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An autophagy-dependent anticancer immune response determines the efficacy of melanoma chemotherapy

Mickaël Michaud[1,2,3,*], Xiaoqi Xie[7], José Manuel Bravo-San Pedro[1,2,3], Laurence Zitvogel[4,5,6], Eileen White[7,8,9], and Guido Kroemer[1,2,3,10,*]

[1]Equipe 11 labellisée par la Ligue Nationale contre le cancer; INSERM U1138; Centre de Recherche des Cordeliers; Paris, France; [2]Metabolomics and Molecular Cell Biology platforms; Gustave Roussy Comprehensive Cancer Center; Villejuif, France; [3]Université Paris Descartes; Sorbonne Paris Cité; Paris, France; [4]INSERM U1015; Villejuif, France; [5]Université Paris Sud-XI; Faculté de Médecine; Le Kremlin Bicêtre, France; [6]Center of Clinical Investigations in Biotherapies of Cancer (CICBT); Villejuif, France; [7]Rutgers Cancer Institute of New Jersey; New Brunswick, NJ USA; [8]Department of Molecular Biology and Biochemistry; Rutgers University; Piscataway, NJ USA; [9]University of Medicine and Dentistry of New Jersey; Robert Wood Johnson Medical School; Piscataway, NJ USA; [10]Pôle de Biologie; Hôpital Européen Georges Pompidou; Paris, France

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There is ample experimental and clinical evidence that chemotherapies are more efficient if they succeed in (re) activating immune surveillance, hence triggering a long-term immune response against residual tumor cells. Most of the preclinical evidence supporting this notion has been obtained with transplantable cancers, for which it has been shown that chemotherapy-induced autophagy in cancer cells is mandatory for the recruitment of myeloid cells into the tumor bed and the subsequent T lymphocyte-mediated reduction in tumor growth. Here, we characterized the chemotherapeutic response of melanomas caused by 4-hydroxy-tamoxifen-induced expression of the Cre recombinase in melanocytes that results in the activation of oncogenic *Braf* together with the inactivation of the tumor suppressor *Pten*, as well as the optional inactivation of the essential autophagy gene *Atg7*. Systemic chemotherapy with the anthracycline Mitoxantrone (MTX) reduced the growth of autophagy-competent melanomas (genotype: $Braf^{Ca/+}$; $Pten^{fl/fl}$; $Atg7^{+/+}$), yet failed to affect the progression of autophagy-deficient melanomas (genotype: $Braf^{Ca/+}$; $Pten^{fl/fl}$; $Atg7^{fl/fl}$). The growth-inhibitory effect of MTX on autophagy-competent melanomas was abolished by the combined depletion of $CD4^+$ or $CD8^+$ T lymphocytes. In conclusion, it appears that the success of chemotherapy against "spontaneous," genetically induced cancers is governed by the same rules as those applicable to transplantable tumors.

Introduction

A selected panel of chemotherapeutic agents is able to induce Immunogenic Cell Death (ICD) of malignant cells, thus converting the tumor into a therapeutic vaccine that elicits an anticancer immune response against residual cancer cells.[1-4] Thus, anthracyclines,[5-7] oxaliplatin,[8,9] cyclophosphamide,[10,11] cardiac glycosides,[12,13] taxanes,[14] vorinostat,[15,16] ionizing radiation[17-19] and photodynamic therapy[20,21] can induce ICD. Apart from its cell biological characteristics (endoplasmic reticulum stress leading to the exposure of calreticulin on the cell surface, premortem autophagy allowing for ATP release during the blebbing phase of apoptosis, secondary necrosis resulting in the exodus of HMGB1 from cells and nuclei),[22-26] ICD is usually monitored by 2 complementary in vivo experiments. First, mouse tumor cells are treated with the ICD inducer in vitro until ~70% of the cells demonstrate signs of apoptosis, washed and injected subcutaneously in the absence of any adjuvant into histocompatible, immunocompentent mice. After one week the mice are challenged by a second injection of live tumor cells into the contralateral flank, and the absence of tumor growth is interpreted as the sign of a protective anticancer immune response elicited by dying tumor cells.[27,28] Second, immunodeficient or immunocompetent mice bearing established cancers are treated with the ICD inducer in vivo. If the treatment is more efficient in the context of an intact immune system than in its absence, the result is interpreted to indicate that the therapeutic effect depends on the active contribution of the immune system.[27,28]

Defects affecting specifically the recognition of ICD-related danger-associated molecular patterns (DAMPs) by the host, as well as broader immune defects (such as the complete absence of dendritic cells or T cells), strongly reduce the efficacy of chemotherapy with ICD inducers.[27,29,30] In addition, specific defects in tumor cells that lead to their incapacity to produce ICD-related DAMPs can cause therapeutic failure. Thus, cancer cells that are unable to mount a premortem autophagic response are unable to secrete ATP[6,23,31,32] and hence have a reduced ability to attract myeloid cells into the tumor bed after chemotherapy.[29,33] As a result, tumors from which essential autophagy genes (such as *Atg5* or *Atg7*) have been deleted, can exhibit a reduced anticancer

*Correspondence to: Guido Kroemer; Email: kroemer@orange.fr; Mickaël Michaud; Email: mickael_michaud@yahoo.fr
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immune response and growth in response to chemotherapy with ICD inducers.[6]

Most of the experiments demonstrating the capacity of anticancer agents to induce ICD have been performed on transplantable cancer cell lines including CT26 colorectal carcinomas, EG7 and EL4 lymphomas, LLC lung adenocarcinomas, MCA205 fibrosarcomas and MC38 colon adenocarcinomas.[27] Driven by the argument that "spontaneous" cancers induced by oncogenes (contrasting with transplantable tumors) would provide a more realistic setting for the evaluation of the immune contribution to chemotherapeutic effects,[34,35] we decided to evaluate the therapeutic effects of the prototypic ICD inducer, mitoxantrone (MTX),[7] on a mouse model of spontaneous *Braf*-driven melanoma. Here we reveal that melanomas only reduce their growth in response to MTX in the presence of a functional cellular immune system. Moreover, we show that melanomas lacking the essential autophagy gene *Atg7* fail to reduce their growth after chemotherapy.

Results and Discussion

Autophagy-deficient BRAF-induced melanomas

C57BL/6 mice bearing a tamoxifen-activable *Cre* recombinase transgene (i.e., a Cre recombinase fused to a G400V/M543A/L544A triple mutation of the human estrogen receptor ligand binding domain, yielding a Cre-ERT2 fusion protein) expressed under the control of the melanocyte-specific *tyrosinase* promoter/enhancer regions (yielding the *Tyr::CreERT2* transgene) were crossed with mice bearing a constitutively active oncogene *Braf* ($Braf^{CA}$) (in which Cre expression lead to oncogenic V600E Braf expression), and mice in which tumor suppressor gene *Pten* is floxed ($PTEN^{fl/fl}$) (and hence can be inactivated by Cre). Optionally, both alleles coding for the *Atg7* gene were also floxed ($Atg7^{fl/fl}$). In this constellation, painting the dorsal skin with 4-hydroxy-tamoxifen results in the nuclear expression of Cre in melanocytes. Cre then causes the activation of $Braf^{CA}$, as well as the inactivation of Pten and optionally that of Atg7 in melanocytes (**Fig. 1**). As the result, the mice developed one or several melanomas in the tamoxifen-painted area that were autophagy-proficient (because they expressed Atg7) or autophagy deficient (because they lacked Atg7). Such tumors usually developed within 3–6 weeks after 4-hydroxy-tamoxifen treatment in 85–95% of the mice, irrespective of the status of the *Atg7* gene (not shown) and proliferated in a linear fashion (**Figs. 2A, 3A**). Moreover, depletion of $CD4^+$ and $CD8^+$ cells from mice that were bearing established melanomas (with an approximate surface of 25 mm^2) failed to affect tumor growth (**Figs. 2B, 3B**), suggesting that T lymphocyte-mediated immunosurveillance does not play any major role in the progression of such tumors, be they autophagy-competent or autophagy-deficient.

Immune-dependent and autophagy-dependent chemotherapeutic responses of BRAF-induced melanomas

To evaluate the chemotherapeutic response, melanoma-bearing mice were biweekly treated by intraperitoneal injections of the prototypic ICD inducer mitoxantrone (MTX), a regime that had no major side effects on control mice (and hence failed to cause a significant weight loss or other manifest alterations in animal behavior). MTX failed to reduce the growth of *Atg7*-deficient melanomas (**Fig. 2A**), and this effect was not altered in conditions in which $CD4^+$ and $CD8^+$ lymphocytes were depleted (**Fig. 2B**). In contrast, MTX did reduce the growth of autophagy-competent (*Atg7* expressing) melanomas, although this effect was partial (**Fig. 3A**). The relative success of this MTX-based chemotherapeutic regime was completely lost upon depletion of $CD4^+$ and $CD8^+$ cells (**Fig. 3B**), in line with idea that the anticancer effects of MTX against autophagy-competent melanomas rely on a cellular immune response.

Concluding Remarks

Here, we provide evidence that chemotherapy of a spontaneous (non-transplanted) model of melanoma involves the active contribution of the cellular immune system. This observation adds to previous reports demonstrating that anthracycline-based chemotherapies of carcinogen-induced skin tumors[36] and transgene (MMTV-Neu) induced HER2-positive breast cancers[37] are abolished upon depletion of $CD8^+$ T cells. Altogether, these data underscore the critical importance of the anticancer immune response for the efficacy of chemotherapies, in line with extensive epidemiological evidence demonstrating that the pre-established immune infiltrate determines the long-term fate of cancer patients[38-40] and influences the reduction of the tumor mass by neoadjuvant chemotherapy, as exemplified by the treatment of locally invasive breast cancer with anthracyclines.[14,41-44]

Beyond these confirmatory aspects, our results reveal for the first time that spontaneous cancers have a reduced ability to respond to MTX if their autophagic machinery has been disabled, presumably because they are unable to elicit natural or chemotherapy-induced immunosurveillance mechanisms.[6,45] Reportedly, autophagy can be deficient in tumor cells,[46-48] and it is hence tempting to speculate that the fraction of cancers that are autophagy-deficient may be refractory to conventional therapies. This possibility requires further preclinical and clinical exploration.

Material and Methods

Chemicals and antibodies

Mitoxantrone and 4-hydroxy-tamoxifen were purchased from Sigma-Aldrich (St Louis, MO USA). Specific antibodies for the elimination of $CD4^+$ and $CD8^+$ lymphocytes, respectively GK1.5 (BE0003-1) and 2.43 (BE0061), were purchased from BioXcell.

Animal experimentation

Mice were maintained in specific pathogen-free conditions, and experiments followed the Federation of European Laboratory

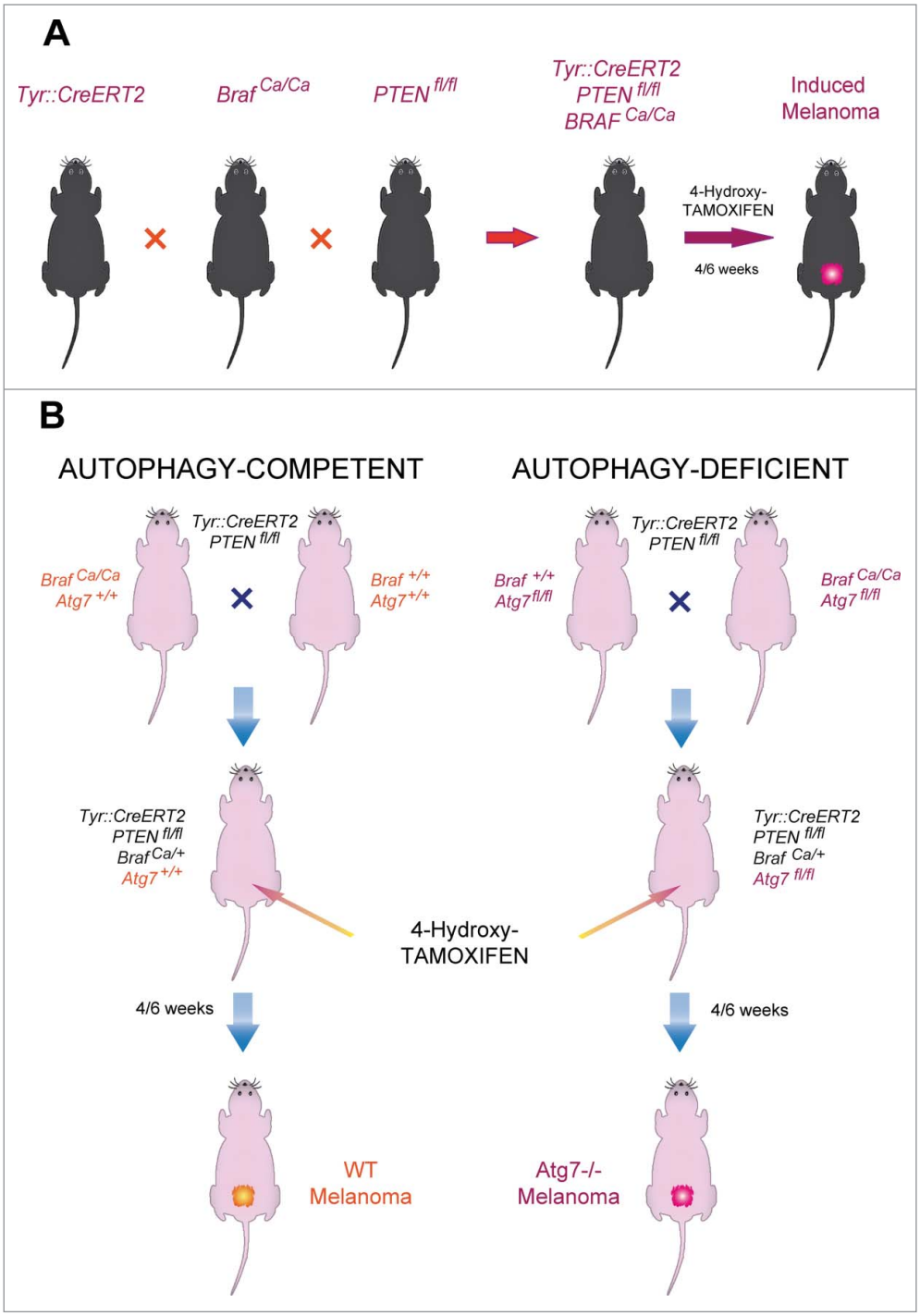

Figure 1. Breeding strategy for the generation of an inducible melanoma mouse model. (**A**) Schematic representation of the breeding. *Tyr::CreERT2* mice were repeatedly crossed with *Pten*$^{fl/fl}$, then *BRaf*$^{CA/CA}$ and optionally *Atg7*$^{fl/fl}$ mice until the floxed loci were homozygous (**A**). Topical tamoxifen administration on the dorsal skin locally induced melanoma in *Tyr::CreERT2;Pten*$^{fl/fl}$*;BRaf*$^{CA/+}$*;Atg7*$^{+/+}$ and *Tyr::CreERT2;Pten*$^{fl/fl}$*;BRaf*$^{CA/+}$*;Atg7*$^{fl/fl}$ mice (**B**).

Animal Science Association (FELASA) guidelines. Animal experiments were approved by the local Ethics Committee (CEEA IRCIV / IGR n°26, registered with the French Ministry of Research) and were in compliance with EU 63/2010 directive. Animals were used between 6 and 30 weeks of age and those bearing tumors exceeding 20–25% of the body mass were euthanatized.

Animal breeding was also performed using Institutional Animal Care and Use approved protocols, I10-064-8 "Use of mice for tumorigenicity," which was approved by IACUC committee

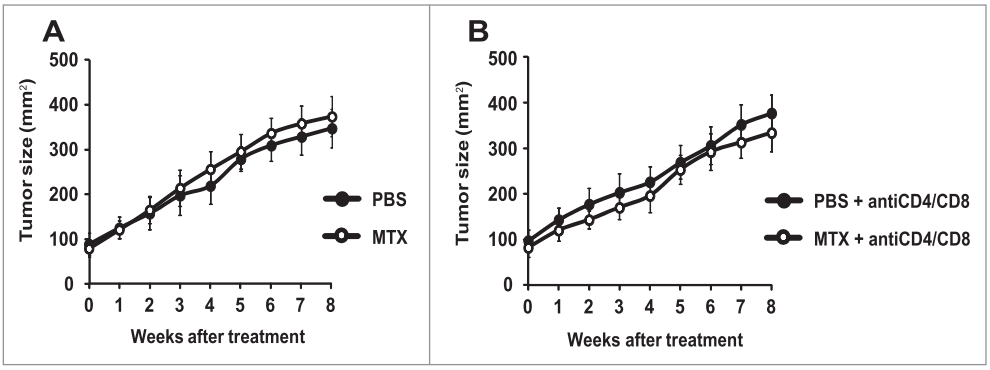

Figure 2. Failure of autophagy-deficient melanomas to respond to chemotherapy. Melanomas were induced by adding tamoxifen on the shaved back of mice with the *Tyr::CreERT2;Pten*$^{fl/fl}$*;BRaf*$^{CA/+}$*;Atg7*$^{fl/fl}$ genotype. Once the tumors had reached a surface of approximately 100 mm^2 (day 0), melanomas were treated by intraperitoneal injections of mitoxantrone (MTX) or vehicle (PBS) every 2 weeks, starting on day 0. Tumor growth was monitored with a caliper for 8 weeks. To assess the impact of the cellular immune system, mice were treated i.p. with PBS (**A**) or a mix of αCD4 and αCD8 antibodies (**B**) at different times days −3, 0, 1, 3, 5, 8, 15, 22, 29, 36, 43, 50). Experiments were done on 15 mice per group. Results are reported as means ± SEM. *$p < 0.05$ (unpaired Student's *t* test).

members at the University of Medicine and Dentistry of New Jersey (UMDNJ).

Mouse breeding and genotyping

Breeding was performed in the IGR or CINJ animal facilities. *Tyr::CreERT2* and *Pten*$^{fl/fl}$ mice were purchased from JAX (JAX012328 and JAX004597, respectively). BRaf$^{CA/CA}$ mice were obtained from McMahon's laboratory.[49] *Atg7*$^{fl/fl}$ mice were provided by Kamatsu's laboratory.[50]

By the age of 3 weeks, pup tails were partially cut for genotyping. DNA was extracted from using the DNAeasy Blood and Tissue Kit (Quiagen). DNA was then amplified by PCR in Micro-Amp 96-well reaction plate (Applied Biosystems), using REDExtract-N-Amp PCR ready Mix (Sigma). The primers used were 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′, 5′-GTG AAA CAG CT TGC TGT CAC TT-3′, 5′-CTA GGC CAC AGA ATT GAA AGA TCT-3′ and 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′ for Tyr-CRE, 5′-AAG CAC TCT GCG AAC TGA G-3′ and 5′-AAG TTT TTG AAG GCA AGA TGC-3′ for PTEN, 5′-TGA GTA TTT TTG TGG CAA CTG C-3′ and 5′-CTC TGC TGG GAA AGC GGC-3′ for BRAF, 5′-TGG CTG CTA CTT CTG CAA TGA TGT-3′ and 5′- CAG GAC AGA GAC CT CAG CTC CAC-3′ for ATG7 (JAX database).[49,50] PCR products were finally loaded on agarose gels and revealed, as previously described (JAX database).

Induction and treatment of melanomas

Mice were shaved on their back. One day later, 1 μl 4-hydroxy-tamoxifen (5 mM in ethanol) was applied and dried on the mice skin. Melanomas appeared 3–6 weeks later. Such melanomas could be directly treated or cut into 1 mm^3 pieces for further transplantation into the flank of syngenic mice. Once

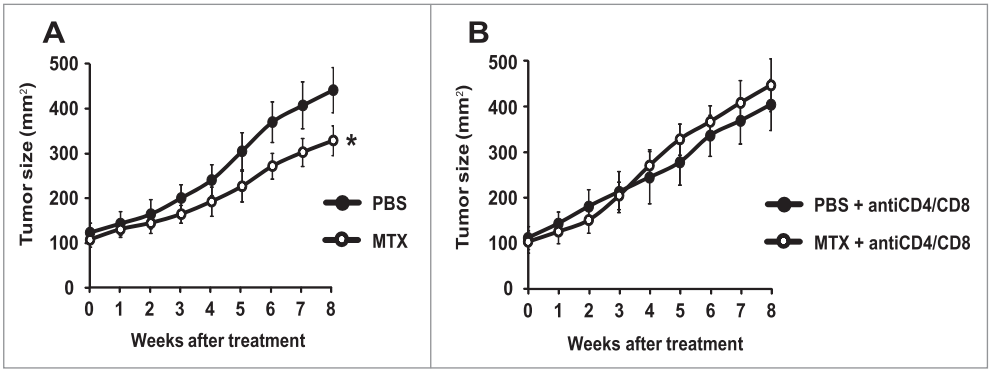

Figure 3. Mitoxantrone reduces the growth of autophagy-competent melanomas in mice with an intact immune system. Once the melanomas from *Tyr::CreERT2;Pten*$^{fl/fl}$*;BRaf*$^{CA/+}$*;Atg7*$^{+/+}$ mice had reached a surface of approximately 100 mm^2 (day 0), they were treated by intraperitoneal injections of MTX or vehicle (PBS) every 2 weeks. Tumor growth was monitored by means of a caliper for 8 weeks. Additionally, to assess the involvement of the immune system, mice were treated i.p. with PBS (**A**) or a mix of αCD4 and αCD8 antibodies (**B**) at different times (days −3, 0, 1, 3, 5, 8, 15, 22, 29, 36, 43, 50). Experiments were done on groups of 15 mice. Results are reported as means ± SEM. *$p < 0.05$ (unpaired Student's *t* test).

melanomas had reached a surface of ~100 mm^2, mice were treated i.p. either with PBS (day 0) or 5.17 mg/kg MTX (in 200 μl sterile water), and this treatment was repeated every 2 weeks. Additionally, mice were treated i.p. by PBS or a mix of anti-CD4/anti-CD8 (200 μL, 0.5 mg/mL) antibodies at different time (days -3, 0, 1, 3, 5, 8, 15, 22, 29, 36, 43, 50). The tumor surface was then monitored with a caliper every week for up to 8 weeks.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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