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The Helicobacter pylori Protein HP-NAP: from a bacterium a new hope for the treatment of metastatic melanoma

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Background: Immune checkpoint inhibitors and small-molecule targeted drugs have significantly improved the prognosis of patients with advanced melanoma. However, the toxicity is severe and patients nearly invariably develop resistance. Several studies show that the Helicobacter Pylori Neutrophil Activating Protein (HP-NAP) has pro-inflammatory and immunomodulatory properties that make it a potential candidate for the therapeutic applications, including vaccine and drug development [1]. By activating cytotoxic Th1 responses, HP-NAP inhibits the growth of bladder cancer [2]. In addition, expression of secreted HP-NAP by oncolytic measles virus and adenovirus has been shown to enhance the anti-tumor activity of the viruses in the treatment of metastatic breast cancer and neuroendocrine tumors, respectively [3-4]. Methods: In the zebrafish model, we examined the therapeutic efficacy of HP-NAP against human metastatic melanoma. Specifically, human melanoma cells were xeno-transplanted into zebrafish embryos and tracked in the presence or absence of HP-NAP. Macrophages behaviour and their drug-induced depletion were analysed exploiting macrophage-expressed transgenes. Results: We observed that HP-NAP administration in zebrafish embryos efficently inhibited tumor growth and metastasis (fig 1) and this was accompanied by a strong recruitment of macrophages at the tumor site (fig 2-3). Considering that the adaptive immune system is not completely developed in embryos until three weeks after fertilization, we hypothesized that HP-NAP exerted its antitumor effect through the activation of pro-inflammatory/anti-tumor macrophages (fig 4-5). Our hypothesis found confirmation in the observation that the depletion of macrophages in zebrafish, with clodronate liposomes (L-CLOD) or L-Leucine Methyl Ester (L-LME), almost completely abrogated the ability of HP-NAP to counteract tumor growth. Conclusions: Our results show that HP-NAP exerts a robust anti-tumor activity not only by modulating a cytotoxic adaptive immune response, but also by recruiting anti-tumor macrophages and support the notion that HP-NAP might become a new biological therapeutic agent for the treatment of metastatic melanomas.

1) HP-NAP counteracts melanoma growth and metastasis in vivo

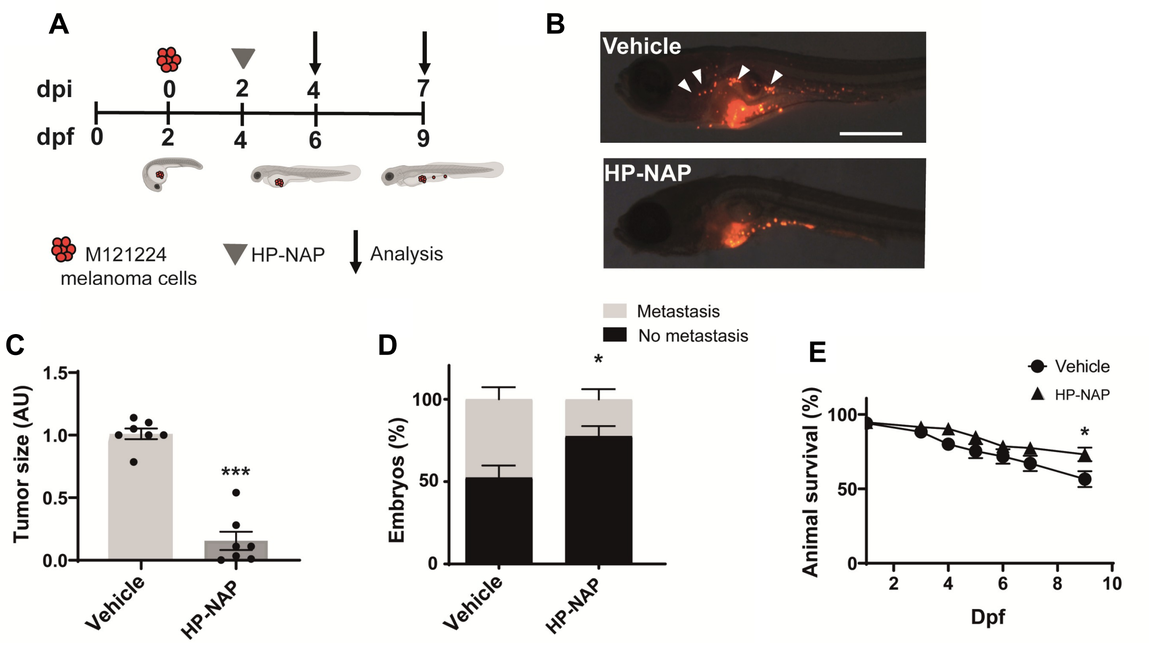

Figure 1: Effect of HP-NAP on melanoma growth and metastasis in zebrafish model. A. Scheme of the experimental workflow. **B.** Representative fluorescence stereoscope images of embryos injected with M121224 cells and treated with HP-NAP or vehicle at 9dpf/7dpi . Arrowheads indicate metastases. Scale bar: 500 µm. The tumor mas s and metastasis is reduced in HP-NAP treated embryos. **C.** Scatter plots show that the tumor size (AU: Arbitrary Unit) is significantly reduced in HP-NAP treated embryos. At 9dpf **D.** The percentage of metastasis at 9 dpf is lower in larvae treated with HP-NAP with respect to vehicle. **E.** Kaplan-Meier curves show that the survival of animals at 9dpf treated with HP-NAP is higher compared to control.
*, p< 0.05; ***, p<0.001.

2) The anti-tumor effect of HP-NAP does not rely on a direct action on melanoma cells *in vitro*

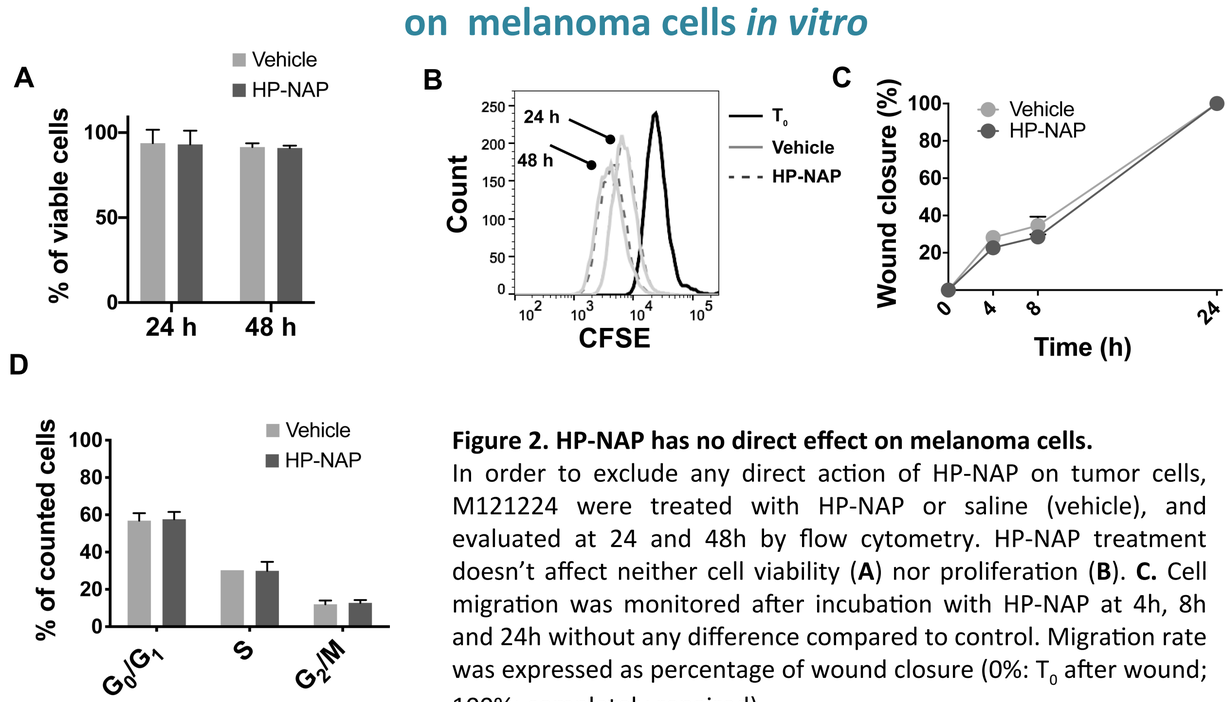

Figure 2. HP-NAP has no direct effect on melanoma cells.
In order to exclude any direct action of HP-NAP on tumor cells, M121224 were treated with HP-NAP or saline (vehicle), and evaluated at 24 and 48h by flow cytometry. HP-NAP treatment doesn't affect neither cell viability (**A**) nor proliferation (**B**). **C.** Cell migration was monitored after incubation with HP-NAP at 4h, 8h and 24h without any difference compared to control. Migration rate was expressed as percentage of wound closure (0%: T_0 after wound; 100%: completely repaired).
D. For cell cycle analysis, cells were seeded in 24-well plate and incubated in medium with or without HP-NAP for 48 h; after DNA staining with propidium iodide, fluorescence was measured by flow cytometry and data are expressed as percent of G1, S, and G2/M cells . No difference in cell cycle between HP-NAP treatment and controls was revealed.

3) HP-NAP promotes the recruitment of macrophages at the tumor site.

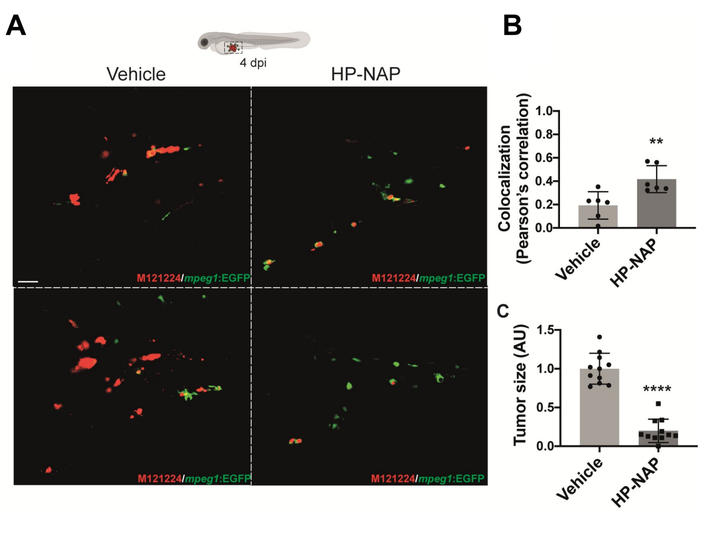

Figure 3. HP-NAP treatment favors the interaction between tumor cells and macrophages and early affects tumor size. To understand if HP-NAP could promote an antitumor environment, *Tg(mpeg1:EGFP)gl22* zebrafish embryos (with green fluorescent macrophages) were xenotrasplantated with melanoma cells (red), injected or not with HP-NAP at 2 dpi, and observed at 4 dpi. **A.** Representative 2D projections of confocal single plane images of the yolk-sac region of embryos at 4 dpi reveal a higher green signal (macrophages) around red melanoma cells in fish treated with HP-NAP. **B.** Scatter plots show the quantification of green and red signals showing that in HP-NAP-injected fishes the colocalization of signals is higher compared to vehicle.
C. Scatter plots show that the tumor size at 4 dpi is significantly reduced in HP-NAP treated fish with respect to vehicle. *, p< 0.05 , ** p<0.005,****, p<0.0001.

4) HP-NAP modulates the macrophages polarization

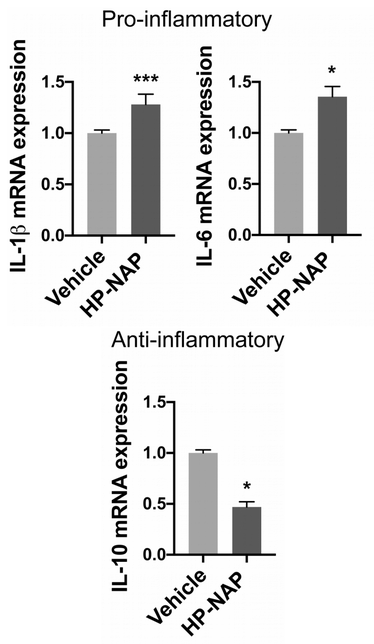

Figure 4. HP-NAP administration in zebrafish promotes the acquisition of a pro-inflammatory profile by macrophages. *Tg(mpeg1:EGFP)gl22* zebrafish embryos were xenotransplantated with melanoma cells and treated with HP-NAP or saline (vehicle) at 2 dpi. After 24 h GFP-tagged macrophages were FACS-sorted and the expression of il1β, il6 and il10 genes was evaluated by qRT-PCR. Each gene in macrophages isolated from HP-NAP-treated fishes was relative to that in macrophages isolated from vehicle animals, taken as reference and set as 1 and data were normalized to the housekeeping gene 18S. As assumed, the RNA levels of proinflammatory cytokines il1β and il6 increased in macrophages from larvae treated with HP-NAP whereas the levels of anti-inflammatory il10 decreased. Data are expressed as the mean ± SEM of 2 independent experiments and analysed by Student's t test; *, p<0.05; ***, p<0.001.

5) Macrophages are essential for the anti-tumor activity of HP-NAP

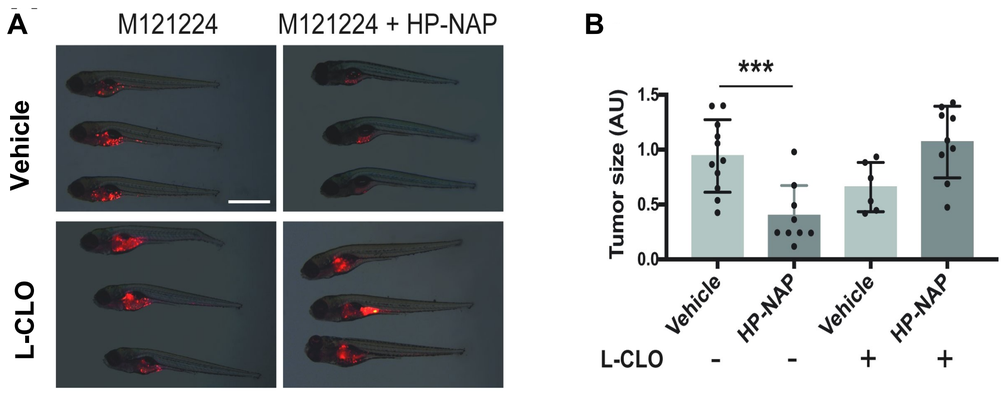

Figure 5. The anti-tumor activity of HP-NAP is strictly dependent on macrophages: effect of macrophage depletion by liposome-encapsulated clodronate (L-CLO). To reinforce thar HP-NAP has action on macrophages to fight tumor, we treated *Tg(mpeg1:EGFP)gl22* xenotransplanted zebrafish embryos with macrophage depleting Clodronate Liposome (L-CLO). Larvae were treated with empty liposomes + saline (vehicle), or empty liposomes + HP-NAP, L-CLO + saline (L-CLO) or L-CLO + HP-NAP at 2 dpi and observed at 4 dpi. ***A.*** Fluorescence stereoscope images of the total tumor mass in fishes at 4 dpi. In the condition with L-CLO (macrophage absence) HP-NAP doesn't exert antitumor activity. Scale bar: 1 mm. ***B.*** Scatter plots show the quantification of the total tumor size (AU: Arbitrary Unit) at 4 dpi
***, p<0.001

References:
[1] de Bernard M, D'Elios MM. Toxicon. 2010;56(7):1186-92.
[2] Codolo G, et al. Cancer Immunol Immunother. 2012;61(1):31-40.
[3] Iankov ID, et al. Mol Ther. 2012;20(6):1139-47.
[4] Ramachandran M, et al. Mol Ther. 2013;21(11):2008-18.

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