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ORIGINAL RESEARCH

## TLR4/IFN $\gamma$ pathways induce tumor regression via NOS II-dependent NO and ROS production in murine breast cancer models

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### ABSTRACT

Toll-like receptor (TLR) 4 agonists have emerged as a new group of molecules used for cancer therapy. They have been exploited to enhance the immunogenicity of current chemotherapeutic regimens. However, their effects on cancer cells remain elusive. Here, we showed that a TLR4 agonist, namely a synthetic lipid A analog (ALA), OM-174, exhibits antitumor effects in several mammary tumor mouse models. We also showed that immune components are involved in such effects, as attested to by the failure of ALA to induce tumor regression or an increase of animal survival in mice knocked-out for interferon  $\gamma$  (IFN $\gamma$ ) or TLR4. TLR4 and IFN $\gamma$  receptor (INFR2) expressed by cancer cells are involved in the antitumor efficacy of ALA since this last did not inhibit tumor growth in mice bearing a tumor but lacking TLR4 or IFN $\gamma$  receptor 2 (IFNR2). Mechanistic investigations revealed that nitric oxide (NO), superoxide and peroxynitrite produced by uncoupling of inducible NO synthase (NOS II) in cancer cells are key mediators of ALA and IFN $\gamma$ -mediated tumor growth inhibition. We present here a comprehensive picture of tumor cell death induction, *in vivo* and *in vitro*, by immunotherapy and for the first time the involvement of the TLR4/IFN $\gamma$ /NOS II pathway in immunotherapy was investigated.

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Interferon gamma; lipid A; mammary or breast cancer; nitric oxide; NOS II uncoupling; OM-174; peroxynitrite; ROS

### Introduction

TLRs are a family of evolutionarily conserved pattern recognition receptors. Originally identified as controllers of the dorsal-ventral embryonic polarity of *Drosophila melanogaster*,<sup>1</sup> orthologs have been characterized in humans<sup>2</sup> and implicated in innate immune responses.<sup>3</sup> To date, 10 TLRs have been identified in humans (TLR1-TLR10) and 13 in mice (Tlr1-tlr13).<sup>4,5</sup> These protein receptors are characterized by their ability to respond to invading pathogens by recognizing conserved molecular structures known as microbe-associated molecular patterns (MAMPs), including flagellin, bacterial lipopolysaccharide (LPS), nucleic acids derived from viruses and zymosan of fungi. Moreover, several TLRs recognize the so-called “damage-associated molecular patterns” (DAMPs), including heat shock proteins,<sup>6</sup> and the high-mobility group box 1 (HMGB1),<sup>7</sup> which both operate as TLR2/TLR4 agonists. TLRs are mainly expressed by immune cells including monocytes and macrophages, mast cells and dendritic cells (DCs).<sup>8</sup> Recently, elevated TLR expression was observed in cancer cells and this was linked to oncogenesis and cancer progression.<sup>9</sup> However, TLR and cancer interrelation remains controversial. On the one hand, TLRs appear able to suppress cancer

progression,<sup>10-12</sup> but on the other hand, they have been reported to enhance cancer progression.<sup>13,14</sup> Enhanced TLR expression within tumors has made these molecules attractive therapeutic targets. The most efficacious TLR ligand in cancer therapy is the Bacillus Calmette and Guérin (BCG) TLR2/TLR4 agonist, which was used successfully for the treatment of bladder cancer for more than three decades.<sup>15</sup> During the following 10 y, other TLR agonists besides BCG were the subject of an intense wave of preclinical investigations.<sup>16</sup> The safety in patients and ability to induce therapeutic effects against a variety of tumors was confirmed for some of these, for example, monophosphoryl lipid A (MPL),<sup>17</sup> imiquimod,<sup>18</sup> and OM-174.<sup>19</sup>

Even if the molecular mechanisms of TLR agonist-mediated tumor regression are not well understood, several mechanisms have been characterized. For instance, dying tumor cells that release specific DAMPs elicited by precise chemotherapeutic agents exert immune-stimulator functions by activating TLR4 on DCs. Hence, the cross-priming of antigen-specific T lymphocytes is promoted.<sup>20</sup> However, how tumor cells are killed to provide DCs with antigen has not been determined. It is known that the controlled activation of TLR4 by ALA induces the

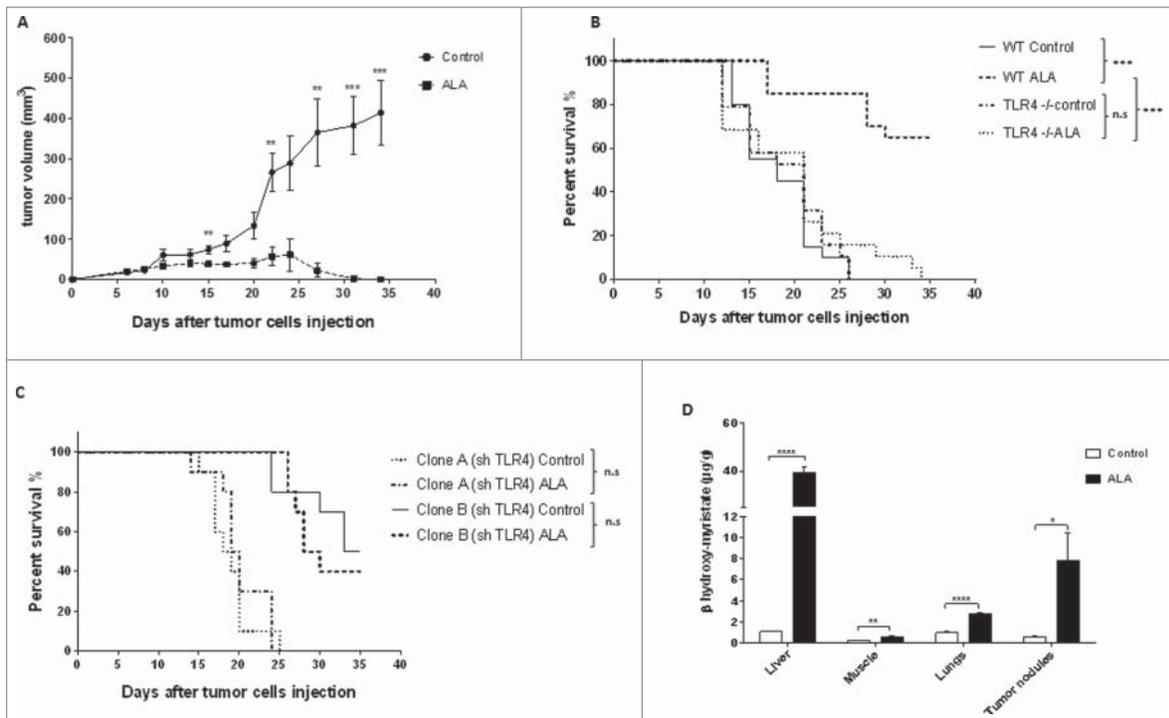
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\*Equal contribution to the manuscript.

#Equal contribution to the manuscript.

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**Figure 1.** ALA-induced tumor growth inhibition depends on both cancer and host TLR4. (A) Tumor growth curve of FvB mice bearing subcutaneous NT2 cells after treatment or not with ALA (8 mg/kg) (8/group). Statistical test: Mann–Whitney *U*-test. \*\**p* < 0.01, \*\*\**p* < 0.001. (B–C) mice survival curves of (B) Balb/c mice bearing EMT-6H cells, in wild-type (wt) mice or mice with the TLR4 gene knocked-out (TLR4<sup>-/-</sup>) after treatment or not with ALA (8 mg/kg) (20/group) or (C) mice bearing EMT-6H cells knocked-down for TLR4 (clone A and B) after treatment or not with ALA (8 mg/kg) (10/group). Survival curves were statistically different (\*\*\**p* < 0.001 Log–Rank (Mantel–Cox) test). (D) Quantification of ALA, as measured by β-hydroxymyristic acid analysis, in different mice tissues 24 h after the 3rd ALA injection. Organs and lung nodules were taken from 27 treated and 12 control mice. Data are representative of at least two experiments. Student’s *t* test \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001.

production of several pro-inflammatory cytokines such as IFN $\gamma$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), as well as NO produced by NO synthases (NOS). These mediators induce apoptosis of chemotherapy-resistant tumor cells,<sup>12,21</sup> or tumor cells sensitized to ionizing radiation<sup>22</sup> and to chemotherapeutic agents such as oxaliplatin.<sup>23</sup> However, the precise mechanisms at the origin of ALA-mediated toxicity remain incompletely understood.

Here, we report the investigation of the antitumor efficacy of an agonist of TLR4, ALA OM-174, in mouse models of mammary cancer. We also studied mechanisms leading to this anti-tumor activity. Our results indicated that ALA inhibited *in vivo* mammary tumor growth. The antitumor efficacy of ALA involved host TLR4 and IFN $\gamma$ , but not NOS II, and TLR4, IFNR2 and NOS II of mammary cancer cells. NOS II-derived NO and ROS, and peroxynitrite produced within tumor cells were found to be key mediators in ALA-induced tumor regression.

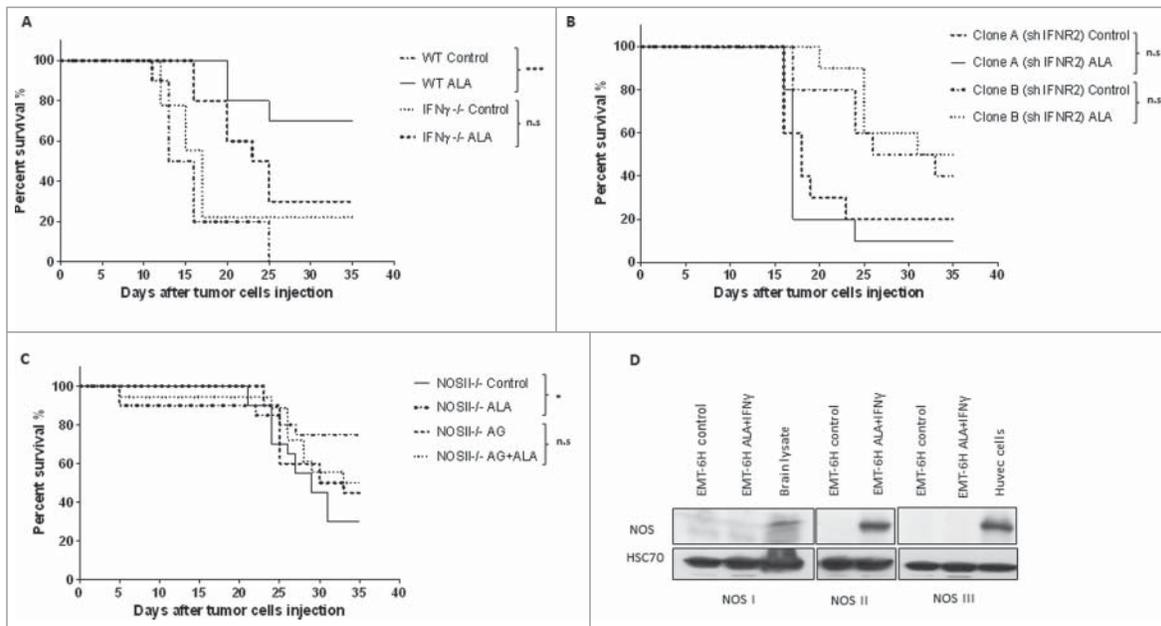
## Results

### The analog lipid A, OM-174 had an *in vivo* antitumor effect, depending on cancer, host TLR4<sup>+</sup> cells and host IFN $\gamma$

The synthetic derivative lipid A OM-174 (ALA) exerts an antitumor effect in different models of cancers.<sup>11,24</sup> However, the molecular mechanisms involved remain unclear. Here, we showed that ALA was also effective in two models of breast

cancer in mice. In NT2 (Fig. 1A) or EMT-6H (Fig. 1B) tumor-bearing mice, ALA induced tumor regression and raised mouse survival, respectively. Seventy percent of EMT-6H tumor-bearing mice were still alive 35 d after *i.v.* tumor cell injection when treated with ALA, while all the mice treated with saline solution died before day 35 (Fig. 1B). Similarly, 100% of mice with *s.c.* NT2 tumors became tumor-free after 35 d upon ALA treatment (Fig. 1A). Mechanistically, ALA was ineffective in TLR4<sup>-/-</sup> mice, since all such mice died before day 35, whether or not they were treated with ALA (Fig. 1B). This indicates that host TLR4-expressing cells are involved in ALA antitumor activity.

To determine whether a direct effect of ALA on tumor cells was involved in its antitumor effect *in vivo*, we down regulated the expression of TLR4 in EMT-6H cells using sh-RNA. This leads to a membrane reduction of 76% and 57% of TLR4 in clone A and B, respectively, as analyzed by flow cytometry. The survival of mice injected with either clone A or clone B, was not significantly different between vehicle and ALA-treated mice (Fig. 1C). We observed an increase in mice survival with clone B, which could be explained by a potential immune-driven effect. We also demonstrated that ALA could reach tumors and interact with cancer cells. The concentration of β-hydroxymyristic acid, a specific component of ALA,<sup>25</sup> was measured in various organs of mice and shown to be higher in tumor nodules and lung tissues of ALA-treated mice than in their non-treated counterparts. In treated mice, β-hydroxymyristic acid was also found in the liver and at very low concentration in muscles (Fig. 1D). Together, all these results suggested



**Figure 2.** ALA-induced tumor growth inhibition depends on both  $\text{IFN}\gamma$  and NOS II. (A) Wild-type (wt) Balb/c mice or mice with the  $\text{IFN}\gamma$  gene knocked-out ( $\text{IFN}\gamma^{-/-}$ ) bearing EMT-6H tumors were treated with ALA (8 mg/kg) or not (control) and mice survival was monitored for 35 d (10/group) (\*\* $p < 0.001$ , Log-Rank (Mantel-Cox) test). (B) Clones A and B of shIFNR2 EMT-6H cells were injected into wild-type mice before treatment or not with ALA (8 mg/kg). Mice survival was monitored for 35 d (10/group). Survival curves were statistically non-significant ( $p > 0.05$  Log-Rank (Mantel-Cox) test). Data are representative of two experiments. (C) Balb/c mice with the  $\text{NOS II}$  gene knocked-out ( $\text{NOS II}^{-/-}$ ) and bearing EMT-6H tumors were treated as in (B), in the presence or absence of the NOS II inhibitor aminoguanidine (AG, 50 mg/kg). Mice survival was monitored for 35 d (20/group). \* $p < 0.05$  Log-Rank (Mantel-Cox) test). (D) Western blot analysis of the protein expression of NOS isoforms in EMT-6H cells treated or not with ALA (500 ng/mL) and  $\text{IFN}\gamma$  (33 ng/mL). Brain lysate and human umbilical vein endothelial cells (HUVEC) were used as positive controls. HSC70 served as a loading control.

that the antitumor effect of ALA required TLR4 expression in tumor cells.

Considering that the activation of immune cell TLR4 induced the production of pro-inflammatory cytokines including  $\text{IFN}\gamma$ , that increased tumor immunogenicity,<sup>22</sup> we investigated whether the antitumor effect of ALA requires  $\text{IFN}\gamma$ .

We tested this hypothesis using  $\text{IFN}\gamma$  knock-out ( $\text{IFN}\gamma^{-/-}$ ) mice. As expected, the ALA-treated wild-type mice (wt ALA) survived longer than mice in the other groups, but there was no difference between  $\text{IFN}\gamma^{-/-}$  mice treated with ALA or not (Fig. 2A). This indicates that the  $\text{IFN}\gamma$  produced by the host was involved in the antitumor effect of ALA. In an attempt to discriminate between a direct effect of  $\text{IFN}\gamma$  on tumor cells and an indirect one, we evaluated the antitumor efficacy of ALA in mice bearing clone A or B tumors in which the expression of the IFNR2 was reduced by sh-RNA. This leads to a membrane reduction of 37% and 38% of  $\text{IFN}\gamma$ R in clone A and B, respectively, as analyzed by flow cytometry. When the two IFNR2 defective clones were injected into mice, no survival difference was found within clone A or clone B groups in ALA treated or untreated mice (Fig. 2B). Suppression of the ALA effect when tumor cells were less receptive to  $\text{IFN}\gamma$  suggested that  $\text{IFN}\gamma$  was necessary for the effectiveness of ALA in this model by acting directly on tumor cells.

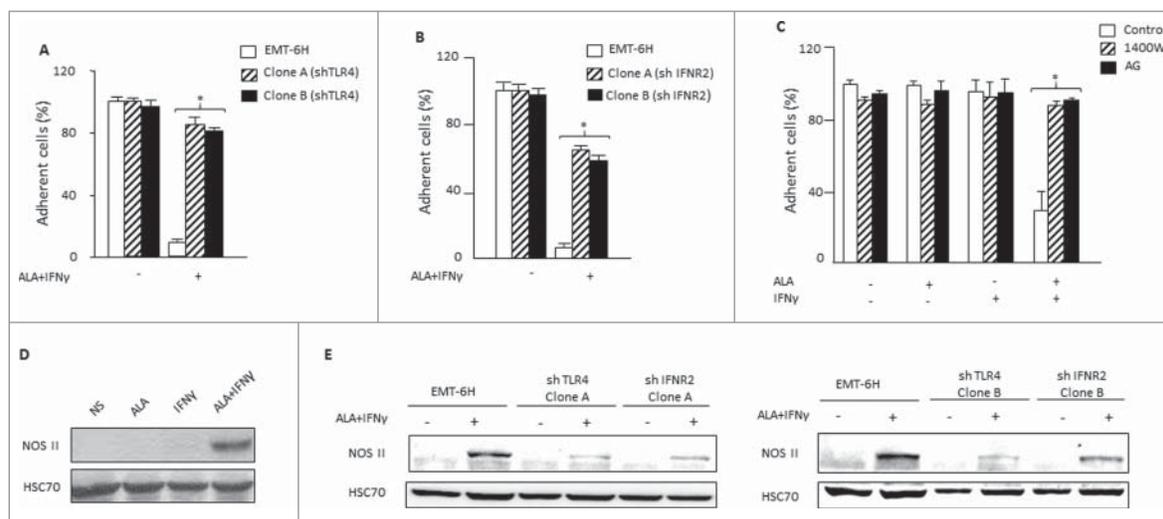
#### Inducible NOS II is involved in the ALA-mediated antitumor effect

We further investigated the molecular mechanisms mediated by TLR4 and  $\text{IFN}\gamma$  at the origin of the antitumor effect of ALA. It is well known that  $\text{IFN}\gamma$  interacts synergistically with LPS to

induce various pro-inflammatory mediators including transcription of the inducible NOS II.<sup>26</sup> To test the involvement of NOS II in the antitumor efficacy of ALA, we analyzed the effect of ALA in mice lacking NOS II. EMT-6H tumor-bearing  $\text{NOS II}^{-/-}$  mice treated with ALA survived significantly longer than  $\text{NOS II}^{-/-}$  control mice (Fig 2C), indicating that ALA efficacy did not involve host cell NOS II. To test whether this enzyme, which was expressed by tumor cells stimulated *in vivo* with ALA and  $\text{IFN}\gamma$ , could explain the antitumor effect,  $\text{NOS II}^{-/-}$  mice with tumors were treated with ALA and aminoguanidine (AG), known to inhibit NOS II of cancer cells that did not express NOS I or NOS III (Fig. 2D). ALA increased the number of mice alive on day 35 in knockout mice ( $\text{NOS II}^{-/-}$  with ALA versus control  $\text{NOS II}^{-/-}$ ) but not in knockout mice treated with AG ( $\text{NOS II}^{-/-}$  with ALA + AG vs. control  $\text{NOS II}^{-/-}$  with AG) (Fig. 2C). These results indicated that NOS II of cancer cells but not host NOS II was indispensable for the ALA-mediated antitumor effect.

#### In vitro induction of cancer cell death by ALA plus $\text{IFN}\gamma$ was dependent on TLR4, $\text{IFN}\gamma$ and NOS II

To further investigate the molecular mechanisms involved in the ALA-inducing antitumor effect, we explored the cytotoxic potential of ALA and  $\text{IFN}\gamma$  *in vitro*. To this end, EMT-6H, shTLR4-EMT-6H and sh $\text{IFN}\gamma$ R2-EMT-6H cells were treated with OM-147,  $\text{IFN}\gamma$  or both these compounds and viable cells (adherent cells) were measured by the methylene blue colorimetric test. ALA alone or  $\text{IFN}\gamma$  alone had no cytotoxic effect on EMT-6H cells (Fig. S1), but the combination of the two compounds had a strong cytotoxic effect on EMT-6H tumor



**Figure 3.** ALA and IFN $\gamma$  induce cell death in EMT-6H cells *in vitro* through TLR4, IFNR and NOS II. (A) Cell viability analysis of clones A and B of TLR4 deficient EMT-6H cells or not after 48 h of cell incubation with ALA (500 ng/mL) and IFN $\gamma$  (33 ng/mL). (B) Cell viability analysis of clones A and B of EMT-6H cells deleted or not for IFNR2 after 48 h treatment with ALA plus IFN $\gamma$  as in (A). (C) Effects of NOS II inhibitors (aminoguanidine [AG; 0.5 mM] and 1400 W; 10  $\mu$ M) on cell viability induced by ALA plus IFN $\gamma$ . Data (A–C) are the mean of at least three independent experiments. Student's t test \* $p < 0.05$ . (D–F) Western blot analysis of NOS II expression induced by ALA and/or IFN $\gamma$  as in (A) in WT EMT-6H cells (D) or those deleted for TLR4 (sh TLR4) or INFR2 (sh IFNR2) (E). Data are representative of three independent experiments. HSC70 served as a loading control.

cells (Fig. 3A, Fig. S1). It should be noted that another pro-inflammatory cytokine, TNF $\alpha$ , failed to synergize with ALA to induce tumor cell death (Fig. S1). However, the ALA plus IFN $\gamma$  combination failed to induce cell death in TLR4-depleted cells (shTLR4-EMT-6H) (Fig. 3A), IFN $\gamma$ R2-depleted cells (shIFN $\gamma$ R2-EMT-6H) (Fig. 3B) or in cells in which NOS II activity was inhibited by two specific inhibitors, AG and 1400W (Fig. 3C). It should be noted that there is some cell death in shINFR2 treated with ALA+IFN $\gamma$ . This could be a consequence of depletion efficacy. Indeed, more than 60% of IFN $\gamma$ R remain expressed in shIFN $\gamma$ R clones as showed before. These results confirmed those obtained *in vivo* that demonstrated the involvement of TLR4, IFN $\gamma$  and NOS II in ALA-induced tumor regression. We also showed that ALA-dependent TLR4 activation and IFN $\gamma$  are at the origin of NOS II induction, as observed *in vivo*. Indeed, treatment of EMT-6H cells with ALA and IFN $\gamma$  induced NOS II expression, as detected by Western blot (Fig. 3D). NOS II expression was significantly inhibited in EMT-6H cells depleted for either TLR4 or IFN $\gamma$ R2 (Fig. 3E) as evaluated by Western blot and NO production attested by nitrite accumulation (Fig. S2). We concluded that ALA plus IFN $\gamma$ -mediated induction of NOS II and NO represents a crucial pathway in mammary cancer cell death.

#### **NO alone was not sufficient to induce the antitumor effect of ALA plus IFN $\gamma$ but required reactive oxygen species (ROS)**

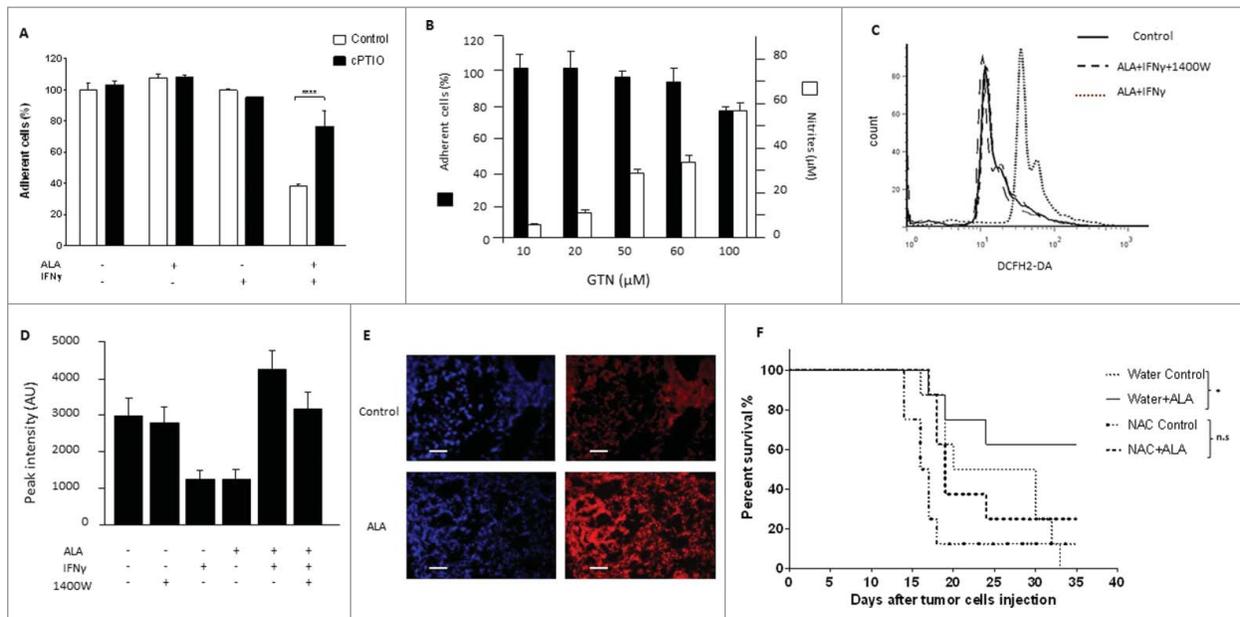
To test the role of NO in ALA and IFN $\gamma$ -induced cell death, EMT-6H cells were pretreated with CPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide), an NO scavenger, before adding ALA + IFN $\gamma$ . Under these conditions, cytotoxicity decreased 3-fold (Fig. 4A). The intracellular NO donor GTN at 50–60  $\mu$ M produced the same amount of nitrite as NOS II, but was not toxic to EMT-6H cells (Fig. 4B).

Furthermore, different NO donors, such as GTN and SNP (sodium nitroprusside), when delivered at concentrations that produced the same amount of nitrite as NOS II did, were not cytotoxic even in the presence of IFN $\gamma$  (Fig. S3). Therefore NO produced by NOS II seemed necessary but not sufficient to induce cytotoxicity, implying that another mediator was required to act with NO.

ROS were candidate cytotoxic agents. As shown in Fig. 4C, the combination of ALA and IFN $\gamma$  induced ROS production in EMT-6H cells as detected by flow cytometry with the DCFH $_2$ -DA ROS-sensitive probe. Addition of the NOS inhibitor 1400 W to the cells abolished the increase in ROS production (Fig. 4C). Superoxide was also detected by electron paramagnetic resonance (EPR) spectroscopy (Fig. 4D). The detection of ROS in cryo-sections of lungs and tumor nodules with DHE (dihydroethidium), a second ROS-sensitive probe, showed an increase of ROS in ALA-treated mice (Fig. 4E). Strikingly, the inhibition of NOS II activity reduced ROS production irrespective of the inhibitor used, whether it was 1400 W (Figs. 4C, D) AG, NMMA (N(G)-monomethyl-L-arginine) or NAME (NG-Nitro-L-arginine methyl ester) (data not shown). Our results suggested that ALA and IFN $\gamma$  together induced NO and ROS production in EMT-6H cells via NOS II activity. To confirm the involvement of ROS in the antitumor effect of ALA, EMT-6H tumor-bearing mice were given N-acetyl cysteine (NAC), a ROS inhibitor, in their drinking water during ALA treatment. Fig. 4F shows a group effect: mice treated with ALA survived significantly longer than mice from other test groups. As NAC reduced the antitumor activity of ALA, ROS production likely has an antitumor effect *in vivo*.

#### **The toxic effect of ALA plus IFN $\gamma$ on EMT-6H cells was due to peroxynitrite**

NO and superoxide are known to react together to give rise to highly cytotoxic peroxynitrite. We thus searched for evidence



**Figure 4.** ALA plus IFN $\gamma$  induce NO and ROS production necessary but not sufficient by themselves to induce tumor cell toxicity. (A) Analysis of EMT-6H cell death after treatment with ALA (500 ng/mL), IFN $\gamma$  (33 ng/mL) or cPTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; 50  $\mu$ M) for 48 h. The data of one experiment representative of five independent ones are shown. Student's t test \*\*\*\* $p$  < 0.0001. (B) Cell death analysis (in black) of EMT-6H cells and NO production (in white) as attested by nitrite accumulation after treatment for 48 h with different concentrations of GTN (Glyceryltrinitrate). (C) EMT-6H cells were treated with ALA (500 ng/mL) and IFN $\gamma$  (33 ng/mL) in the presence or absence of 10  $\mu$ M 1400 W for 24 h. ROS production was detected by flow cytometry after adding the DCFH2-DA probe for 30 min. (D) EMT-6H cells were treated as in (C). Production of superoxide was detected by Electronic paramagnetic resonance after adding CMH for 15 min. Data show the mean light intensity emitted and are representative of two independent experiments. (E) Mice were treated as described in Fig. 1B. Lung tissues were harvested after the 3rd ALA injection and cryo-sections were incubated with DHE (dihydroethidium), which fluoresces red in the presence of ROS. Cell nuclei were stained with DAPI (blue). Scale bars: 20  $\mu$ m. (F) Survival curve of Balb/c mice bearing EMT-6H tumors were given drinking water *ad libitum* containing 3.5 mg/mL N-acetyl cysteine (NAC) or not throughout the treatment with ALA (8/group) (8 mg/kg) (\* $p$  < 0.05 Log-Rank (Mantel-Cox) test). The data from one experiment representative of two are shown.

of peroxynitrite formation in the tumor cells, as detected by luminescence in the presence of luminol, a non-specific compound to measure peroxynitrite. The association of ALA and IFN $\gamma$  had a potent effect on the amount of luminescence produced in EMT-6H cells (Fig. 5A), whereas IFN $\gamma$  alone increased the luminescence only slightly and ALA alone had no effect (not shown). The production of peroxynitrite in tumors from ALA-treated mice was assessed *in vivo* using tumor sections and anti-NO-tyrosine antibodies (Fig. 5B). When 1400 W or FeTPPS (5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride), a catalyst for peroxynitrite degradation,<sup>27</sup> was added to EMT-6H cells treated with ALA and IFN $\gamma$ , less luminescence was detected than in the absence of these former molecules (Fig. 5A) and the cytotoxicity of ALA plus IFN $\gamma$  was inhibited (Fig. 5C).

#### ALA plus IFN $\gamma$ induced uncoupling of NOS II activity to produce NO plus superoxide anion

NADPH oxidases (NOX) are the main enzymes that catalyze the production of superoxide by cells. However, the inhibition of NOX with various inhibitors (apocynin, R6G, oxypurinol) did not decrease ALA plus IFN $\gamma$  cytotoxicity (Fig. 6A), showing that NOX were not involved in the ALA plus IFN $\gamma$  antitumor effect.

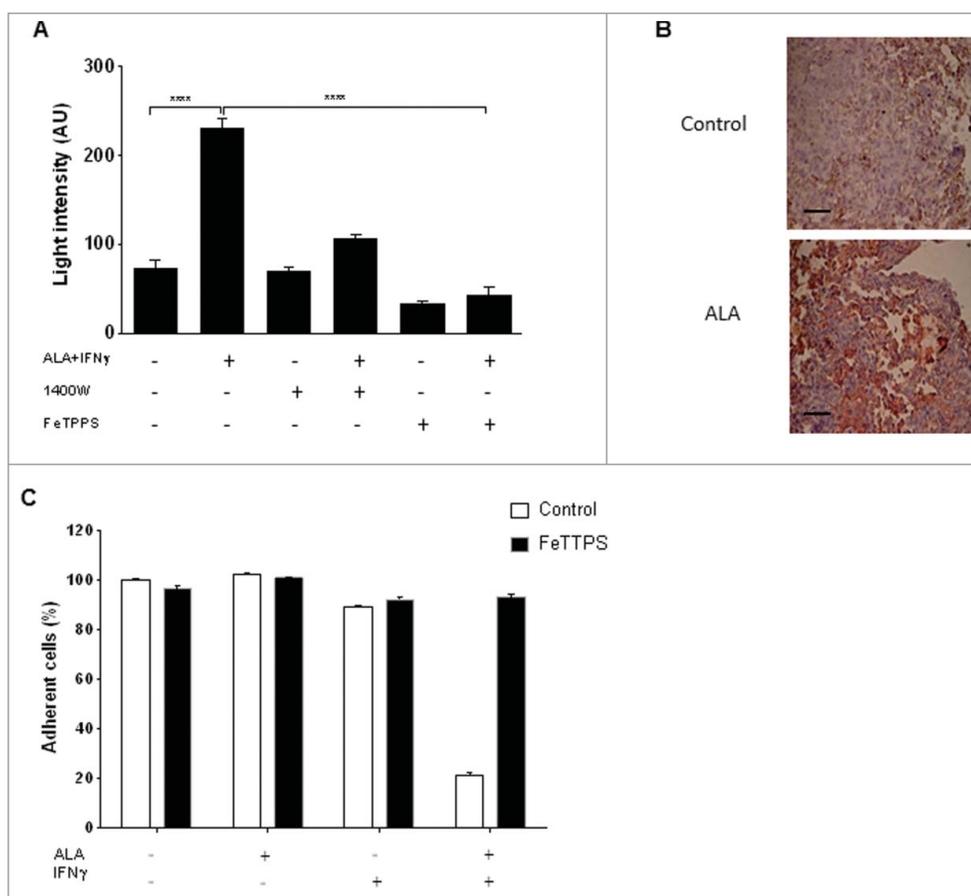
EMT-6H cells did not produce nitrites when treated with ALA plus IFN $\gamma$  and NOS II inhibitors such as AG and 1400 W (Fig. 6B). Interestingly, 1400 W (Figs. 4C, D), like AG (data not shown), also suppressed ROS and superoxide accumulation in tumor cells. These data led us to examine whether NOS II itself

was both an NO and ROS producer, in a so-called “uncoupling process.” NOS II uncoupling is regulated by the availability of its substrate, L-arginine (L-Arg) and the co-factor tetrahydrobiopterin (H<sub>4</sub>B). Modifying the amounts of H<sub>4</sub>B and L-Arg, particularly decreasing the L-Arg concentration, can lead to the generation of superoxide anions and hydrogen peroxide by NOS II.<sup>28</sup> We therefore measured the concentrations of H<sub>4</sub>B and L-Arg in EMT-6H cells treated with ALA and IFN $\gamma$ . The amount of H<sub>4</sub>B in treated EMT-6H cells was not significantly different from that in control cells (data not shown). In contrast, the amounts of L-Arg decreased as much as 3-fold in treated cells compared to untreated cells (Fig. 6C). We thus concluded that a reduced availability of L-Arg caused NOS II uncoupling in EMT-6H cells treated with ALA and IFN $\gamma$  and that under these conditions NOS II of EMT-6H cells produced both NO and ROS.

Altogether, our results clearly demonstrated for the first time that the antitumor effect of two immune-stimulators in a model of mammary cancer are mediated by NOS II via its capacity to produce both NO and ROS in cancer cells, a so-called “NOS uncoupling process,” infrequently observed in physiology.

#### Discussion

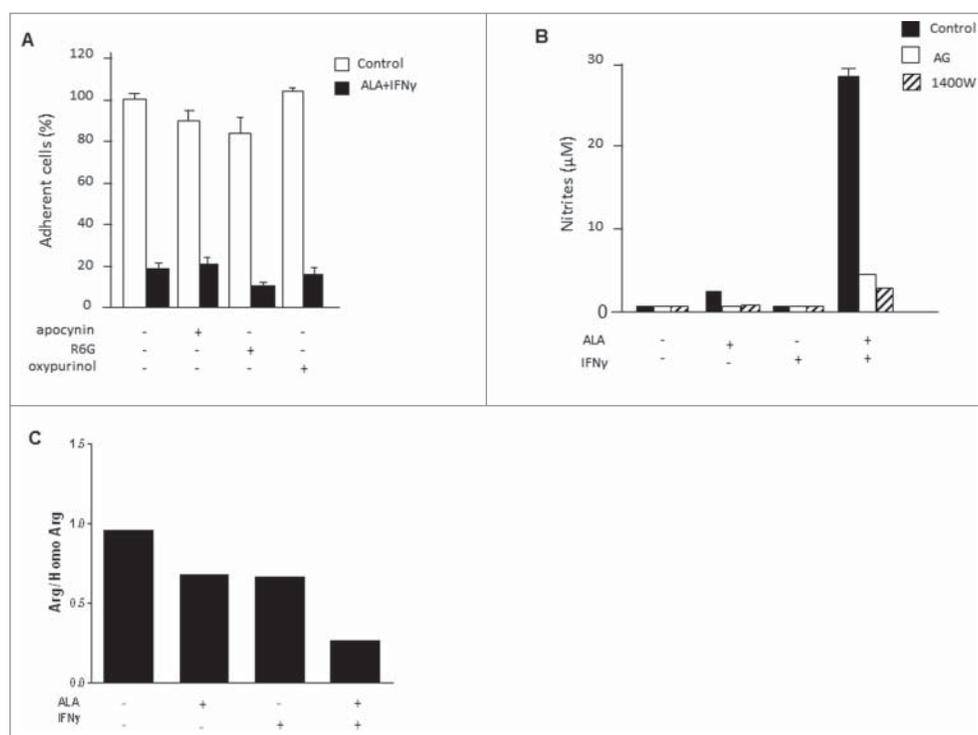
Cancer immunotherapy was revealed in the late 19th century, when Coley observed that bacterial extracts exhibit antitumor activity. Subsequent work demonstrated that several TLR agonists induce anticancer responses in different types of cancer.<sup>29</sup> Here, in two different mammary cancer models we showed that treatment with a TLR4 agonist, a synthetic lipid A OM-



**Figure 5.** The toxic effect of ALA plus IFN $\gamma$  on EMT-6H cells is due to peroxynitrite. (A) EMT-6H cells were treated with or without ALA (500 ng/mL) plus IFN $\gamma$  (33 ng/mL) and 10  $\mu$ M 1400 W or FeTTPS (5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride; 150  $\mu$ M), for 48 h. Peroxynitrite production was detected by adding luminol to cells and immediately measuring luminescence. Student's t test \*\*\*\* $p$  < 0.0001. (B) Lung tissues were taken after the 3rd injection of ALA or saline solution as Fig. 4E. The sections were stained with an anti-NO-tyrosine antibody (red). Scale bars: 20  $\mu$ m. (C) EMT-6H cells were treated with or without ALA, IFN $\gamma$  or FeTTPS for 48 h and cell viability was measured as described in Materials and Methods.

174 (ALA), controlled tumor growth. This effect involved TLR4 and IFN $\gamma$  of immune cells and TLR4 and NOS II of cancer cells. Although TLR4 signaling in immune cells is critical for the reduction of tumor growth,<sup>20</sup> the role it plays in cancer cells remains controversial. Stimulation of TLR4 expression on tumor cell surfaces has been demonstrated to have a positive role in tumorigenesis,<sup>30,31</sup> but other data have shown that the TLR4 pathway activation on tumor cells contributes positively to inhibition of tumor growth.<sup>32,33</sup> These discrepancies are not fully understood. Our results indicated that the simple activation of TLR4 in cancer cells failed to induce cell death, but its combination with IFN $\gamma$  triggered cell demise and tumor growth inhibition. This suggested that the sensitivity of cancer cells to TLR4-mediated cell death needs the presence of a second signal; in the case examined activation of the IFN $\gamma$  receptor by IFN $\gamma$ . Previously, along with Ridder et al., we reported that this cytokine could be produced by immune cells when they were activated by OM-174.<sup>22,34</sup> Nunez et al.<sup>33</sup> evoked such a scenario. They demonstrated that a TLR-activated melanoma produces IFN $\beta$ , which contributes positively to the antitumor immune response. We anticipate that systematic application of the TLR4 ligand will engage TLR4 to produce IFN $\gamma$  on both host immune cells and cancer cells, where IFN $\gamma$  cooperates with activated TLR4 to induce cell death and tumor growth inhibition. Based on our findings, we hypothesize that those

tumors such as IFN $\gamma$ -secreting immune cell infiltration, i.e. with a certain immune environment, are sensitive, in terms of cell death, to treatment with exogenous or endogenous TLR4 analogs such as lipid A and HMGB1 or HSP60, respectively. In the current study, we also showed, both *in vitro* and *in vivo*, that NO and ROS produced by tumor cell NOS II, but not by the host enzyme, mediate the cytotoxic effect of the ALA-IFN $\gamma$  combination. The capacity of NOS II to produce both NO and ROS rarely occurs except when substrate and cofactor availability are reduced, referred to as NOS uncoupling.<sup>35</sup> In our investigation, we showed that ALA plus IFN $\gamma$  induced a decrease in the intracellular L-Arginine concentration, which could explain NOS II uncoupling. Alternatively, the drop in intracellular arginine could be due to NOS II-dependent consumption and to some enzymes involved in the arginine recycling or degradation pathways; such as arginine decarboxylase, arginine:glycine amidinotransferase, or arginase, respectively.<sup>36</sup> We did not find appreciable changes in arginase activity between control and ALA plus IFN $\gamma$ -treated cells (data not shown). Proving an involvement of the arginine recycling pathway enzymes in arginine depletion needs further investigation. Considering the key role of arginine in survival and proliferation of various malignancies, L-arginine depletion has been considered a promising approach for the treatment of these diseases. Accordingly, recent clinical trials have been performed or are ongoing to



**Figure 6.** NOS II uncoupling was observed when EMT-6H cells were activated with ALA plus IFN $\gamma$ . (A) EMT-6H cells were treated with or without NOX inhibitors: 300  $\mu$ M apocynin, 0.3  $\mu$ M rhodamine 6G, or 150  $\mu$ M oxypurinol, for 1 h before adding ALA (500 ng/mL) plus IFN $\gamma$  (33 ng/mL) for 48 h. Cell viability was detected as described in Materials and methods. Data from one representative of three independent experiments in triplicate are shown. (B) EMT-6H cells were treated for 1 h with 0.5 mM AG or 10  $\mu$ M 1400 W, then for 48 h with or without ALA and IFN $\gamma$ . Nitrite accumulation was measured by the Griess assay. (C) EMT-6H cells were treated with or without ALA or IFN $\gamma$ . L-arginine was measured in cell extracts by HPLC using homo-L-arginine as the internal standard. Data from one experiment representative of two are shown.

examine the use of arginine deprivation as a treatment for advanced malignancies.<sup>37</sup> Our findings have also uncovered a new tool for depleting arginine using lipid A and IFN $\gamma$ . Further studies are needed to confirm the impact of arginine depletion in lipid A plus IFN $\gamma$ -mediated cancer cell death.

Collectively, our data imply that combining the activation of TLR4 and IFN $\gamma$  pathways in cancer cells can trigger an anticancer response in mammary cancer cells. Mechanistically, our results highlight the major role that NOS II plays in cancer cell death. Whatever the methods, any therapeutic strategy that permits the activation of TLR4 and IFN $\gamma$  in a tumor microenvironment should be deleterious for cancer cell survival.

## Materials and methods

### Cells, animals and treatment

Murine mammary EMT-6H cells were obtained after cloning of the EMT-6 cell line<sup>34</sup> and the mammary NT2 cancer cell line was purchased from the ATCC. Balb/c mice, Fvb mice, IFN $\gamma$ , TLR4, and NOS II knockout mice were purchased from Charles River Laboratory International. All experiments involving animals were performed in accordance with institutional guidelines and approved by the University of Burgundy's Ethics Committee.

On day 0, EMT-6H cells ( $10^5$  cells) were injected intravenously (*i.v.*) into syngenic Balb/c mice giving rise to lung tumor nodules that killed the mice within 5 weeks. NT2 cells ( $5 \times 10^5$  cells) were injected subcutaneously (*s.c.*) into syngenic Fvb mice on day 0. For mice bearing EMT-6H tumors, treatment

consisted of five *i.v.* injections of ALA OM-174 (OM PHARMA) at 5 d intervals from day 5 until day 25 at the dose of 8 mg/kg. For mice bearing NT2 tumors, treatment consisted of three *i.v.* injections of 8 mg/kg ALA at day 8 and every week thereafter. AG, an inhibitor of NOS II, was injected *i.p.* at the dose of 50 mg per kg, two times per week, whereas N-acetyl cysteine (NAC) (3.5 mg/mL), a ROS inhibitor, was given orally, in drinking water. On day 35, all surviving mice were euthanized and autopsied.

### Measurement of live cell density

EMT-6H cells ( $10^4$  cells) were seeded into a 96-well plate for 24 h, treated with or without 500 ng/mL ALA, 33 ng/mL IFN $\gamma$ , or 10  $\mu$ M Glyceroltrinitrate (GTN) and incubated for a further 48 h. The density of live cells was determined by a methylene blue colorimetric test.

### Determination of NO and ROS production

NO production was estimated using the Griess microassay to determine nitrite accumulation in the cell culture medium. The intracellular ROS production was measured using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH<sub>2</sub>-DA) probe. Tumor cells were incubated in the dark with the DCFH<sub>2</sub>-DA probe. Fluorescence was measured with a FACScan flow cytometer.

## Lipid A quantitation

Organs from mice were collected and immediately stored in liquid nitrogen. Samples were ground up and the amounts of ALA were determined by direct quantitation of  $\beta$ -hydroxymyristic acid in the extracts by gas chromatography-mass spectrometry (GC-MS).<sup>38</sup>

## Statistics

Unless otherwise stated in the figure legends data from one experiment representative of three experiments are shown. *In vitro* data are presented as the mean  $\pm$  SEM and paired groups were compared with the Student's *t* test. Tumor sizes between two groups were compared using Mann-Whitney *U*-test. Survival analysis was assessed by Mante-Cox log-rank test;  $p < 0.05$  was considered significant compared with controls.

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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## Author contributions

Myriam Lamrani, Nejia Sassi, Jean-Luc Boucher, Jérôme Labbé, Cédric Seigneur, Cindy Racœur, Anne Athias, Catherine Vergely conceived and carried out experiments and analyzed data, Romain Guerreiro analyzed data, Luc Rochette conceived experiments and discussed data. Catherine Paul conceived and carried out experiments, analyzed and discussed data and edited the manuscript; Ali Bettaieb conceived experiments, analyzed and discussed data and edited the manuscript; Jean-François Jeannin conceived experiments, analyzed and discussed data, coordinated the experiments, prepared and edited the manuscript.

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