Autophagy-Dependent Anticancer Immune Responses Induced by Chemotherapeutic Agents in Mice

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Science **334**, 1573 (2011);
DOI: 10.1126/science.1208347

Our results offer an explanation for how long-term memories can be stably embedded into networks as quiescent and overlapping Hebbian assemblies. Unlike previous studies, our network does not exhibit the behavior of an attractor network, in which activated cell assemblies will compete with each other and the winning pattern often exhibits persistent elevated activity. Instead, the network remains quiet unless the balance of one or more assemblies is modulated in favor of the excitation and returns to the background state when the modulation is turned off. We have shown this effect here by driving a subset of cells with an external stimulus, but there are several conceivable methods to modulate the balance of excitation and inhibition (SOM). The possibility to activate several patterns simultaneously allows the analog combination of patterns into larger composite memories. The capacity of storable and retrievable patterns is likely to depend on complex interactions between dynamics, size, and connectivity of the assemblies and the host network, as well as several other parameters.

We show that a simple, Hebbian plasticity rule on inhibitory synapses leads to robust and self-organized balance of excitation and inhibition that requires virtually no fine-tuning (figs. S6 to S9) and captures an unexpected number of recent experimental findings. The precision of the learned balance depends on the degree of correlation between the excitatory and the inhibitory inputs to the cell, ranging from a global balance in the absence of correlated inputs to a detailed balance for strong correlations. The phenomenon is robust to the shape of the learning rule, as long as it obeys two fundamental requirements: Postsynaptic activity must potentiate activated inhibitory synapses, whereas in the absence of postsynaptic firing inhibitory synapses must decay. Because the balance is self-organized, inhibitory plasticity will most likely maintain balance also in the presence of excitatory plasticity, as long as excitation changes more slowly than inhibition or when excitatory plasticity events are rare.

The mammalian brain hosts a wide variety of inhibitory cell types with different synaptic time scales, response patterns, and morphological target regions. Presumably, these cell types serve different functions, and consequently their synapses may obey several different plasticity rules (*31*). In our simplified model, the dynamics of inhibitory plasticity powerfully contributes to the functional state of cortical architectures and may have a strong impact on cortical coding schemes.

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Acknowledgments: Research was supported by Swiss National Science Foundation grant no. 200020 13287 (Coding Characteristics) and CRSIK0 122697 (Sinergia). Additionally, T.P.V. was supported by the European Community's Seventh Framework Marie Curie International Reintegration grant no. 268436, and H.S. and F.Z. by the European Community's Seventh Framework Program under grant agreement no. 243914 (BRAIN-I-NETS) and 237955 (FACETS-ITN), respectively. C.C. received additional support from a French National Science grant ANR-08-SYSC-005. Thanks to G. Hennequin and A. Woodruff for helpful discussions.

Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1211095/DC1
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13 July 2011; accepted 20 October 2011
Published online 10 November 2011;
10.1126/science.1211095

Autophagy-Dependent Anticancer Immune Responses Induced by Chemotherapeutic Agents in Mice

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Antineoplastic chemotherapies are particularly efficient when they elicit immunogenic cell death, thus provoking an anticancer immune response. Here we demonstrate that autophagy, which is often disabled in cancer, is dispensable for chemotherapy-induced cell death but required for its immunogenicity. In response to chemotherapy, autophagy-competent, but not autophagy-deficient, cancers attracted dendritic cells and T lymphocytes into the tumor bed. Suppression of autophagy inhibited the release of adenosine triphosphate (ATP) from dying tumor cells. Conversely, inhibition of extracellular ATP-degrading enzymes increased pericellular ATP in autophagy-deficient tumors, reestablished the recruitment of immune cells, and restored chemotherapeutic responses but only in immunocompetent hosts. Thus, autophagy is essential for the immunogenic release of ATP from dying cells, and increased extracellular ATP concentrations improve the efficacy of antineoplastic chemotherapies when autophagy is disabled.

Transplantable or primary murine cancers respond to chemotherapy with anthracyclines or oxaliplatin much more efficiently when they grow in syngenic immunocompetent mice than in immunodeficient hosts (*1*, *2*). Similarly, clinical studies indicate that severe lymphopenia negatively affects the chemotherapeutic response of solid cancers (*3*), and immune defects are negative predictors of the response to chemotherapy with anthracyclines or oxaliplatin (*2*, *4*, *5*). Apparently, some successful chemotherapeutics can induce a type of tumor cell stress and death that is immunogenic (*6*–*8*), implying that the patient's dying cancer cells serve as a therapeutic vaccine that stimulates an antitumor immune response, which in turn can control residual cancer cells (*9*, *10*). Immunogenic cell death is characterized by the preapoptotic exposure of calreticulin (CRT) on the cell surface (*11*), postapoptotic release of the chromatin-binding protein high mobility group B1 (HMGB1) (*2*), and secretion of adenosine triphosphate (ATP) (*4*).

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CRT, HMGB1, and ATP interact with the receptors CD91, toll-like receptor 4 (TLR4), and purinergic P2RX7 receptors, respectively, which are present on the surface of dendritic cells (DCs). CD91, TLR4, and P2RX7 are present on DCs and promote engulfment of dying cells (*12*, *13*), presentation of tumor antigens (*2*, *14*), and production of interleukin-1β (IL-1 β), respectively (*4*, *15*).

CRT exposure results from an endoplasmic reticulum (ER) stress response induced by chemotherapy (*16*), whereas HMGB1 release occurs when cells undergo postapoptotic necrosis (*2*). The mechanism accounting for chemotherapy-elicited ATP release has been elusive. Because autophagy may be connected to the ER stress response, as well as to xenophagy (*17*), an innate immune response, we wondered whether autophagy might participate in immunogenic signaling in the context of anticancer chemotherapies. To investigate this possibility, we studied the emission of immunogenic signals by autophagy-deficient ($Atg5^{KD}$ or $Atg7^{KD}$) CT26 tumor cells. Mass spectrometric analyses of supernatants from mitoxantrone (MTX)-treated CT26 colorectal carcinoma cells revealed that, as compared to their autophagy-competent counterparts, autophagy-deficient CT26 cells released lower amounts of ATP (Fig. 1A). Accordingly, autophagy-competent tumor cells released more ATP when treated with MTX or oxaliplatin than did cells rendered

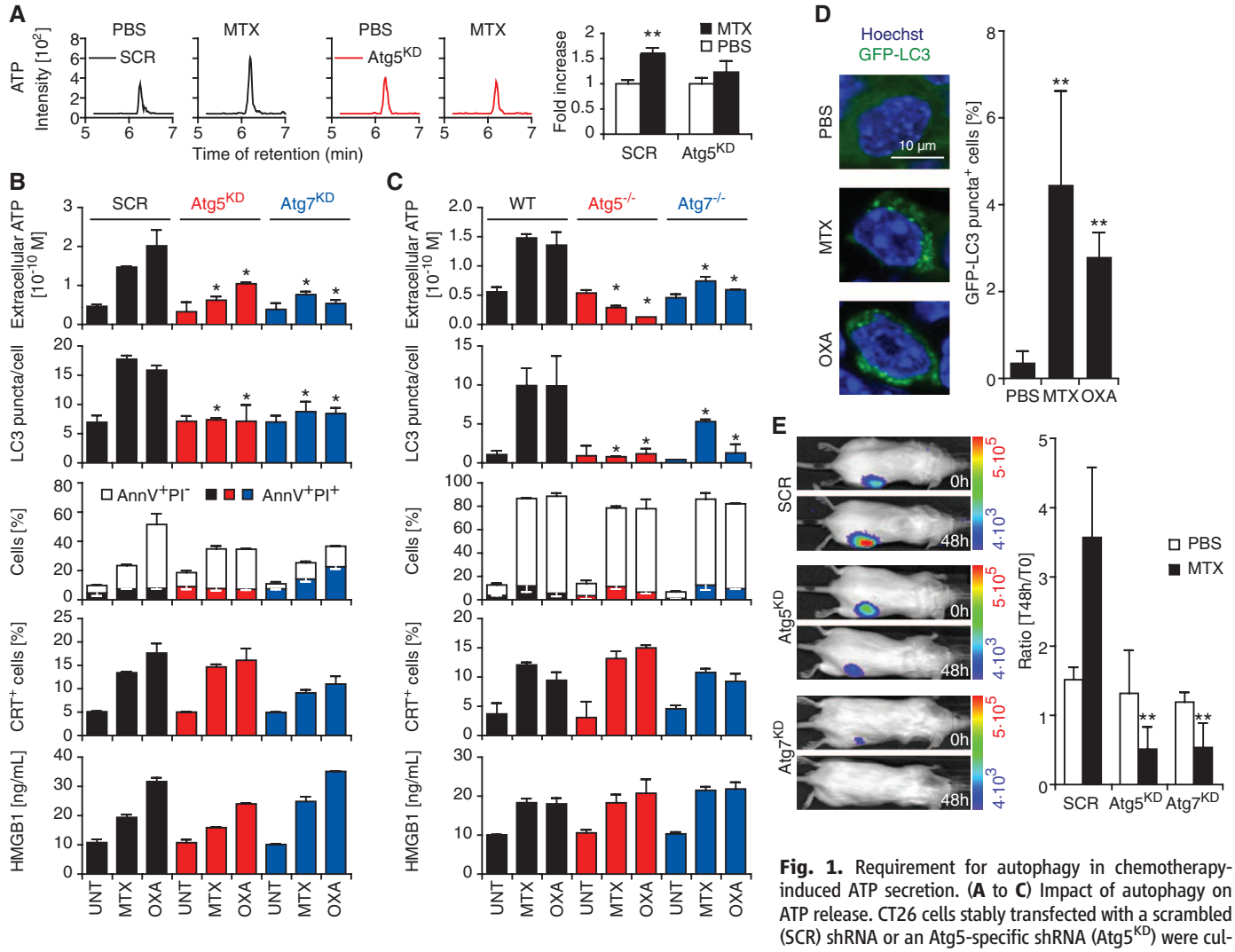

Fig. 1. Requirement for autophagy in chemotherapy-induced ATP secretion. (**A** to **C**) Impact of autophagy on ATP release. CT26 cells stably transfected with a scrambled (SCR) shRNA or an Atg5-specific shRNA ($Atg5^{KD}$) were cultured with MTX or left untreated (PBS, phosphate-buffered saline), and extracellular ATP was determined by mass spectrometry (A). Alternatively, ATP release induced by MTX or oxaliplatin (OXA) in $Atg5^{KD}$ and $Atg7^{KD}$ CT26 cells (B) or *Atg5*$^{-/-}$ and *Atg7*$^{-/-}$ MEFs (C) was measured by enzymatic methods, and autophagy, cell death, CRT exposure, and HMGB1 release were determined. Results are reported as means ± SEM of triplicates, and asterisks indicate significant ($P < 0.05$, unpaired Student's *t* test) differences as compared to autophagy-competent cells cultured in similar conditions. (**D**) Induction of autophagy by chemotherapy in vivo. Subcutaneous GFP-LC3–expressing CT26-derived tumors were treated by intraperitoneal chemotherapy, and the percentage of tumor cells with cytoplasmic GFP-LC3 dots was determined 48 hours later (means of 10 determinations ± SEM). Asterisks indicate significant ($P < 0.01$, unpaired Student's *t* test) autophagy induction as compared to control conditions (PBS). (**E**) Extracellular ATP concentration before and after chemotherapy in vivo. Mice bearing palpable luciferase-expressing CT26-derived tumors that had been engineered to express a control shRNA (SCR) or shRNAs targeting Atg5 or Atg7 were treated with PBS or MTX, and D-luciferin–dependent chemoluminescence was measured before treatment (0 hour) or 48 hours later. Results are reported as means ± SEM of triplicates. Asterisks indicate significant (*$P < 0.01$, unpaired Student's *t* test) inhibition of ATP release as compared to autophagy-competent controls.

autophagy-deficient by the knockdown (KD) or the knockout (KO) of an array of essential autophagy proteins including Atg5 and Atg7 (Fig. 1, B and C, and figs. S1 to S4). CT26 or MCA205 fibrosarcoma cells underwent apoptosis in response to MTX or oxaliplatin in vitro irrespective of their autophagic competence. Moreover, autophagy-deficient and autophagy-competent cancer cells similarly exposed CRT and released HMGB1 in response to chemotherapy (Fig. 1B and figs. S1 and S2). *Atg5*$^{-/-}$ *or Atg7*$^{-/-}$ mouse embryonic fibroblasts (MEFs) also failed to release ATP, yet exposed CRT and released HMGB1, in response to anticancer agents (Fig. 1C and fig. S3). In human osteosarcoma U2OS cells stably expressing a green fluorescent protein (GFP)–LC3 fusion protein, the depletion of several *Atg* gene products (ATG5, ATG7, ATG10, Beclin 1) significantly decreased the MTX-driven accumulation of autophagosomes containing GFP-LC3 as well as ATP release, yet failed to reduce the release of HMGB1 or the exposure of CRT at the plasma membrane surface (fig. S4). Thus, autophagy is required for optimal ATP release but dispensable for the emission of other immunogenic signals.

Pharmacological inhibition of autophagy with bafilomycin A1 or 3-hydroxychloroquine limited the chemotherapy-induced release of ATP, as did the inhibition of caspases with Z-VAD-fmk (fig. S5). However, pharmacological induction of autophagy with rapamycin (fig. S5) or other agents (not shown) failed to trigger ATP release. CT26 tumors showed the autophagic aggregation of a GFP-LC3 reporter fusion protein 48 hours after chemotherapy in vivo, indicating that MTX can induce autophagy in vivo (Fig. 1D). Moreover, CT26 cancers engineered to express the ATP sensor luciferase on the cell surface (*18*) exhibited a significant increase in ATP-dependent luminescence 48 hours after systemic chemotherapy with MTX, which was absent in their autophagy-deficient (Atg5^{KD} or Atg7^{KD}) counterparts (Fig. 1E). Together, these results indicate that, in the context of apoptosis induction, autophagy is required for chemotherapeutics to induce ATP release.

Autophagy-competent CT26 tumor cells [class I major histocompatibility complex (MHC) haplotype H-2^{d}] treated with MTX in vitro and then subcutaneously injected in the absence of any adjuvant elicited a protective anticancer immune response and vaccinated syngenic BALB/c mice against further inoculation with live tumor cells of the same type (Fig. 2A). CT26 cells that were rendered autophagy-incompetent by the depletion of Atg5 or Atg7 with small interfering RNA (siRNA, fig. S6) or a short hairpin RNA (shRNA, figs. S2 and S7) mounted apoptotic responses to MTX similar to those of autophagy-competent control cells, in vitro (Fig. 1B and fig. S2), yet largely failed to induce antitumor immunity in vivo, in prophylactic vaccination experiments (Fig. 2A and fig. S7). These results were confirmed with MCA205 cells (class I MHC haplotype H-2^{b}) in syngenic C57BL/6 mice and ultraviolet treatment (fig. S8). When combined with MTX, bafilomycin A1 and 3-hydroxychloroquine also reduced the immunogenic potential of tumor cells (fig. S8). Moreover, autophagy-deficient cells failed to prime T cells in vivo, as determined by experiments in which dying cells were inoculated into footpads, and T cells were recovered from draining lymph nodes 6 days later, restimulated with tumor antigens, and assessed for secretion of interferon-γ (IFN-γ) (Fig. 2B). The failure of

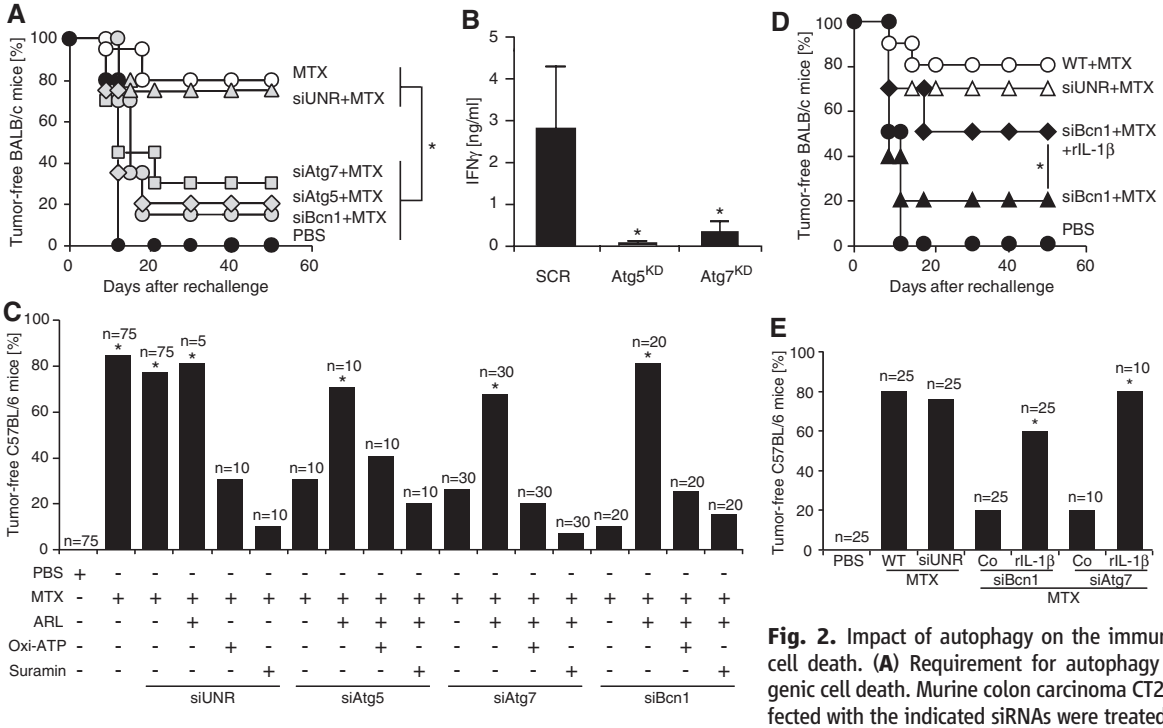

Fig. 2. Impact of autophagy on the immunogenicity of cell death. (**A**) Requirement for autophagy in immunogenic cell death. Murine colon carcinoma CT26 cells transfected with the indicated siRNAs were treated with MTX in vitro and then subcutaneously injected into BALB/c mice (20 mice per group) that were inoculated with living CT26 cells 7 days later. (**B**) Requirement for autophagy in T cell priming. MTX-treated autophagy-competent (SCR) or deficient (Atg5^{KD} or Atg7^{KD}) CT26 cells were injected into the footpad, followed by restimulation of popliteal lymph node cells by CT26 lysates in vitro and quantification of IFN-γ secretion. Results are reported as means ± SEM of triplicates. (**C**) Implication of ATP and purinergic receptors in the reduced immunogenicity of autophagy-deficient cells. Murine fibrosarcoma MCA205 cells were transfected with the indicated siRNAs and then treated with MTX, followed by subcutaneous injection in the presence or absence of the ecto-ATPase inhibitor ARL67156 (ARL) and/or purinergic receptor antagonists (oxidized ATP or suramin). One week later, mice were rechallenged with live MCA205 cells, and the absence of tumor growth was scored 60 days later as the indication of an anticancer immune response. (**D** and **E**) Recombinant IL-1β restores the defective immunogenicity of autophagy-deficient cells succumbing to chemotherapy. CT26 (D) or MCA205 cells (E) were transfected with the indicated siRNAs, cultured with MTX, and subcutaneously injected, either alone or in the presence of recombinant IL-1β (rIL-1β), into syngenic mice. Seven days later, mice were subcutaneously inoculated with the same tumor cell type. Tumor incidence was monitored for 60 days. Kaplan-Meier curves were statistically evaluated by the logrank test (*P < 0.05, as compared to the same siRNA-transfected MTX-treated cells without rIL-1β). [*P < 0.05, unpaired Student's t test in (B); logrank test in (A) and (D); χ^2 test in (C) and (E) to indicate significant vaccination; n = 10 unless otherwise indicated.]

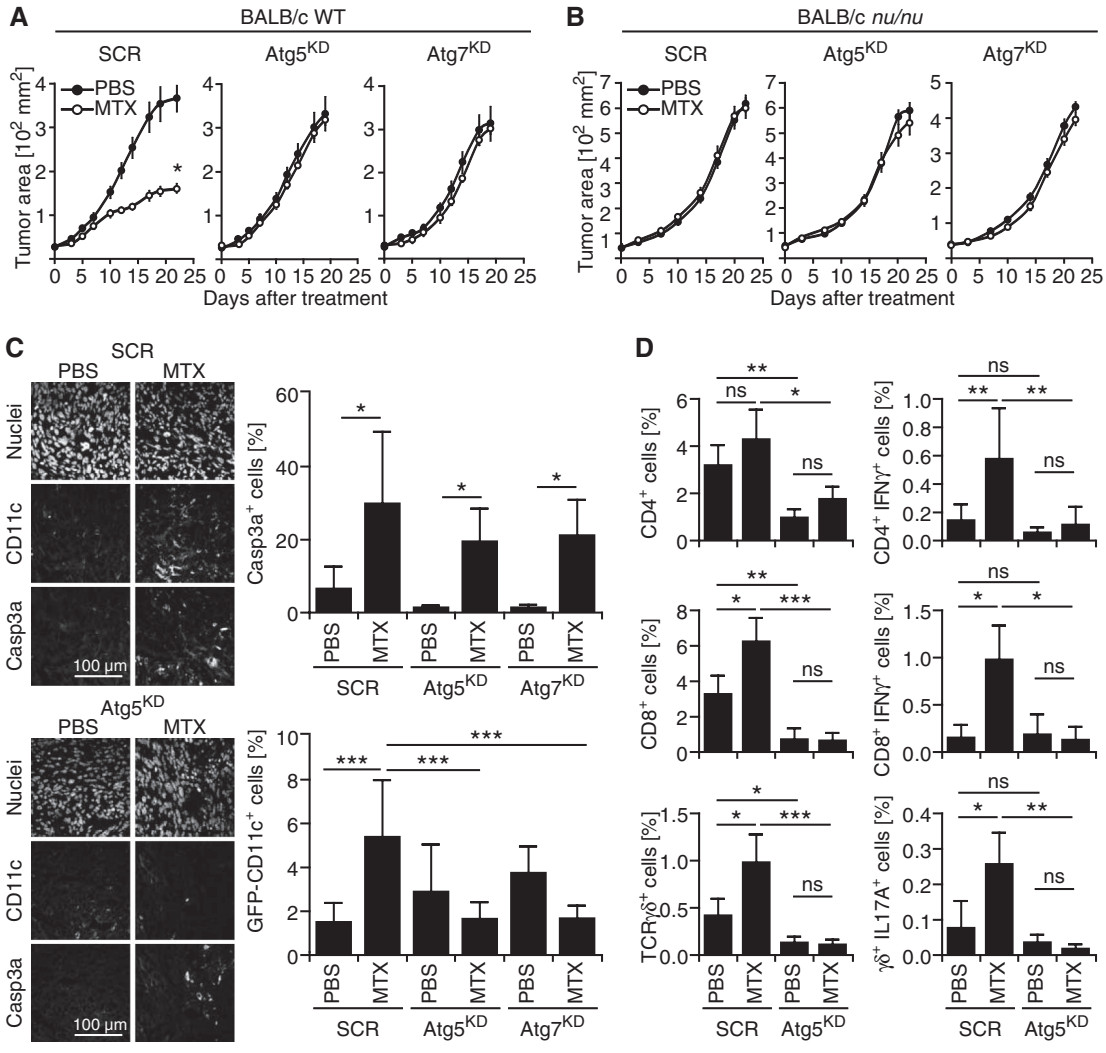

Fig. 3. Failure of autophagy-deficient tumor cells to induce a therapeutic immune response. (**A** and **B**). Reduced T cell–dependent therapeutic response of autophagy-incompetent tumors. Palpable CT26-derived tumors stably transfected with the indicated constucts growing on immunocompetent BALB/c mice (A) or immunodeficient *nu/nu* hosts (B) (n = 10 per group) were treated with one intraperitoneal injection of MTX or vehicle (PBS) (day 0), and tumor growth was monitored. (**C**) Failure of autophagy-deficient tumors to recruit DCs upon chemotherapy. Control (SCR) or autophagy-deficient ($Atg5^{KD}$) tumors were implanted on transgenic mice expressing GFP under the control of the CD11c promoter. Once tumors became palpable, mice were treated with MTX or vehicle (PBS), and 48 hours later tumors were subjected to immunofluorescence detection of apoptotic cells (that stain positively for active caspase-3, Casp3a) and GFP-positive cells. (**D**) Failure of autophagy-deficient tumors to recruit T lymphocytes. Control (SCR) or autophagy-deficient ($Atg5^{KD}$) CT26-derived tumors were recovered 7 days after MTX or PBS injection, and the frequency of cells with the indicated immunophenotypes was determined by cytofluorometry. Results are reported as means ± SEM (*P < 0.05, **P < 0.01, ***P < 0.001, unpaired Student's t test; ns, not significant).

MTX-treated cancer cells depleted from Atg5, Atg7, or Beclin 1 to induce a protective immune response in vivo could be corrected by co-injecting ARL67156 (ARL), an inhibitor of ecto-ATPases (apyrases) that artificially increases extracellular ATP concentrations (*19*). Such an immunogenic activity of ARL was neutralized by the addition of the purinergic receptor antagonists oxidized (oxi)-ATP and suramin (Fig. 2C). Moreover, the immunogenicity of MTX-treated autophagy-deficient cells could be restored by co-injecting recombinant IL-1β (Fig. 2, D and E), whose production depends on the action of ATP on purinergic receptors (*4*, *15*), but not by co-injecting recombinant CRT (fig. S9).

CT26 and MCA205 cells that were rendered autophagy-deficient by depletion of Atg5 or Atg7 normally underwent apoptosis-associated phosphatidylserine exposure and cell death in vitro, in response to MTX or oxaliplatin (Fig. 1B and figs. S2 and S6). Moreover, the proliferation of autophagy-competent and autophagy-deficient tumors growing in immunodeficient mice was inhibited to similar extents by the intratumoral injection of high doses of MTX (fig. S10). By contrast, systemic MTX treatment only limited the growth of autophagy-competent tumors when such tumors were growing in normal, immunocompetent mice (Fig. 3A and fig. S10). This therapeutic response to systemic MTX was lost by all tumors, irrespective of their autophagic competence, when they were grown in immunodeficient, athymic (*nu/nu*) mice (Fig. 3B). Although autophagy-deficient tumor cells (fig. S11) exhibited an apoptotic response to systemic MTX in vivo (and hence stained positively for activated caspase-3, and accumulated apoptotic as well as HMGB1-negative nuclei) (Fig. 3C and fig. S12), they failed to attract DCs that were rendered visible by transgenic expression of GFP under the control of the CD11c promoter (Fig. 3C). In addition, autophagy-deficient tumor cells failed to elicit a local immune response characterized by the recruitment of $CD4^+$ or $CD8^+$ T cells, which produce IFN-γ, and of γδ T cells, which secrete IL-17 (*20*) (Fig. 3D). Thus, autophagy deficits abolish the capacity of cancer cells to elicit an immune response.

Intratumoral injections of ARL performed in the context of systemic MTX treatment enhanced pericellular ATP concentrations around autophagy-deficient tumor cells (as assessed by tumor cells engineered to express surface luciferase) (Fig. 4A), enhanced the recruitment of DCs (as shown in CD11c-GFP transgenic mice) (Fig. 4B), stimulated the recruitment of IFN-γ–producing $CD4^+$ or $CD8^+$ αβ T cells and of IL-17A–producing γ/δ T lymphocytes into the tumor bed (Fig. 4, C to E, and fig. S13), and restored T cell priming by autophagy-deficient tumor cells (Fig. 4F). Although ARL did not increase the cytotoxic potential of MTX, ARL injections restored the efficacy of MTX-based chemotherapy against autophagy-deficient CT26- or MCA205-derived cancers, hence significantly reducing tumor growth (fig. S14). Similarly, autophagy-deficient MCA205 fibrosarcomas failed to respond to oxaliplatin in vivo, unless chemotherapy was accompanied by ARL injections (fig. S15). Intratumoral ARL

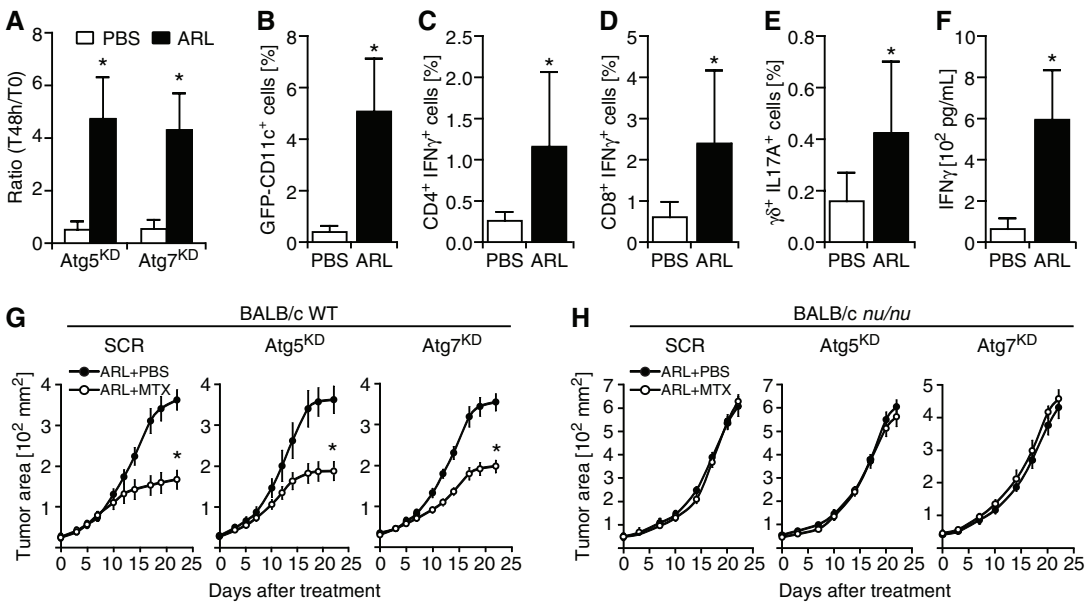

Fig. 4. Restoration of therapeutic immune responses by injection of ecto-ATPase inhibitors into autophagy-deficient tumors. (**A**) Restoration of ATP levels. Extracellular ATP was measured in Atg5-deficient ($Atg5^{KD}$) and Atg7-deficient ($Atg7^{KD}$) CT26 cell-derived tumors (n = 3 per group) engineered to express luciferase, 48 hours after the intraperitoneal injection of MTX in combination with the intratumoral injection of PBS or of the ecto-ATPase inhibitor ARL67156 (ARL). (**B** to **E**) Restoration of DC and lymphocyte recruitment by ARL. $Atg5^{KD}$ tumors were treated with MTX as they became palpable and injected with either PBS or ARL. Then, the percentage of infiltrating dendritic or T cells among all cells was determined on day 9, as in Fig. 3, C and D. Results are means ± SEM (n = 10 mice per group). (**F**) ARL-mediated restoration of T cell priming by Atg5-deficient tumor cells, determined as in Fig. 2B (n = 5 per group). (**G** and **H**) Effect of ecto-ATPase inhibition on immune-dependent chemotherapy responses in vivo. Autophagy-competent or -deficient CT26-derived tumors (n = 10 per group) growing on immunocompetent BALB/c (G) or athymic *nu/nu* mice (H) were treated with MTX or PBS (day 0) alone or in combination with intratumoral ARL (on days 0 and 3), followed by tumor growth monitoring. Results are reported as means ± SEM (*P < 0.05, unpaired Student's t test).

administration also improved the defective chemotherapeutic response of tumors engineered to overexpress the ecto-ATPase CD39 (fig. S16), but did not enhance the response of normal CT26- or MCA205-derived cancers to chemotherapy with MTX or oxaliplatin (Fig. 4G and figs. S13 to S15), presumably because in this latter context extracellular ATP concentrations are already optimal. Another apyrase inhibitor, NGXT191, recapitulated the effects of ARL in that it ameliorated the growth-inhibitory effect of MTX on autophagy-deficient cancers (fig. S16). The ARL- or NGXT191-mediated amelioration of the therapeutic response to MTX or oxaliplatin was clearly T cell–dependent, as it was lost in mice lacking T lymphocytes (Fig. 4H and figs. S14 to S16). Together, these results, which have been obtained with immunogenic cancers, indicate that restoring extracellular ATP concentrations suffices to reestablish the chemotherapeutic response of autophagy-deficient tumors.

The activation of several oncogene products (in particular phosphatidylinositol 3-kinase, PI3K; the protein kinase AKT1; and antiapoptotic proteins from the Bcl-2 family) and the inactivation of multiple tumor suppressor proteins (such as DAPK1, PTEN, TSC1, TSC2, LKB1/STK11, and proapoptotic "BH3-only" proteins from the BCL-2 family) result in disabled autophagy (*21*). Moreover, Beclin 1, which is required for autophagy induction, acts as a haploinsufficient tumor suppressor protein, and additional essential mediators of autophagy (such as ATG4C, UVRAG, and BIF-1) are bona fide tumor suppressors (*22*–*24*). Failing autophagy contributes to oncogenesis through several potential mechanisms, such as increased genomic instability (*25*), increased nuclear factor κB signaling (*26*), reduced senescence (*27*), or increased survival in conditions of oncogenic stress (for instance, upon the activation of oncogenic *RAS*) (*28*). As a result, autophagy is often disabled in (pre-)malignant cells, especially during early oncogenesis. By contrast, tumor progression is frequently coupled to enhanced autophagy, likewise as an adaptation to stressful conditions (*22*–*24*). This implies that tumors are highly heterogeneous with regard to their autophagic potential (SOM Text). If our findings could be translated to humans, patients with autophagy-deficient cancers might profit from pharmacological strategies designed to compensate defective immunogenic signaling. Beyond the development of cancer-specific autophagy inducers (*29*), one such compensatory strategy might be to enhance local ATP concentrations through the application of apyrase inhibitors.

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Acknowledgments: G.K. and L.Z. are supported by the Ligue Nationale contre le Cancer (Equipes labelisées), Agence Nationale pour la Recherche (ANR), European Commission (Active p53, Apo-Sys, ChemoRes, ApopTrain INFLACare), Fondation pour la Recherche Médicale (FRM), Fondation Bettencourt-Schueller Institut National du Cancer (INCa), and Cancéropôle Ile-de-France and the LabEx OncoImmunology. G.M. is supported by Association pour la Recherche sur le Cancer. F.d.V. and P.P. are supported by Italian Association for Cancer Research (AIRC). M.M. is supported by FRM. I.M., S.A., M.T. are supported by La Ligue Nationale contre le Cancer and Y.M. by China Scholarship Council. A.Q.S. is supported by Higher Education Commission of Pakistan. S.R.-V. is supported by Fondation de France. L.Z. has served as a paid consultant for Innate Pharma S.A., a biopharmaceutical company developing immunotherapy drugs for cancer.

Supporting Online Material

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13 May 2011; accepted 27 October 2011
10.1126/science.1208347