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ROLE OF miRNA FROM MELANOMA CELL-DERIVED EXOSOMES IN TUMOR IMMUNE ESCAPE

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BACKGROUND

Among regulation mechanisms, the role of extracellular vesicles, such as the exosomes, released by cancer cells in tumor microenvironment appears to be a mechanism used by tumor cells to disseminate bioinformation in autocrine and paracrine manner. This communication can be achieved by direct contact or over long distances and could help cancer proliferation and metastases and to alter immune cell responses^{1,2}. These endosome-derived vesicles of 30–100 nm are formed in the multivesicular bodies and then released from the cell into the extracellular space. They carry a broad range of cargo, including proteins, noncoding RNAs (miRNAs, lncRNAs...), mRNAs, and DNA fragments, to target cells. Upon entering the target cell, the exosomes induce modulation of cell function and even identity switch (phenotypic and epigenetic).

Multiple miRNAs have been described to play key roles in T lymphocytes development, differentiation and function. Upon activation, T cells undergo global gene expression remodeling to support cell growth, proliferation, and effector functions. All changes in this pattern of expression could have consequences on T cell activation and response to melanoma.

Although specific immune responses are often amplified in melanoma patients, several mechanisms could contribute to the failure of T cell responses, inducing tumor escape. Tumor-derived exosomes are major participants of this immune evasion. We postulate that most of these effects are carried by miRNAs encapsulated in exosomes that could influence the properties of recipient cells present in the tumor microenvironment.

METHODS

Exosome purification

Exosomes were recovered from the supernatant of melanoma cells cultured for 24 hours in depleted medium (*i.e.* medium depleted from FCS-derived exosomes). Exosomes were purified using Exoquick TC (SBI). In each exosome preparation, the concentration of total proteins was quantified by BCA (ThermoFisher).

miRNA assay

Total RNA including miRNA was isolated from melanoma cell lines and exosomes using the miRNeasy kit (Qiagen). The miRNA expression profiles were analyzed by GeneChip miRNA 4.0 array (Affymetrix) and validated by RT-qPCR using MystiCq microRNA quantitation system (Sigma).

T cell assay

Melanoma exosomes (4µg) were incubated with 2.10⁵ CD8+ T cells in coated with anti-CD3 96-well plated for 5 or 24 hours. TNFα and IFNγ secretions were measured by ELISA kits (Affymetrix).

Confocal microscopy

T cells were incubated with exosomes as described in "T cell assay". Cells were fixed in 4% paraformaldehyde. We used PKH67 and WGA647 to stain respectively the exosomes and the cytoplasmic membrane. Nuclei were counterstained with Hoechst. Cell observations and images processing were performed with a Nikon A1 N-SIM (MicroPicell platform).

T cell gene expression analysis

Total RNA was isolated from CD8+ T cells using the miRNeasy kit (Qiagen). To generate sufficient material, we used the whole transcriptome amplification kit (WTA2, sigma). The final cDNA product was used for the RT² Profiler PCR Array "Human T-cell & B-cell activation" using Maxima SyBR Green/ROX qPCR Master Mix (ThermoFisher).

RESULTS

miRNA content

Three human melanoma cell lines were established from surgical specimens obtained from primary or metastatic tumors called M113, M117 and M28. We analyzed the miRNA content of melanoma cell-derived exosomes and identified 216 miRNAs enriched in exosome compare to donor melanoma cell lines. The miRNA content was highly similar between exosomes ($r^2=0.98$), independently of their donor cell origins (Figure 1). Among these 216 miRNAs, some have been previously associated with metastasis or angiogenesis, and 2 with T cell response (miR-122 and miR-149).

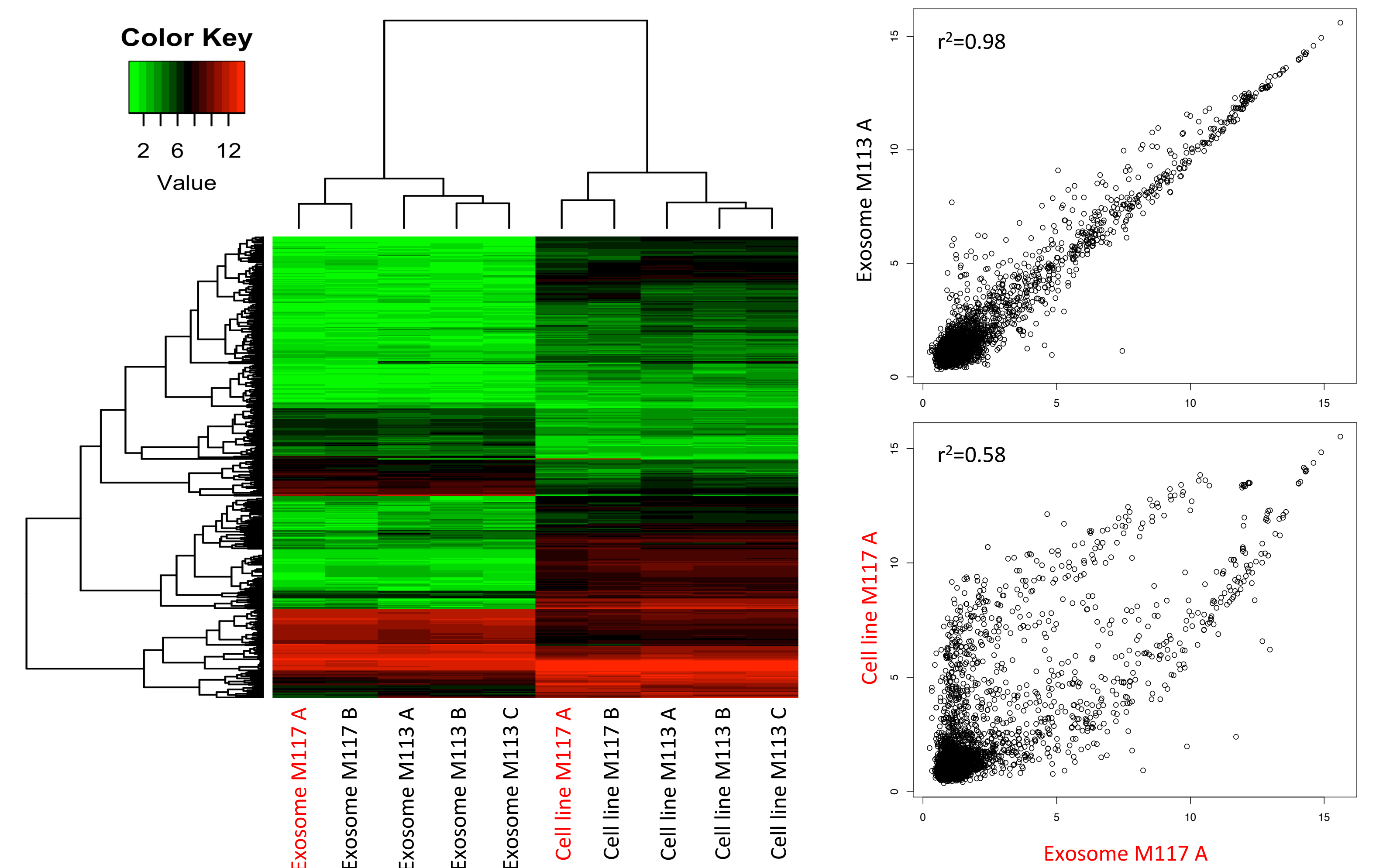


Figure 1. Hierarchical clustering of miRNA expression from exosomes and donor cells. The color scale bar (green-black-red) indicate increasing expression levels. On the left, correlation graph across exosomes and donor cells or exosomes/exosomes.

Immune recipient cells

To determine whether the exosomes and their cargo could be taken up by CD8+ T cells, we utilize an *in vitro* experimental model where exosomes isolated from Melanoma cell lines were incubated with T cells during 5 or 24 hours. To visualize the internalization of the vesicles into CD8+ T cells, exosomes were stained with PKH67. The internalization of exosomes was evidenced by a green fluorescent punctuate signal inside the cytoplasm of recipient T cells (Figure 2A).

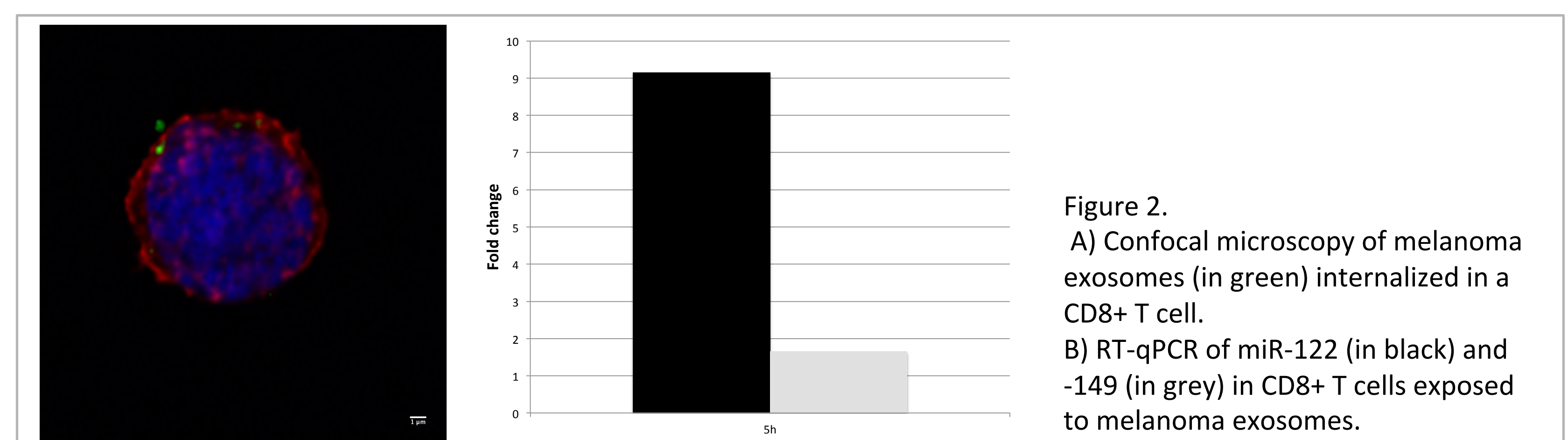


Figure 2. A) Confocal microscopy of melanoma exosomes (in green) internalized in a CD8+ T cell. B) RT-qPCR of miR-122 (in black) and -149 (in grey) in CD8+ T cells exposed to melanoma exosomes.

The transfer content was confirmed by specific qRT-PCR for two enriched miRNAs in exosomes: miR-149 and miR-122. We observed a two- to six-fold enrichment of these miRNAs in the exposed T cells (Figure 2B).

Exosome and immune response

We next investigated whether melanoma exosomes containing miR-122 and miR149 would have an impact on the activated T cells. The melanoma exosomes have no impact on T cell proliferation or death (data not shown). We showed a downregulation of several messenger RNAs coding for proteins implicated in the T cell receptor signaling pathway (Kegg hsa04660, Figure 3) after 24h of co-culture. We observed a decreasing expression of co-stimulatory molecule CD3, CD28 and CD45, and several cytokines (interleukin-2 (IL-2), IL-5, IL-10, IFNγ and GM-CSF). For now, using ELISA assays, we replicated these results by the observation of a reduce secretion of IL-2 ($p<0.05$) and a trend to a reduced secretion of IFNγ (not significant) by the CD8+ T cells exposed to melanoma exosomes (Figure 4). Since miR-149 targets *TNFα*, we also evaluated the *TNFα* secretion by T cells exposed or not to melanoma exosomes, and found a significant decrease of *TNFα* ($p<0.05$) in exposed cells (Figure 4).

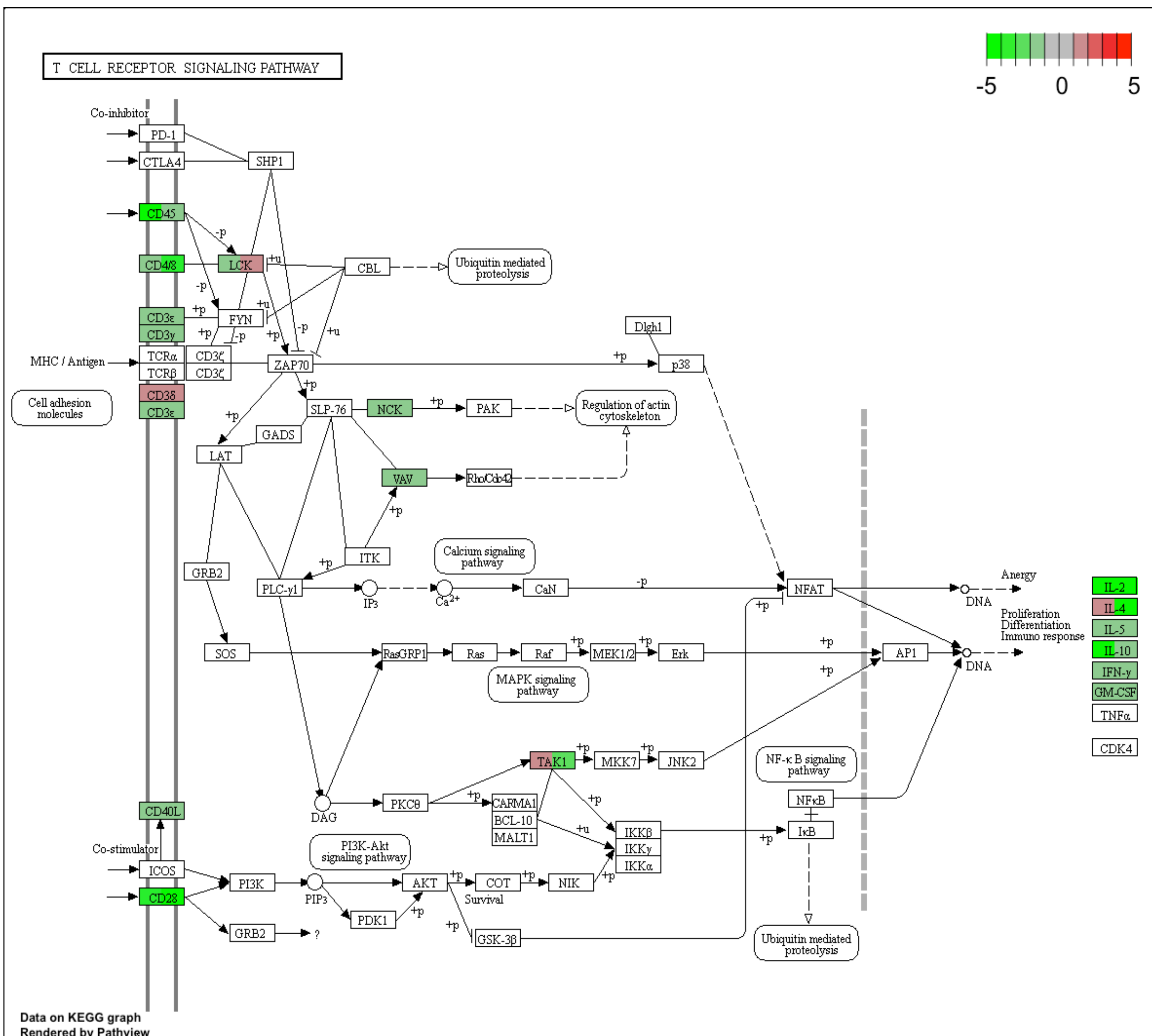


Figure 3. Log2 fold changes of gene expression level mapped onto the KEGG pathway module « T cell receptor signaling pathway ». Most genes in this pathway showed decreased expression in CD8+ T cells exposed to melanoma exosomes (in green). In white, non tested genes.

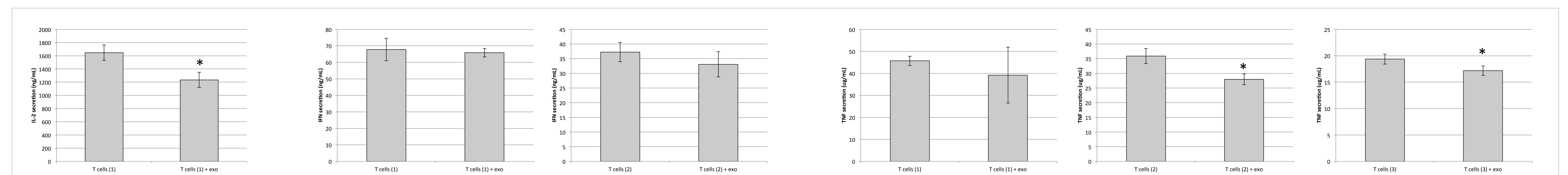


Figure 4. Cytokine secretion by CD8+ T cells 24h after exposition to melanoma exosomes. For each plot, n=3. (1) CD8+ T cell clone 1 against melanoma ; (2) CD8+ T cell clone 2 against melanoma ; (3) polyclonal CD8+ T cell population

CONCLUSION

Although exosomes have been studied for a number of years, the biological roles of exosomal miRNAs are just beginning to be investigated³⁻⁶. Our data demonstrate that miRNA are released from melanoma cells in exosomes and are taken up by recipient CD8+ T cells and subsequently could modulate target gene expression.

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