ALK+ Anaplastic Large Cell Lymphoma (ALCL)-Derived Exosomes Carry ALK Signalling Proteins and Interact With Tumor Microenvironment

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Short report

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Abstract

The oncogenic pathways activated by the NPM-ALK chimeric kinase of ALK+ anaplastic large cell lymphoma (ALCL) are well characterised, however, the potential interactions of ALK signalling with the microenvironment are not yet known. Here we report that ALK+ ALCL-derived exosomes contain critical components of ALK signalling as well as CD30 and that exosome uptake by lymphoid cells led to increased proliferation and expression of critical antiapoptotic proteins by the recipient cells. The bone marrow fibroblasts highly uptake ALK+ ALCL - derived exosomes and acquire cancer-associated fibroblast (CAF) phenotype. Moreover, exosome-mediated activation of stromal cells altered the cytokine profile of the microenvironment. These interactions may contribute to tumor aggressiveness and possibly resistance to treatment.

Main Text

Anaplastic lymphoma kinase-positive (ALK+) anaplastic large cell lymphoma (ALCL) is a distinct T-cell lymphoma type characterized by overexpression and activation of ALK due to chromosomal translocations of ALK gene locus at 2p23.(1) The most common translocation is the t(2;5)(p23;q35)[2] resulting in the chimeric NPM-ALK, which activates multiple oncogenic pathways including the Ras/ERK, JAK/STAT3, PI3K/AKT/MTOR, JNK/Jun, Sonic Hedgehog and others.(2) ALK+ ALCL is also characterised by expression of CD30 receptor, a common feature for both ALK+ and ALK- ALCL.

The role of tumor microenvironment (TME) in lymphoma progression and drug resistance has been recognized over the past few years. TME is characterized by stromal cells such as fibroblasts, mesenchymal stem cells, follicular dendritic cells, and inflammatory cells such as macrophages, T- and B-lymphocytes. Various components of the TME may interact with the tumor cell, thus contributing to lymphoma cell survival and proliferation. The cross-talk between the neoplastic cells and the TME is mediated by autocrine as well as paracrine signaling by cytokines, growth factors and secreted extracellular vesicles. Exosomes, the best characterized type of extracellular vesicles, represent the newest family of bioactive vesicles that originate from the endosome and are actively secreted by virtually all cell types under normal as well as pathological conditions.(3) Exosomes range between 30-100 nm in diameter, and contain a set of characteristic exosomal proteins (e.g. tetraspanins) as well as molecules (proteins, coding and non-coding RNA and DNA) specific for the cell type of origin. In certain hematologic malignancies such as chronic lymphocytic leukemia, exosomes play a pivotal role in shaping the TME in favor of tumor growth.(4) However, the molecular content and functional properties of ALCL-derived exosomes are not yet known.

Results And Discussion

Molecular characterisation of ALK+ ALCL - derived exosomes

Here we analyse for the first time the molecular content of the ALK+ and ALK- ALCL-derived exosomes using an *in vitro* model of ALK+ (Karpas 299, SUP-M2) and ALK- (Mac1, Mac2) ALCL. Exosomes were isolated using well established ultracentrifugation protocols (**Suppl. methods**). Abundant exosome secretion was seen in both ALK+ and ALK- ALCL cell lines by Nanoparticle Tracking Analysis (NTA), which measures the size and the relative particle concentration, and by transmission electron microscopy (**Figure 1a-b**). CD30 receptor was detected in the ALCL-derived exosomes by immuno-electron microscopy and Western blot analysis (**Figure 1c**). Hansen and co-workers have demonstrated a CD30 vesicle-containing network in lymphoid tissue of classical Hodgkin lymphoma (cHL), which might facilitate the communication between distant cell types in cHL tissue and allow a functional CD30–CD30L interaction *in trans*.(5) Better understanding of the potential role of CD30 in tumor cell microenvironment may have therapeutic implications in the era of CD30-targeted therapy.

In this study, we also found for the first time, that activated ALK and critical components of the ALK signaling are present in the exosomes secreted by ALK+ ALCL cells indicating activation of Ras/ERK, JAK/STAT3, PI3K/AKT/mTOR and Sonic Hedgehog pathways. (**Figure 1d-f**). Of note, JunB(6), known to be upregulated by NPM-ALK, was enriched at a higher level in ALK+ compared to ALK- ALCL exosomes. Activated STAT3 and AKT, which represent common signaling components in both ALK+ and ALK- ALCL are detected at a variable level in all ALCL-derived exosomes (**Figure 1d-f**). Interestingly, PD-L1, a crucial regulator of the immune checkpoint, which is differentially expressed among the ALK+ and the ALK- ALCL cell lines and tumors(7) is present in the exosomes (**Figure 1d-f**). Whether exosomal PD-L1 functionally interacts with the PD-1 on the T cells in TME and may affect the response to immune checkpoint immunotherapy certainly merits further investigation. Mac2A cells expressed higher levels of exosomal PD-L1 than Mac1, and notably, Mac2A were derived from the same patient (as Mac1) cells at a later, more advanced stage of disease. To the best of our knowledge, detection of PD-L1 has not been demonstrated in any lymphoma-derived exosomes to date.

ALCL-derived exosomes can be uptaken by lymphoid cells

The ALCL-derived exosomes were readily uptaken by various cell types including murine pro-B lymphoid cells, Ba/F3 (**Figure 2a-b**). The uptake was time-dependent and reached a level of 75% at 6 hours. Proteins present in the exosomes such as the CD30 receptor were detected in Ba/F3 cells following exosome uptake and, furthermore, the recipient cells acquired anti-apoptotic properties such expression of cFLIP, which has been previously shown to confer resistance to FAS-mediated apoptosis in ALCL (**Figure 2c**).(8) Notably, proliferation of Ba/F3 cells was increased in the presence of ALCL-derived exosomes with the highest level being observed at 8 hours, and at this point the proliferation fraction exceeded that of IL-3 – treated cells (**Figure 2d**). Taken together, these findings suggest that the ALK+ ALCL-derived exosomes are enriched in oncogenic signals, and their uptake by recipient cells leads to anti-apoptotic and cell proliferation effects. Export of oncogenic signaling by exosomes with functional properties has been described in aggressive B-cell lymphomas as well.(9) Moreover, we show that

exosomes secreted by the Ba/F3 cell line stably transfected with NPM-ALK are potently uptaken by paternal Ba/F3 cells in a time dependent manner (**Figure 2e-j**). These findings provide evidence of functional exosome uptake by non-neoplastic lymphoid cells (Ba/F3) as well.

ALCL-derived exosomes interact with the tumor microenvironment cells

To investigate the role of ALCL-derived exosomes on the tumor stroma, we examined the uptake of ALCL derived exosomes by the bone marrow stroma-derived L88 fibroblasts. We found that incubation of the L88 cells with ALCL-derived exosomes confers morphological and molecular changes (expression of aSMA) in the fibroblasts that undergo transformation to cancer-associated fibroblasts (CAF) (**Figure 3a-c**). It is well-established that the tumor cells induce CAF phenotype, and the activated fibroblasts in turn, produce a number of growth factors and cytokines that promote, among other cellular functions, cell proliferation and angiogenesis.

Having shown the functional interaction of ALCL-secreted exosomes with L88 fibroblasts, we sought to investigate whether exosome-mediated activation of stromal cells altered the cytokine profile of the TME. Using a cytokine array, the level of certain cytokines including MCP-1/CCL2, CCL5/RANTES, IL-6 and IL-8 was substantially increased in the medium following incubation with ALK+ ALCL-secreted exosomes (**Figure 3d**) with the highest relative increase being observed for IL-8 and MCP-1/CCL2. Notably, MCP-1/CCL2 and IL-6 levels were found to be elevated in the plasma of SCID/beige mice injected with two ALK+ (SUPM2, Karpas 299) and one ALK- (Mac1) ALCL cell lines (**Fig 3e-g**). In a previous study, Dejean and co-workers (10) showed that high-mobility-group box-1 (HMGB-1), a proinflammatory cytokine, is released by ALK+ ALCL cells, and extracellular HMGB-1 stimulated secretion of the IL-8 chemokine by other cell types as epithelial cells of the skin (keratinocytes). IL-8 was capable of inducing invasiveness and dissemination of ALK+ ALCL cells. This is of particular interest, since ALK+ ALCL express the IL-8 receptors CXCR1 and CXCR2. Therefore, it is tempting to speculate that ALK+ ALCL-secreted exosomes induce IL-8 production by the CAFs, which are secreted in the microenvironment and IL-8, in turn, interacts with the tumor cells bearing the IL-8 receptors. The role of monocyte chemoattractant protein-1 / CCL2 (MCP-1/CCL2) in ALCL pathogenesis and progression is not yet known. The MCP-1/CCL2 and its receptor CCR2 regulate migration from the blood across the vascular endothelium and tissue infiltration of monocytes and macrophages, which play a significant role in immunological surveillance and inflammation.

Conclusions

We have shown that ALK+ ALCL cells secrete exosomes that bear critical molecules of ALK signalling, which can be uptaken by lymphoid cells with apparent biologic effects. Functional interactions of the ALK+ ALCL cells with bone marrow stroma cells lead to fibroblast activation (CAFs) and alter the cytokine profile of the TME that may contribute to tumor aggressiveness and possibly resistance to treatment.

List Of Abbreviations

ALK: Anaplastic lymphoma kinase

ALCL: Anaplastic large cell lymphoma

cHL: Classical Hodgkin lymphoma

NPM: Nucleophosmin

aSMA: a-Smooth muscle actin

CAF: Cancer-associated fibroblasts

TME: Tumor microenvironment

MCP-1 / CCL2: Monocyte chemoattractant protein-1 / Chemokine C-C motif ligand 2

CCL5 / RANTES: Chemokine (C-C motif) ligand 5 / Regulated on Activation, Normal T Cell Expressed and Secreted

IL-6: Interleukin 6

IL-8: Interleukin 8

PBS: Phosphate buffer saline

PD-1: Programmed-Death 1

PD-L1: Programmed-Death Ligand 1

Declarations

Ethics approval and consent to participate

The study does not involve patient samples or any patient information and, therefore, consent to participate is not applicable. The study was approved by the institutional ethics board.

Consent for publication

The study does not involve patients and, therefore, consent for publication is not applicable.

Availability of data and materials

The materials and constructs used in the study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

D.C, J.B, G.B, P.F, N.T, and I.V. carried out the experimental work, V.L and E.D contributed to data analysis, G.Z.R and T.P supervised the research and wrote the manuscript. All authors read and approved the final manuscript.

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All authors read and approved the final manuscript.

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Figures

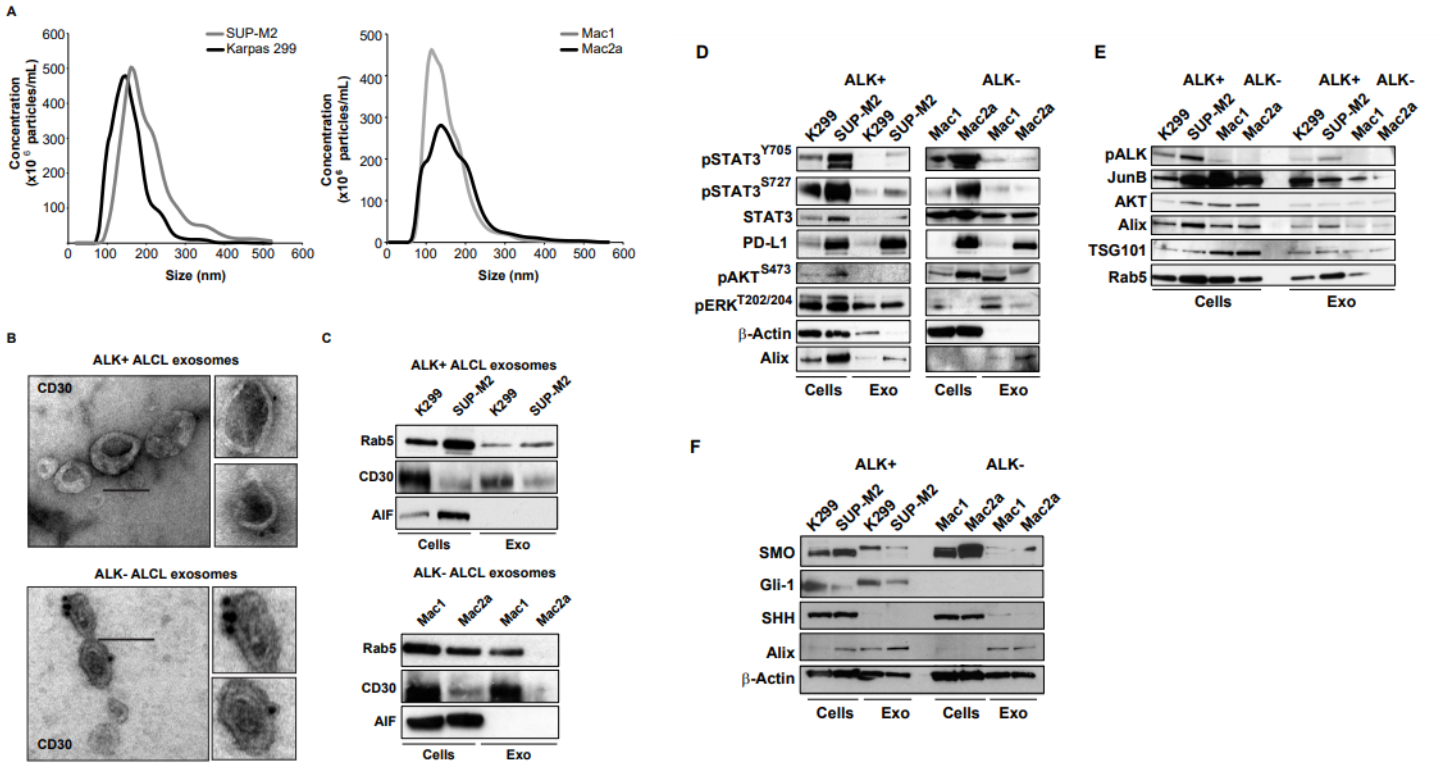

Figure 1

Molecular profiling of ALK+ and ALK- ALCL-derived exosomes A) Nanoparticle tracking analysis (NTA) for the ALK+ (Karpas 299, Sup-M2) and ALK- (Mac1, Mac2A) ALCL exosomes. The mean size and particle concentration of the preparations are shown. B) Immuno-electron microscopy (iEM) staining with gold particles for CD30 on the ALCL-derived exosomes. C) Western blot analysis of the ALK+ (upper panel) and ALK- (lower panel) ALCL whole cell and exosomal lysates shows the protein levels of Rab5 (exosomal mark-er), CD30, and AIF (quality control for the exosomal preparations). D) Protein composition of the ALK+ and ALK- cell lines and exosomes. The whole cell and exosomal lysates were probed for known exosomal markers as well as for key proteins of the ALK oncogenic pathway. Both ALK+ and ALK- derived exosomes bear proteins of the activated oncogenic pathways such as pSTAT3Y705, pAktS473 and pERKT202/204. Alix is one of the exosomal marker. E) Alongside the ALK+ ALCL cells and their respective exosomes carry the NPM-ALK oncoprotein. The AP-1 transcription factor, known to be unregulated in ALK+ ALCL by NPM-ALK, is detected at a higher level in exosomes derived from ALK+ than ALK- ALCL cells.11 Alix, Tsg101 and Rab5 are characteristic exosomal proteins. F) Two crucial components of the sonic hedgehog pathway, SMO and Gli-1, which are known to be upregulated by NPM-ALK (ref), are detected at a higher level in ex-osomes secreted by ALK+ than ALK- ALCL cell lines.

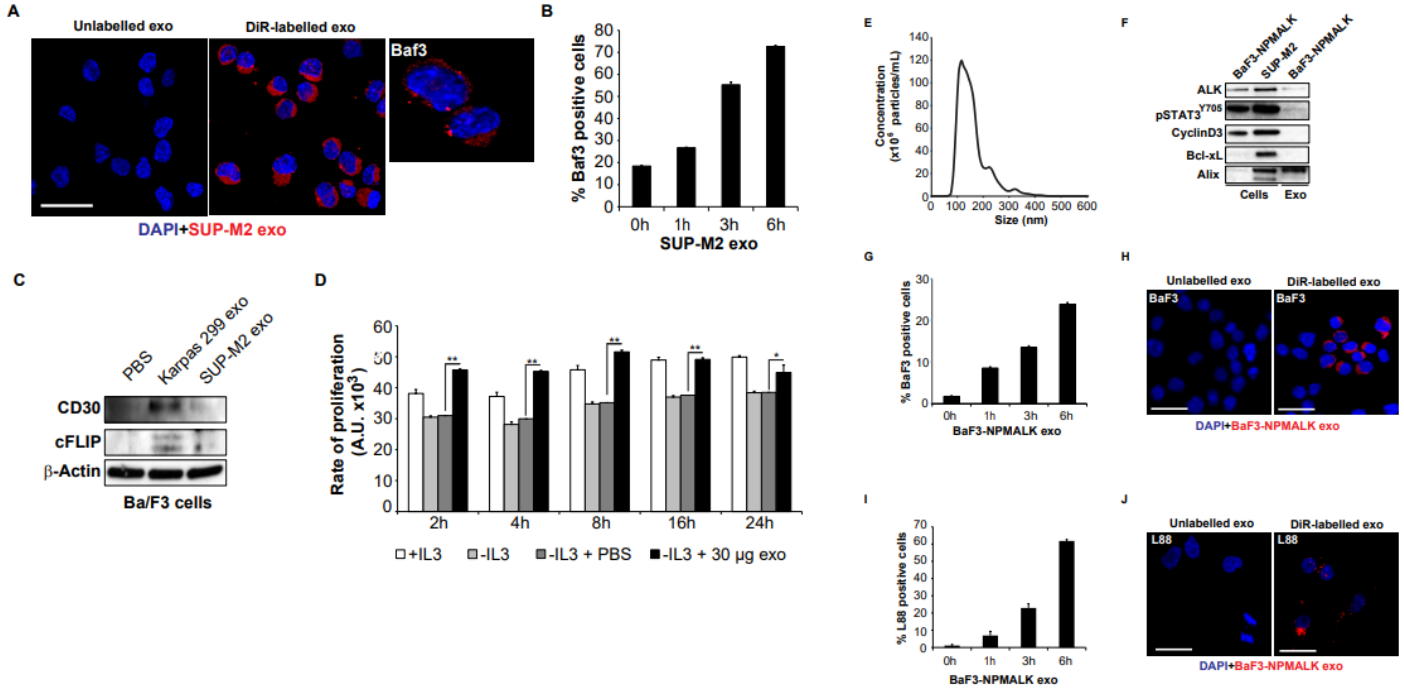

Figure 2

ALK+ ALCL-derived exosomes are uptaken by Ba/F3 cells with apparent biologic ef-fects. A, B) DiR-labelled SUP-M2 secreted exosomes (20 µg/ml) were added to the Ba/F3 cells for the time points as indicated and the uptake was quantified by flow cytome-try and depicted by confocal microscopy. After 6 hours, the exosome uptake reached a level of approximately 75% as shown (B). C) Immunoblots showing detection of CD30 and the anti-apoptotic protein cFLIP in Ba/F3 cells following uptake of exosomes derived from two ALK+ ALCL cell lines, Karpas 299 and SUPM2. D) Proliferation of Ba/F3-cells with or without IL-3 (10 ng/mL) and 30 µg SUP-M2-exosomes. 1x104 Ba/F3-cells in a volume of 100 µl were cultured in the settings as shown for 48 hours. To determine the proliferation of the cells, 20 µl CellTiter-Blue® Reagent (Promega, Madison, WI, USA) was added and the fluorescence at 560/ 590 nm was determined at the indicated timepoints. The highest level of proliferation fraction in the cells treated with 30 µg SUP-M2 exosomes (no IL-3) was observed at 8 hours and exceeded that of IL-3 treated cells (** $p<0.05$). E) Nanoparticle tracking analysis (NTA) for the BaF3-NPM-ALK - derived exosomes. F) The immunoblots (Western blot) show total ALK, pSTAT3Y705, CyclinD3, Bcl-xL and Alix protein levels for the BaF3-NPM-ALK cells and exosomes compared to the SUP-M2 cells. The BaF3-NPM-ALK is a BaF3 clone stably transfected with NPM-ALK construct as described in Suppl. Methods section. Expression of pALK and pSTAT3Y705 confirmed the presence of an active NPM-ALK kinase in BaF3-NPM-ALK cells. The activated (phosphorylated) form of STAT3, pSTAT3Y705 and cell cycle regulator CyclinD3 but not BCL-XL were detected in the exosomes derived from BaF3-NPM-ALK. Alix was used as an exosomal marker. G, H) DiR-labelled Ba/F3-NPM-ALK exosomes (20 µg/ml) were added to the Ba/F3 cells for the time points as indicated and the exosome uptake was quantified by flow cytometry (C) and depicted by confocal microscopy (D). At 6 hours, the uptake reached a level of 25% as shown.

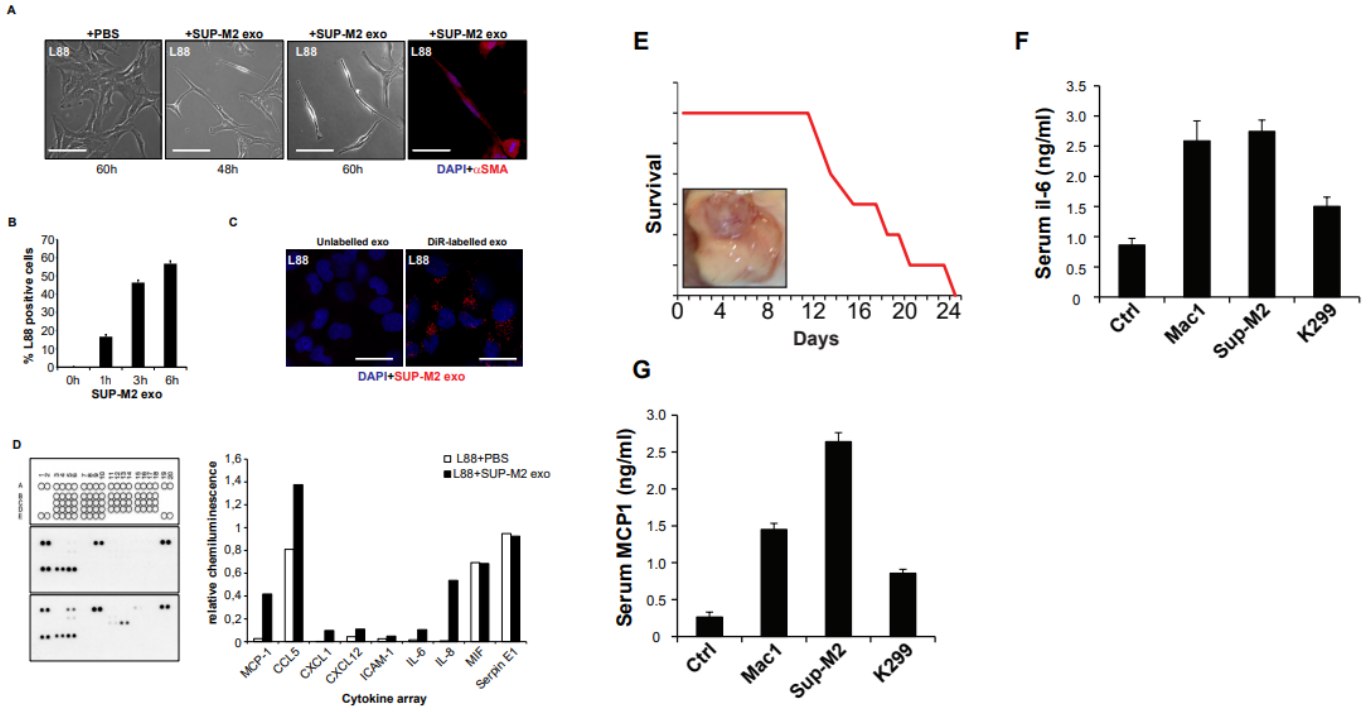

Figure 3

Effects of ALK+ ALCL derived exosomes on the bone marrow derived fibroblasts and cytokine profile. A, B) L88 cells were educated with 60 µg/ml of SUPM2-derived and DiR-labelled exosomes at the indicated time points. The positive population was quantified by flow cytometry (A) and depicted by confocal microscopy (B) after 6 hours of uptake. C) Morphological changes of bone marrow-derived L88 fibroblasts due to education with SUPM2-derived exosomes (60 µg/ml) for 60 hours. Incubation of the L88 cells with ALCL-derived exosomes also confers molecular changes such as expression of aSMA in the fibroblasts characteristic of the transformation to a cancer-associated fibroblast (CAF) phenotype. D) The cells' supernatant was recovered and used for the cytokine array assay as described in Suppl. Methods. The cytokine expression was captured on X-ray films (left panel) and the differences were visualized by chart bars after normalization (white bars for PBS/control and black bars for SUP-M2 exosomes). The levels of cer-tain cytokines including MCP-1, CCL5/RANTES, IL-6 and IL-8 were substantially increased in the medium following incubation with ALK+ ALCL-secreted exosomes. E) 10X106 cells from two ALK+ ALCL cell lines, Karpas 299 and SUPM2, and one ALK- ALCL cell line, Mac1, were injected subcutaneously in SCID/beige mice (10 mice for each cell line) and were followed for tumor development. The tumors were measured and the mice were sacrificed when the tumor reached 1.5 cm in max. di-ameter according to the institutional ethical guidelines. A representative example of SUPM2 xenografts and the survival of this group of SCID/beige mice was calculated (Kaplan-Meier curve). F, G) Plasma was isolated from all SCID/beige mice. Plasma samples were thawed on ice, briefly mixed and spun down at 500g for 30 seconds. Supernatant was col-lected in a new tube, diluted 1:1 with ice-cold PBS 1X and spun down at 12000g for 45 minutes at 4oC. Supernatant was collected in a new tube and kept on ice until use (100µl per well). IL-6 and MCP1/CCL2 levels were assessed with ELISA, which was

performed according to the manufacturer's recommended protocol (Mini ELISA Development kits, PeproTech Nordic, Stockhol, Sweden). Results were obtained by using Spark 10M plate reader (Tecan, Männedorf, Switzerland). IL-6 and MCP1/CCL2 levels were significantly increased in the plasma of all three xenograft models (Karpas 299, SUPM2 and Mac1) as compared to control SCID/Beige mice.

Supplementary Files

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