Supplemental Figure Legends

Fig. S1. NK cells from melanoma patients up-regulate inhibitory receptors and down-regulate activating receptors.

(A) The plot on the left represents the gate on NK cells purified from peripheral blood from a melanoma patient. The other three plots show >95% purity in NK cell negative selection (> 95% are CD56⁺ CD3⁻ CD14⁻ CD19⁻ cells). (B) The graph represents the percentage of CD16⁺ (left panel) and CD56⁺ NK (right panel) cells in healthy donors (n=13) and melanoma patients (n=11). (C) The plot shows the percentage of each NK cell subset (CD56dim and CD56bright) in total NK cells from healthy donors (n=34) and from melanoma patients (n=24). (D) The graph shows the percentage of DNAM-1⁺ cells in each NK cell subset (CD56dim and CD56bright) and in total NK cells from healthy donors (n=10) and from melanoma patients (n=10). (E) The plots show the percentage of CTLA-4 (left panel) and PD-1 (right panel) expression in NK cells from healthy donors (n=10) and from melanoma patients (n=5). All experiments were performed in duplicate.

Fig. S2. NK cells from melanoma patients down-regulate the expression of cytokine receptors, rendering them refractory to cytokine stimulation.

(A) The graphs show the expression of IL-2 receptor (α, β and γ chains) in each
NK cell subset (CD56^{dim} and CD56^{bright}) and in total NK cells from healthy donors (n=10) and from melanoma patients (n=10). (B and C) Freshly purified NK cells (HD n=12; MD n=5) were stimulated with 200U/ml of IL-2: (B) expression of CD122 (IL-2R β chain; left panel) and CD132 (IL-2R γ chain; right panel); and (C) expression of KIR3DL1 (left panel) and NKG2D (right panel) were monitored every two days over 6 days (day 0, 2, 4 and 6) by flow cytometry. (D) The plot shows the percentage of IL-15R^{+} cells in each NK cell subset (CD56^{dim} and CD56^{bright}) and in total NK cells from healthy donors (n=10) and from melanoma patients (n=10). All experiments were performed in duplicate.

Fig. S3. MD NK cells are functionally impaired/exhausted.

(A) The plot shows the percentage of Lamp-1^{+} NK cells from healthy (n=11) and from melanoma donors (n=10), stimulated overnight with IL-15 and co-cultured with K562 cells for 4h. (B) The percentage of IFNγ^{+} NK cells stimulated with: (left panel) K562 cells (HD n=9; MD n=6); (right panel) IL-12+IL-18 or IL-15 cytokines (HD n=11; MD n=10). (C) The percentage of proliferating NK cells from healthy (n=11) and from melanoma donors (n=10) after 6 days of culture in the presence of IL-12, IL-15 or IL-18 is shown. (D) The plots show the percentage of Lamp-1^{+}, IFNγ^{+} and proliferation in each NK cell subset and in total NK cells from healthy (n=11) and from melanoma donors (n=10), stimulated with IL-2, after a cytotoxic, IFNγ production and proliferation assay. All experiments were performed in duplicate.
**Fig. S4. Tim-3 is up-regulated in MD NK cells.**

(A) The graphs show the expression of Tim-3 receptor (% - left panel; MFI – right panel) in each NK cell subset (CD56\textsuperscript{dim} and CD56\textsuperscript{bright}) from healthy donors (n=10) and from melanoma patients (n=6), in unstimulated conditions and after stimulation. (B) The plots show the percentage of lamp-1\textsuperscript{+} cells in the Tim-3\textsuperscript{+} vs Tim-3\textsuperscript{-} NK cells in unstimulated conditions (left panel) and after IL-2 stimulation (right panel). (C) The plots show the percentage of IFN\textgamma\textsuperscript{+} cells in the Tim-3\textsuperscript{+} vs Tim-3\textsuperscript{-} NK cells in unstimulated conditions (left panel) and after IL-2 stimulation (right panel). All experiments were performed in duplicate.

**Fig. S5. Tim-3 engagement on NK cells from healthy donors inhibits their cytotoxicity.**

(A) Graphs represent the percentage (left panel) and the MFI (right panel) of LAMP-1\textsuperscript{+} NK cells from healthy donors incubated with IgG-coated beads, or anti-Tim-3-coated beads 2h prior to cytotoxicity assay (n=16). Data were normalized to the values obtained for the condition with IgG-coated beads. (B) Reverse-ADCC assay. Graph represents the % of LAMP-1\textsuperscript{+} NK cells from healthy donors co-cultured with P815 cells, in the presence of anti-Tim-3, anti-CD94 or anti-CD16 antibodies for 4h (n=5). (C) K562 cells were stained with CFSE, coated with IgG or anti-Tim-3, and cocultured with NK cells from healthy donors for 4h.
Data were normalized to values obtained for the condition with K562 cells only. The percentage of CFSE\(^{+}\) 7AAD\(^{+}\) K562 cells (“killed cells”) after a killing assay with NK cells from healthy donors is shown. (D) We performed a viability assay using the reverse-ADCC assay. MD NK cells were co-cultured with P815 cells, and anti-Tim-3, anti-CD94 or anti-CD16 antibodies were added to the reaction for 4h (n=5). The graph shows the percentage of Annexin V\(^{-}\) 7AAD\(^{-}\) NK cells (live NK cells) in each condition. All experiments were performed in duplicate.

**Fig. S6.** Tim-3 engagement by Galectin-9 on NK cells from healthy donors and melanoma patients inhibits their cytotoxicity.

(A) Expression of LAMP-1 (MFI, n=20) by NK cells from healthy donors incubated with 25 or 50\(\mu\)g/ml of soluble recombinant human galectin-9 (rh-Gal9) 1h before assessing cytotoxicity (left panel) and NK cell viability (right panel) using K562 cells as targets. (B) Lamp-1 expression (%) by NK cells from healthy donors (n=6): incubated with 50\(\mu\)g/ml of soluble recombinant human galectin-9 (rh-Gal9) alone or in the presence of anti-galectin-9 antibody (10\(\mu\)g/mL) or \(\beta\) lactose (50nM), to block the effect of soluble rh-Gal9. (C) We used another approach to crosslink Tim-3 using Gal9. The melanoma Gmel cells were sorted into Gal9\(^{+}\)-Gmel and Gal9\(^{-}\)-Gmel. **Left panel** shows galectin-9 expression in Gal9\(^{+}\)-Gmel vs Gal9\(^{-}\)-Gmel. Graphs represent the expression of LAMP-1 (MFI) by NK cells from HD (middle panel; n=11) and by NK cells from MD (right panel;
n=4), using Gal9⁺-Gmel or Gal9⁻-Gmel as target cells. Data were normalized to the values obtained for the condition without rh-Gal9 (Fig. S6A and S6B) or for the condition with Gal9⁻-Gmel cells as target cells (Fig. S6C). (D) The graph shows Lamp-1 expression (MFI) in Tim-3⁺ vs Tim-3⁻ NK cells, in the presence of Gal-9⁻-Gmel or Gal-9⁺-Gmel target cells. All experiments were performed in duplicate.

Fig. S7. Tim-3 blockade improves NK cell function.

(A) The plots show the percentage of Lamp-1⁺, IFN-γ⁺ and proliferating NK cell subset from healthy (n=8) and from melanoma donors (n=8) incubated with 10μg/ml of a different soluble Tim-3 blocking antibody (R&D #AF2365) or IgG isotype control 1h before the cytotoxicity, IFNγ production, and proliferation assays. (B) The plots depict the percentage of lamp-1⁺ NK cells (n=5) incubated with 10μg/ml of soluble Tim-3 blocking antibody 1h before the cytotoxicity assay using melanoma cell lines as target cells: WM1552 (upper panel); WM793b, WM3248 or FM29 (lower panel). (C) The graphs show galectin-9 expression in the four melanoma cell lines: FM29, WM793, WM1552 and WM3248. (D) Graphs represent the expression of LAMP-1 (n=5; left panel), IFNγ (n=5; middle panel) and the percentage of proliferating cells (n=5; right panel) in NK cells from melanoma donors incubated with 10 μg/ml of soluble Tim-3 blocking antibody (clone 2E2) or IgG1 isotype control 1h before the functional assays. (E) The graph shows the expression of LAMP-1 (MFI, n=32) in NK cells from healthy
donors incubated with 10 or 20μg/ml of soluble Tim-3 blocking antibody 1h before the cytotoxicity assay using K562 cells as targets. (F) The plot depicts the percentage of CFSE⁺ 7AAD⁺ K562 cells (“killed cells”) after 4h of co-culture with HD NK cells untreated or treated with 10 or 20μg/ml of soluble blocking anti-Tim-3 antibody 1h before the functional assay. (G) To determine if Galectin-9 was involved in our system, we used a blocking antibody for Galectin-9 for 1h (10 and 20ug/ml) and assessed the cytotoxicity in NK cells from healthy donors (n=6) against K562 cells. Graphs represent percentage of LAMP-1⁺ cells (left panel) and MFI (middle panel) of LAMP-1⁺ cells. Right panel shows galectin-9 expression in the K562 cells. Data were normalized to the values obtained for the condition without blocking antibody. All experiments were performed in duplicate.

**Fig. S8.** Tim-3 blockade has no effect in NK cell viability but increases the expression of CD16 in these cells.

(A) Viability assay with MD NK cells untreated or treated with 5, 10 or 20μg/ml of soluble Tim-3 blocking antibody for 5h. The graph shows the percentage of Annexin V⁻ 7AAD⁻ NK cells (live NK cells). (B) The graph shows the percentage of lamp-1⁺ MD NK cells after 1h treatment with 10μg/ml CD16 blocking antibody alone or after 1h treatment with 10μg/ml CD16 blocking antibody followed by 1h treatment with 10μg/ml soluble Tim-3 blocking antibody. (C) The plot shows the expression of CD16 receptor (MFI) in MD NK cells untreated or after 1h treatment with 10μg/ml of soluble Tim-3 blocking antibody. (D) The graphs depict
the MD NK cell expression of KIR3DL1 (left panel - MFI) and NKG2D (right panel - %) after two days of culture with 200U/mL of IL-2 untreated or treated with anti-α chain, anti-β chain or anti-γ chain blocking antibodies. (B and C) Data were normalized to the values obtained for the condition without Tim-3 blocking antibody. All experiments were performed in duplicate.