the expansive cost of ipilimumab (about 100,800 dollar to treat one patient (Kaplan, 2011)). Therefore, increasing our knowledge on the immune-mediated effects of chemotherapy is an important step to optimize any immune-based regimen in melanoma. In this regard, our results showing that the combination of DTIC with anti-CTLA4 relies on NK cells hint us about the potential clinical relevance of our observation. Indeed, it is conceivable that some tumors may resist the DTIC-induced NKG2D ligand upregulation, and in these patients the immune effects of DTIC would be reduced or absent. Our results also point that CTLA4 blockade might be effective against tumor growth only in the presence of NK cells, which seems surprising and needs further investigations, according to the fact that CTLA4 inhibition is thought to only act through dampening of T-cell inhibition (Robert and Ghiringhelli, 2009; Tarhini and Iqbal, 2010).

To conclude, our results indicate that DTIC antitumor efficacy relies on NKG2D ligand expression by tumor cells, which results in NK cell activation and IFNγ secretion, leading to increased tumor MHC-I presentation and higher CTL efficacy. We speculate that patients who feature upregulation of NKG2D ligands on tumor cells and NK cell activation after one cycle of DTIC may be better responders to immunomodulation and may be candidates in clinical trials that assess the efficacy of T cell–activating agents such as ipilimumab. As such, NKG2D ligand expression might be a predictive biomarker for DTIC treatment response; however, these data must first be validated in a clinical trial.

MATERIALS AND METHODS

Mice and cell lines

B16F10 melanoma cells, syngeneic of C57BL/6, were obtained from the ATCC (Molsheim, France). B16F10 cells expressing OVA (B16OVA) were a kind gift from Laurence Zitvogel. MelC human melanoma cell line was a kind gift from Anne Caignard. Cells were cultured in RPMI 1640 (Lonza, Levallois Perret, France) with 10% fetal bovine serum enriched with 0.4 mM sodium pyruvate, 4 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), penicillin, streptomycin, and amphotericin B. C57BL/6 and Nude mice were purchased from the Centre d'élevage Janvier (Le Genest St Isle, France); OT-I were from Charles River (Saint Germain sur l'Arbresle, France) and were used at 6–10 weeks of age. Animals were all maintained according to the Animal Experimental Ethics Committee Guidelines.

Tumor model

B16F10 cells (5×10^5 in 100 μl PBS) were subcutaneously injected into the flank of mice. Mice were treated 7 days after tumor injection and received a unique intraperitoneal injection of DTIC at 100 mg kg^{-1} body weight.

Reagents

We used DTIC (Déticène; Sanofi Aventis, Paris, France) and doxorubicin (Pfizer, Paris, France). NKG2D Fc was from R&D Systems (Minneapolis, MN). Anti-TRAIL antibody (clone N2B2) was a kind gift from Laurence Zitvogel. The DAPI (4′,6′-diamidino-2-phenylindole), CFSE, concanamycine A, crystal violet, and lipopolysaccharide were bought from Sigma-Aldrich (Saint-Quentin Fallavier, France). ELISA assay for IFNγ was from BD Pharmingen (Le Pont de Claix, France). To investigate the role of IFNγ in the induction of MHC-I expression, a blocking anti-IFNγ antibody (XMG1.2, BioXCell, West Lebanon, NH) was added to the culture medium at 10 μg ml^{-1}. All magnetic separations were performed with Miltenyi Biotec SAS (Paris, France) reagents, according to the manufacturer's protocol. For the *in vivo* depletion or blocking experiments, we used intraperitoneal injections of 150 μg of anti-NK1.1 (clone PK136), anti-IFNγ (clone XMG1.2), anti-CD4 (GK1.5), anti-CD8 (53-6.72), anti-NKG2D (HMG2D), or anti-CTLA4 (9H10), all from BioXCell, twice a week. IL-2 was from Chiron (Suresne, France).

Flow cytometry

Antibodies anti-Rae1 (CX1), anti-MHC-I H2Kb (AF6-88.5), anti-CD11c (HL3), anti-Gr1 (RB6-8/5), anti-CD3 (145-2C11), anti-CD4 (L3T4), and anti-CD49b (DX5) were purchased from BD PharMingen; anti-CD40 (1C10), anti-CD86 (PO3.1), anti-Foxp3 (236A/E7), anti-MHC -II (M5/114.15.2), anti-CD11b (M1/70), anti-H-2Kb/SIINFEKL (25-D1.16), and anti-Mult-1 (5D10) were from eBioscience (Paris, France); and anti-human NKG2D (1D11) and anti-mouse CD69 (H1.2F3) were from Biolegend (San Diego, CA). Anti-mouse RAE-1(186107), anti-MICA(159227), and anti-MICB(236511) were from R&D system. Anti-human IgG was from Beckman Coulter (Roissy, France). All DAPI-positive events were excluded as dead cells. The data were produced using a LSRII cytometer (BD Pharmingen) equipped with FACSDiva (BD Pharmingen) and analyzed with FlowJo (Tristar, Ashland, OR).

Detection of calreticulin expression on tumor cells

B16F10 cancer cells were cultured for 24 hours in complete culture medium supplemented or not supplemented with DTIC (0.4. g l^{-1}), doxorubicin (25 μM), or PBS. Cells were fixed with 1% paraformaldehyde, and then saturated with 200 μl of PBS supplemented with 30% fetal bovine serum before being stained with a rabbit anti-calreticulin antibody (AbCam, Paris, France) for 20 minutes at 4 °C, followed by Cy5-conjugated anti-rabbit antibody (Amersham, GE Healthcare, Velizy-Villacoublay, France).

Quantitative PCR

Total RNA was extracted using TRIZOL (Invitrogen, Villebon sur Yvette, France) as recommended by the manufacturer. The RNAs were rehydrated and measured with a NanoDrop 1000 (Thermo-Scientific, Illkirch, France). Reverse transcription was performed using the kit ''M-MLV Reverse Transcriptase'' (Invitrogen) as recommended by the manufacturer. The samples were then placed in a Biometra (Archamps, France) thermocycler. The quantitative PCR reaction was performed using the kit ''SYBR Green PCR Master Mix'' (Applied Biosystems, Villebon sur Yvette, France) according to the manufacturer's recommendations, on an Applied 7500 device (Applied Biosystems). Relative messenger RNA levels were determined using the ΔCt method. Values were expressed relative to *Actb*. The sequences of the oligonucleotides used in this study are listed in the Table 1.

NK cell cytotoxic assay

B16F10 were cultured for 24 hours in complete culture medium supplemented or not supplemented with DTIC at 0.4 g l^{-1}. After

Table 1. Sequences of the oligonucleotides used in this study for RT-qPCR

Gene	Forward primer	Reverse primer
Rae1	5′-CCCCAGTATCACCCAGCTTACAT-3′	5′-CCCTCCTCTGGCCTCTCCTT-3′
Mult1	5′-AGCTCATGTTGCACTGGAAA-3′	5′-CATCCAAGAGAGGTGGTGGT-3′
Mouse *Actb*	5′-ATGGAGGGGAATACAGCCC-3′	5′-TCTTTGCAGCTCCTTCGTT-3′
MICA	5′-GGACAGCACCGTGAGGTTAT-3′	5′-ACTGCTTGAGCCGCTGAG-3′
MICB	5′-AACCCTGACTGCACAGATCC-3′	5′-GGTCCTGCTGTTTCTGGC-3′
ULBP1	5′-GGCCTTGAACTTCACACCAC-3′	5′-GCGTTCCTTCTGTGCCTC-3′
ULBP2	5′-CGTGGTCCAGGTCTGAACTT-3′	5′-CAAGATCCTTCTGTGCCTCC-3′
ULBP3	5′-CTCTGGACCTCACACCACTG-3′	5′-ACCTGCTATTCGACTGGTCC-3′
Human *ACTB*	5′-GATCATTGCTCCTCCTGAGC-3′	5′-TGCGCAAGTTAGGTTTTGTC-3′

Abbreviation: RT-qPCR, real-time quantitative PCR.

24 hours, B16F10 cells were harvested and incubated with increasing numbers of NK cells and IL-2 (1,000 UI ml^{-1}). In some wells, NKG2D Fc was added at a concentration of 10 μg ml^{-1}. After 48 hours, cells were collected and B16F10 death was assessed using 4′-6-Diamidino-2-phenylindole staining by flow cytometry. NK cells were excluded as CD49b-positive events.

For human experiments, peripheral blood mononuclear cells were isolated from healthy volunteers' blood, and NK cells were separated using the NK cell isolation kit (Miltenyi). NK cells were incubated overnight with melanoma target MelC, previously treated with PBS or DTIC. In some wells, antihuman NKG2D (BD biosciences) was added at a concentration of 10 μg ml^{-1}. Tumor cells were harvested and death was assayed using DAPI staining by flow cytometry or CellTiter assay (Promega, Lyon, France).

Determination of CD8$^+$ T-cell activation and cytotoxicity

For *in vitro* experiments, B16OVA cells were cultured for 24 hours in complete culture medium supplemented or not supplemented with the supernatant of NK cells cocultured with DTIC-treated B16F10 cells. B16OVA cells were then harvested and incubated for 48 hours with increasing ratio of CD8$^+$ T OT-I cells. Cell viability was assessed using the crystal violet test.

For *in vivo* experiments, B16OVA cells (5×10^5 cells or 5×10^4) were subcutaneously injected into the thigh of C57BL/6 mice. Mice were treated or not treated with DTIC 7 days after injection. CD8$^+$ T cells from OT-I mice spleen and lymph nodes were isolated by magnetic selection, labeled by CFSE (10 μmol l^{-1}), and then injected intravenously 8 days after tumor implantation. Some mice were treated with depleting anti-NK1.1 or anti-IFNγ antibodies. Five days after injection of OT-I cells, mice were killed and tumor-draining inguinal lymph node was removed. OT-I cell activation was assessed by CFSE dilution by flow cytometry.

Statistical analysis

Tests used were Student's *t*-test or Mann–Whitney test for the comparison of parametric and nonparametric means. For comparison of more than two groups, analysis of variance with Bonferroni post-test was used. All tests were performed with the GraphPad Prism software (La Jolla, CA). All *P*-values were two-tailed. A *P*-value of <0.05 was considered statistically significant for all experiments and is indicated with *.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Apetoh L, Ghiringhelli F, Tesniere A *et al.* (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 13:1050–9

Apetoh L, Mignot G, Panaretakis T *et al.* (2008) Immunogenicity of anthracyclines: moving towards more personalized medicine. *Trends Mol Med* 14:141–51

Apetoh L, Vegran F, Ladoire S *et al.* (2011) Restoration of antitumor immunity through selective inhibition of myeloid derived suppressor cells by anticancer therapies. *Curr Mol Med* 11:365–72

Casado JG, Pawelec G, Morgado S *et al.* (2009) Expression of adhesion molecules and ligands for activating and costimulatory receptors involved in cell-mediated cytotoxicity in a large panel of human melanoma cell lines. *Cancer Immunol Immunother* 58:1517–26

Cerwenka A, Bakker AB, McClanahan T *et al.* (2000) Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 12:721–7

Cerwenka A, Baron JL, Lanier LL (2001) Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*. *Proc Natl Acad Sci USA* 98:11521–6

Champsaur M, Lanier LL (2010) Effect of NKG2D ligand expression on host immune responses. *Immunol Rev* 235:267–85

Diefenbach A, Jamieson AM, Liu SD *et al.* (2000) Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat Immunol* 1:119–26

Diefenbach A, Jensen ER, Jamieson AM *et al.* (2001) Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413:165–71

Dranoff G (2011) Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nat Rev Immunol* 12:61–6

Fine JH, Chen P, Mesci A *et al.* (2010) Chemotherapy-induced genotoxic stress promotes sensitivity to natural killer cell cytotoxicity by enabling missing-self recognition. *Cancer Res* 70:7102–13

Fregni G, Perier A, Pittari G *et al.* (2011) Unique functional status of natural killer cells in metastatic stage IV melanoma patients and its modulation by chemotherapy. *Clin Cancer Res* 17:2628–37

Gannage M, Buzyn A, Bogiatzi SI *et al.* (2008) Induction of NKG2D ligands by gamma radiation and tumor necrosis factor-alpha may participate in the tissue damage during acute graft-versus-host disease. *Transplantation* 85:911–5

Gasser S, Orsulic S, Brown EJ *et al.* (2005) The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436:1186–90

Gerner RE, Moore GE, Dickey C (1975) Combination chemotherapy in disseminated melanoma and other solid tumors in adults. *Oncology* 31:22–30

Ghiringhelli F, Apetoh L, Tesniere A *et al.* (2009) Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. *Nat Med* 15:1170–8

Ghiringhelli F, Larmonier N, Schmitt E *et al.* (2004) CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34:336–44

Hodi FS, O'Day SJ, McDermott DF *et al.* (2010) Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 363:711–23

Kadison AS, Morton DL (2003) Immunotherapy of malignant melanoma. *Surg Clin North Am* 83:343–70

Kaplan MG (2011) Ipilimumab plus dacarbazine in melanoma. *N Engl J Med* 365:1256–7; author reply 7–8

Kim JY, Son YO, Park SW *et al.* (2006) Increase of NKG2D ligands and sensitivity to NK cell-mediated cytotoxicity of tumor cells by heat shock and ionizing radiation. *Exp Mol Med* 38:474–84

Lugade AA, Sorensen EW, Gerber SA *et al.* (2008) Radiation-induced IFN-gamma production within the tumor microenvironment influences antitumor immunity. *J Immunol* 180:3132–9

Lui P, Cashin R, Machado M *et al.* (2007) Treatments for metastatic melanoma: synthesis of evidence from randomized trials. *Cancer Treat Rev* 33:665–80

Mistry AR, O'Callaghan CA (2007) Regulation of ligands for the activating receptor NKG2D. *Immunology* 121:439–47

Nice TJ, Deng W, Coscoy L *et al.* (2010) Stress-regulated targeting of the NKG2D ligand Mult1 by a membrane-associated RING-CH family E3 ligase. *J Immunol* 185:5369–76

Obeid M, Tesniere A, Ghiringhelli F *et al.* (2007) Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 13:54–61

Palermo B, Del Bello D, Sottini A *et al.* (2010) Dacarbazine treatment before peptide vaccination enlarges T-cell repertoire diversity of melan-a-specific, tumor-reactive CTL in melanoma patients. *Cancer Res* 70:7084–92

Paschen A, Sucker A, Hill B *et al.* (2009) Differential clinical significance of individual NKG2D ligands in melanoma: soluble ULBP2 as an indicator of poor prognosis superior to S100B. *Clin Cancer Res* 15:5208–15

Pende D, Cantoni C, Rivera P *et al.* (2001) Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. *Eur J Immunol* 31:1076–86

Pietra G, Manzini C, Rivara S *et al.* (2012) Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res* 72:1407–15

Robert C, Ghiringhelli F (2009) What is the role of cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma? *Oncologist* 14:848–61

Robert C, Thomas L, Bondarenko I *et al.* (2011) Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 364:2517–26

Salih HR, Holdenrieder S, Steinle A (2008) Soluble NKG2D ligands: prevalence, release, and functional impact. *Front Biosci* 13:3448–56

Soriani A, Zingoni A, Cerboni C *et al.* (2009) ATM-ATR-dependent up-regulation of DNAM-1 and NKG2D ligands on multiple myeloma cells by therapeutic agents results in enhanced NK-cell susceptibility and is associated with a senescent phenotype. *Blood* 113:3503–11

Sutherland CL, Rabinovich B, Chalupny NJ *et al.* (2006) ULBPs, human ligands of the NKG2D receptor, stimulate tumor immunity with enhancement by IL-15. *Blood* 108:1313–9

Suzuki E, Kapoor V, Jassar AS *et al.* (2005) Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. *Clin Cancer Res* 11:6713–21

Tanaka H, Matsushima H, Nishibu A *et al.* (2009) Dual therapeutic efficacy of vinblastine as a unique chemotherapeutic agent capable of inducing dendritic cell maturation. *Cancer Res* 69:6987–94

Tarhini AA, Iqbal F (2010) CTLA-4 blockade: therapeutic potential in cancer treatments. *Onco Targets Ther* 3:15–25

van Elsas A, Hurwitz AA, Allison JP (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190:355–66

Vincent J, Mignot G, Chalmin F *et al.* (2010) 5-Fluorouracil selectively kills tumor-associated myeloid-derived suppressor cells resulting in enhanced T cell-dependent antitumor immunity. *Cancer Res* 70:3052–61

Walker LS, Sansom DM (2011) The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat Rev Immunol* 11:852–63

Zhang T, Sentman CL (2011) Cancer immunotherapy using a bispecific NK receptor fusion protein that engages both T cells and tumor cells. *Cancer Res* 71:2066–76

Zitvogel L, Apetoh L, Ghiringhelli F *et al.* (2008) Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* 8:59–73

Zoller M, Strubel A, Hammerling G *et al.* (1988) Interferon-gamma treatment of B16 melanoma cells: opposing effects for non-adaptive and adaptive immune defense and its reflection by metastatic spread. *Int J Cancer* 41:256–66