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Molecular mechanisms of DC activation by melanoma cells responding to LTX-315

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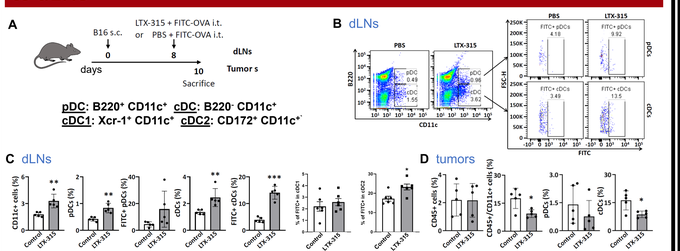
Introduction

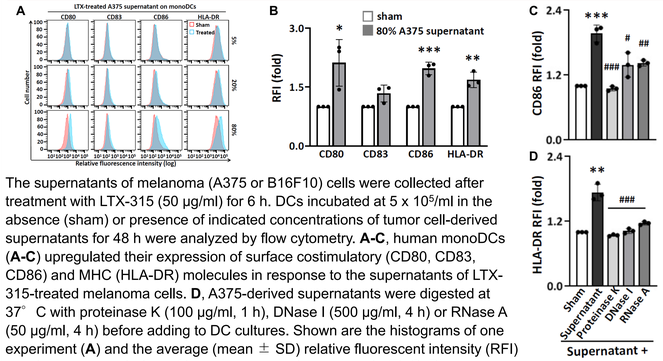
LTX-315 is a synthetic 9-mer cationic oncolytic peptide developed as an analogue of bovine lactoferricin[1,2] and was selected from a series of chemically modified lactoferricin-derived lytic peptides because it displayed superior anticancer activity and lower toxicity on normal cells[2]. LTX-315 induces tumor cell death by rapidly damaging cell membrane integrity[1,3] and permeabilizing mitochondrial membrane[4,5]. Intra-tumoral administration of LTX-315 stimulates the generation of systemic tumor-specific immune responses[6,7], resulting in increased infiltration of cytotoxic CD8$^+$ T cells and decreased regulatory T cell (Treg) infiltration in primary treated tumors[7-10] and in re-challenged secondary tumors[6]. Delivery of LTX-315 into one tumor causes the regression of distal non-treated lesions[6] and the cured mice are protected against a re-challenge with the same tumors[6,7]. A phase I clinical trial showed that LTX-315 converts immunogenically "cold" tumors to "hot" in patients with advanced or metastatic tumors (melanoma, sarcoma, or breast cancer), with increases in CD8+ tumor-infiltrating lymphocytes (TILs) in more than 80% of the patients and regression of distant tumor in some patients[11]. Beside inducing an immunogenic cell death, the mechanism by which LTX-315 treatment stimulates systemic tumor-specific immune responses is not fully elucidated. In tumor-bearing hosts, the generation of tumor-specific immune responses requires effective presentation of tumor antigen(s) to T cells in the draining lymph nodes (dLNs) by antigen-presenting cells (APCs) migrating from the tumor tissue. Dendritic cells (DCs) are the main type of APCs that initiate and control the induction of adaptive (including antitumor) immune responses[12]. For tumor-infiltrating DC (TiDCs) to traffic to dLNs for the induction of antitumor immune responses, they must undergo a process of maturation to acquire the necessary features capable of sufficiently triggering the activation of specific T cells. Given the release of multiple damage-associated molecular patterns (DAMPs) and alarmins (ATP, HMGB1, etc) by tumor cells treated with LTX-315 *in vitro*[5-7] and the known capacities of DAMPs/alarmins to induce DC maturation[13,14], it has been proposed that the DAMPs/alarmins released by LTX-315-treated tumor cells are responsible for triggering the maturation of TiDCs and subsequent tumor-specific immune response[1]. We therefore sought to investigate whether and how LTX-315 induces DC maturation in the context of the generation of anti-melanoma immunity. The results revealed that LTX-315 induced DC maturation *in vivo* and *in vitro*. We also identified two additional pathways by which LTX-315 treatment triggered DC maturation: one involving direct activation of DCs by activating NF-kB, MAPKs, and inflammasome, and the other involving the formation of DC-maturing complexes between LTX-315 and DNA/RNA fragments released by LTX-315-treated melanoma cells. Importantly, LTX-315-induced TiDC maturation and the generation of anti-melanoma immunity relied on the presence of the signal transducer MyD88. Thus, LTX-315 triggers the generation of anti-melanoma immunity by inducing MyD88-dependent maturation of TiDCs.

LTX-315 treatment triggers migration of TiDCs from tumor tissues to dLNs



A, Schematic illustration of the experiments. C57BL/6J mice were subcutaneously (s.c.) injected into the right flank with 1.5 x 10^5 B16F10 cells on day 0. On day 8, the B16-bearing mice were intratumorally (i.t.) injected with a mixture of FITC-OVA (1 μg) and LTX-315 (1 mg) or equal volume of PBS containing 1 μg FITC-OVA alone. Tumors and corresponding dLNs were collected and analyzed on day 10. **B-D**, Flow cytometry analysis of CD45$^+$ leukocytes and various types of DCs (CD11c$^+$ DCs, pDCs, cDCs, FITC$^+$ pDCs, FITC$^+$ cDCs, FITC$^+$ cDC1 and FITC$^+$ cDC2) in dLNs and tumors. Shown are the representative plots (**B**) and average (mean ± SD, n=5) percentages (**C**,**D**) of dLNs (**B**,**C**) and tumors (**D**). *p < 0.05, **p < 0.01, ***p < 0.001 by Student's t test in comparison with the control.

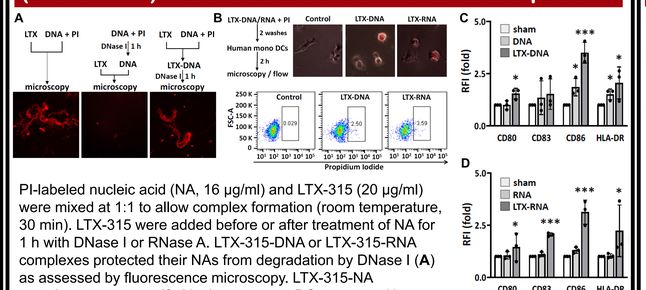
The supernatants of LTX-315-treated melanoma cells contains elements capable of inducing DC maturation



The supernatants of melanoma (A375 or B16F10) cells were collected after treatment with LTX-315 (50 μg/ml) for 6 h. DCs incubated at 5 x 10^5/ml in the absence (sham) or presence of indicated concentrations of tumor cell-derived supernatants for 48 h were analyzed by flow cytometry. **A-C**, human monoDCs (**A-C**) upregulated their expression of surface costimulatory (CD80, CD83, CD86) and MHC (HLA-DR) molecules in response to the supernatants of LTX-315-treated melanoma cells. **D**, A375-derived supernatants were digested at 37° C with proteinase K (100 μg/ml, 1 h), DNase I (500 μg/ml, 4 h) or RNase A (50 μg/ml, 4 h) before adding to DC cultures. Shown are the histograms of one experiment (**A**) and the average (mean ± SD) relative fluorescent intensity (RFI) or cytokine concentration of three independent experiments (**B, C, D**).

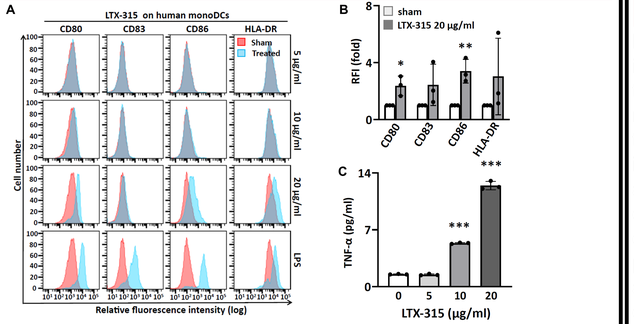
*p < 0.05, **p < 0.01, ***p < 0.001 by Student's t-test in comparison with sham; #p < 0.05, ##p < 0.01, ###p < 0.001 by One-way ANOVA in comparison with native supernatant.

Complexes of LTX-315 (LTX) and A375-derived nucleic acids (DNA or RNA) induced maturation of monoDCs and pDCs



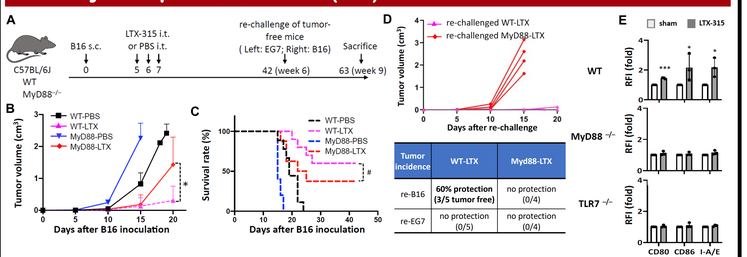
PI-labeled nucleic acid (NA, 16 μg/ml) and LTX-315 (20 μg/ml) were mixed at 1:1 to allow complex formation (room temperature, 30 min). LTX-315 were added before or after treatment of NA for 1 h with DNase I or RNase A. LTX-315-DNA or LTX-315-RNA complexes protected their NAs from degradation by DNase I (**A**) as assessed by fluorescence microscopy. LTX-315-NA complexes were engulfed by human monoDCs assessed by fluorescence microscopy and flow cytometry (**B**). LTX-315-NA complex induction of monoDC (**C, D**) maturation. Human monoDCs (5x10^5/ml) were incubated with LTX-315-NA for 48 h, respectively before they were analyzed for surface markers. Shown are the average (mean ± SD) RFI (**C, D**) of three independent experiments. *p < 0.05, ***p < 0.001 by Student's t-test in comparison with the sham.

LTX-315 (LTX) directly stimulated the maturation of human monoDCs and pDCs.



LTX-315 directly induced the maturation of human monoDCs. Human monoDCs incubated at 5 x 10^5/ml in the absence (sham) or presence of indicated concentration of LTX-315 for 48 h were examined for the expression of surface markers by flow cytometry (**A, B**) and TNFα production by cytokine array (**C**). Data are shown as the representative data of one experiment (**A**) or the average (mean ± SD) of three independent experiments (**B, C**). *p < 0.05, **p < 0.01, ***p < 0.001 in comparison with the sham (by Student's t-test).

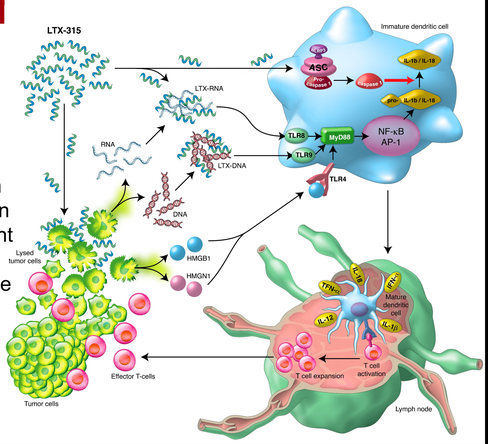
MyD88 knockout compromised the generation of anti-B16 melanoma immunity in response to LTX-315 (LTX) treatment



A, Schedule of the experiment. C57BL/6J mice and MyD88-/- mice (female, 8 wks, n = 8-10) were s.c. inoculated into the right flank with 1.5 x 10^5 B16F10 cells on day 0. Mice were i.t. treated with 3 consecutive daily injections of 1 mg LTX-315 or equal volume of PBS. Tumor-free mice were s.c. re-challenged with 1.5 x 10^5 B16F10 and 4 x 10^5 EG7 cells into the right flank and left flank, respectively. **B-C**, B16 tumor growth (**B**) and survival (**C**). **D**, Incidence of EG7 and B16 tumors on Day 15 after rechallenge and growth of re-challenged B16 tumors in LTX-315-cured WT and MyD88-/- mice. *p < 0.05 and #p < 0.05 by Repeated Measures Analysis of Variance (ANOVA) and Log-rank test, respectively. **E**, LTX-315 upregulated the expression of surface CD80, CD86, and I-A/E in WT, but not in MyD88-/- or TLR7-/-, DCs. *p < 0.05, ***p < 0.001 compared with the sham by Student's t-test.

Conclusions

LTX-315 induces immunogenic cell death through its membranolytic mode of action, leading to the release of potent immunostimulants in addition to a wide spectrum of tumor antigens. Activation of DC via Myd88- dependent pathways underlies the ability of LTX-315 to mediate optimal immunostimulatory effects with subsequent presentation to T cells and execution of effective anti-cancer immune response.



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