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Supplemental Information

IL-10-Engineered Human CD4^{+} Tr1 Cells Eliminate Myeloid Leukemia in an HLA Class I-Dependent Mechanism

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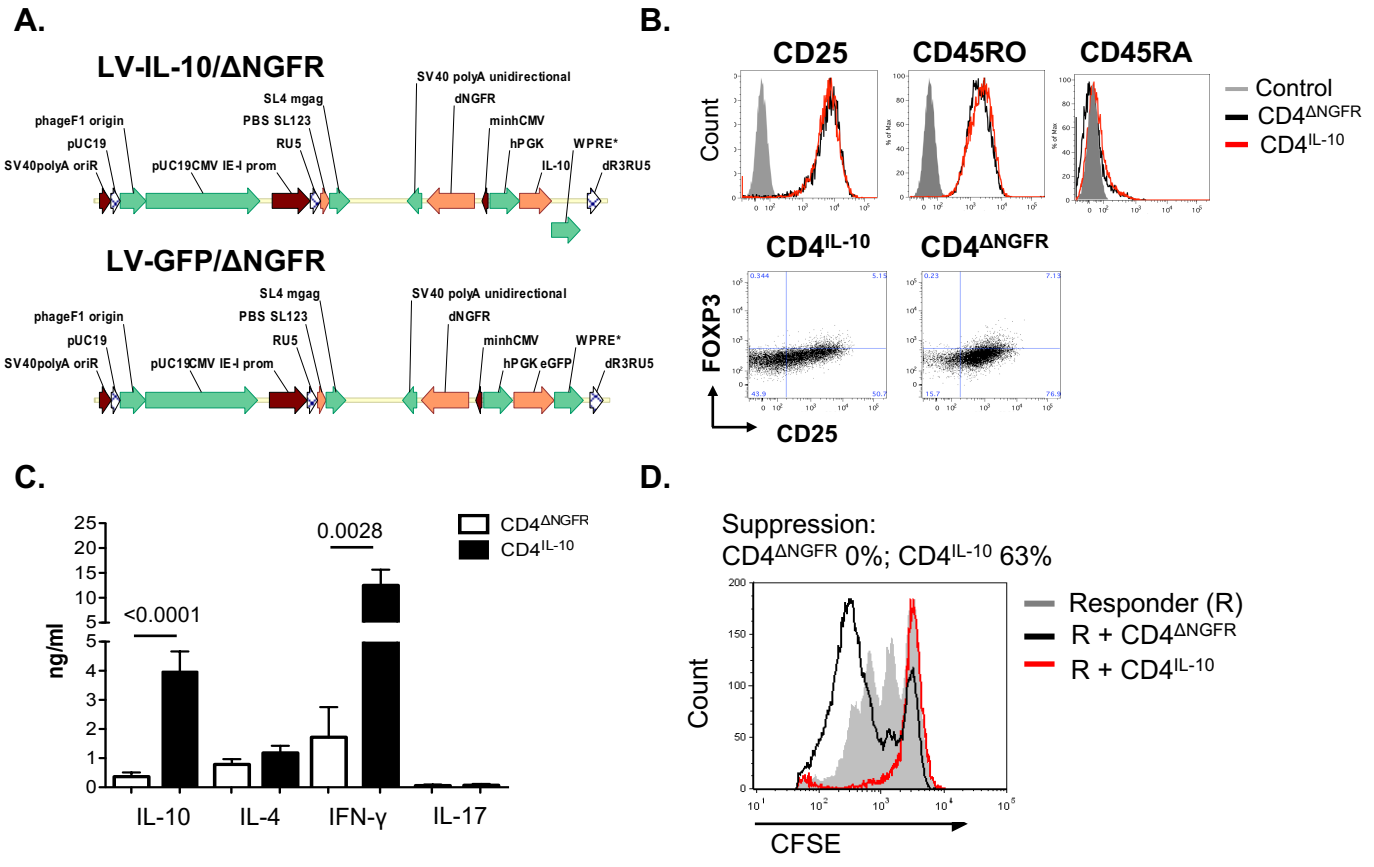
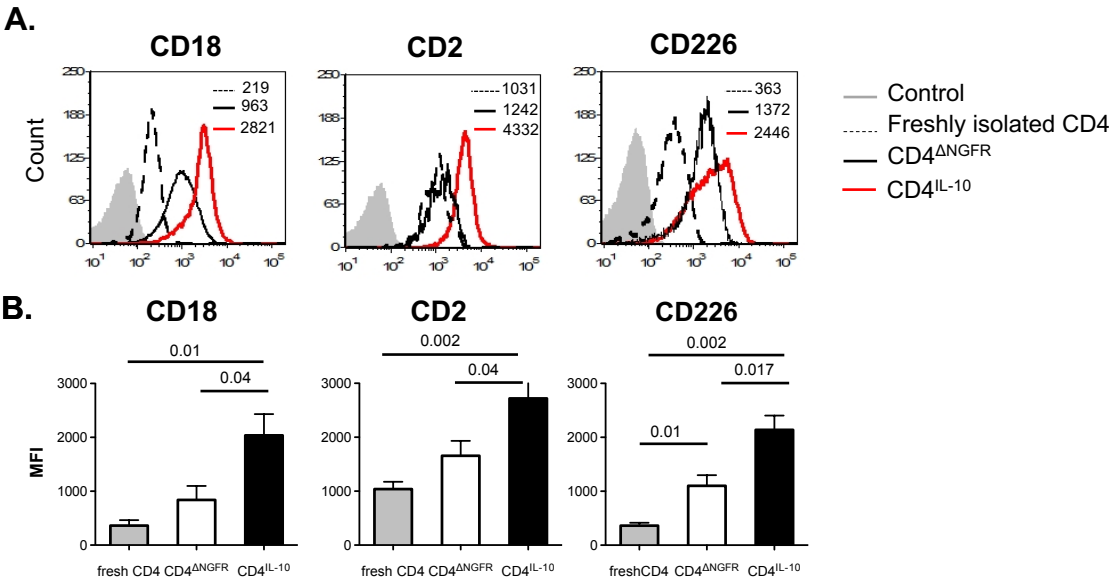


Locafaro G. and Andolfi G. et al., Figure S1

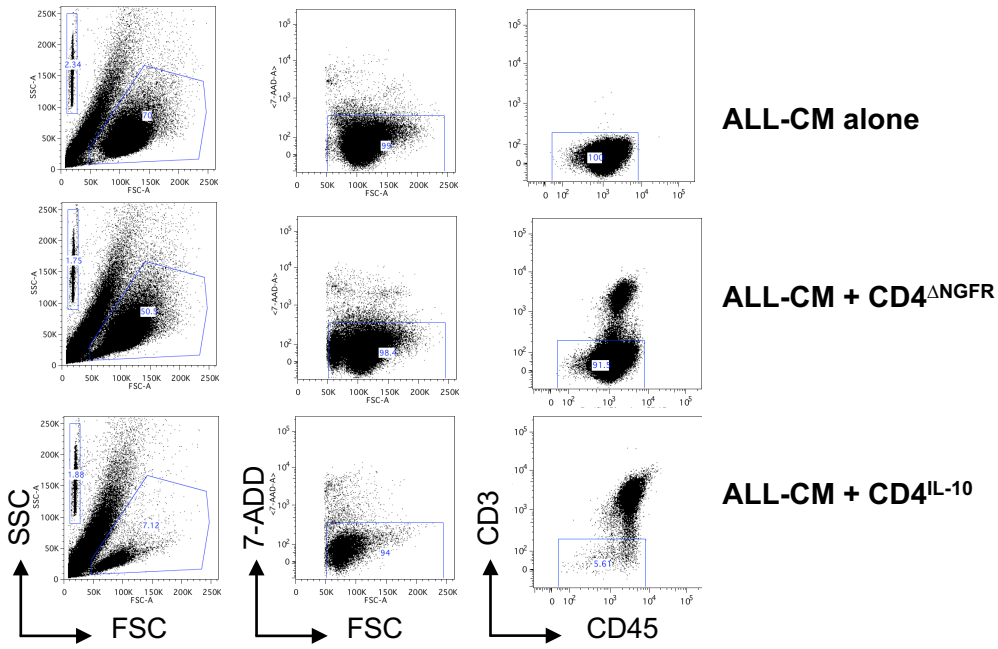
Figure S1. $CD4^{IL-10}$ cells are phenotypically and functionally super-imposable to Tr1 cells. A. Scheme of the LV-IL-10/ΔNGFR and LV-GFP/ΔNGFR vectors. The presence of the bidirectional promoter (mCMV/PGK) allows the co-regulated expression of ΔNGFR and human IL-10 or GFP genes. Ψ, encapsidation signal including the 5' portion of GAG gene (GA); RRE, Rev-responsive element; cPPT, central poly-purine tract; polyA, poly-adenylation site from the Simian Virus 40; CTE, constitutive transport element; WPRE, woodchuck hepatitis virus post-transcription regulatory element. **B**. $CD4^{IL-10}$ cells are $CD25^{+}$, but not $FOXP3^{+}$ memory T cells. $CD4^{IL-10}$ and $CD4^{\Delta NGFR}$ cells were analyzed for the expression of CD25, CD45RO, CD45RA, and FOXP3 by FACS after gating on the $CD4^{+}$ $\Delta NGFR^{+}$ population. One representative donor out of 4 tested is shown. **C.** Cytokine production profile of $CD4^{IL-10}$ and $CD4^{\Delta NGFR}$ cells. $CD4^{IL-10}$ and $CD4^{\Delta NGFR}$ cells were stimulated with immobilized anti-CD3 and soluble anti-CD28 mAbs for 48 hours and IL-10, IL-4, IFN-γ and IL-17 levels were measured by ELISA in culture supernatants. All samples were tested in duplicate-triplicate. Mean±SEM of n=15 for IL-10 and IL-4, n=12 for IFN-γ, and n=5 for IL-17 of $CD4^{IL-10}$ cells and $CD4^{\Delta NGFR}$ cells are presented. *P*, Mann Whitney test. **D**. $CD4^{IL-10}$ cells suppress T cell proliferation *in vitro*. Allogeneic PBMC cells were labeled with CSFE and stimulated with immobilized anti-CD3 and anti-CD28 mAbs alone or in the presence of $CD4^{IL-10}$ cells or $CD4^{\Delta NGFR}$ cells at 1:1 ratio. After 4 days of culture, the percentage of proliferating PBMC was determined by CSFE/eFluor dilution of $CD4^{+}\Delta NGFR^{-}$ cells. One representative donor out of six tested is shown. The suppression mediated by $CD4^{IL-10}$ cells or $CD4^{\Delta NGFR}$ cells was calculated as follows: ([proliferation responder-proliferation transduced)/proliferation responder] x 100).



Locafaro G. and Andolfi G. et al., Figure S2

Figure S2. $CD4^{IL\text{-}10}$ cells express CD2, CD226 and CD18. $CD4^{IL\text{-}10}$, $CD4^{\Delta NGFR}$, freshly isolated $CD4^+$ T cells were analyzed for the expression of CD18, CD2, and CD226 by FACS after gating on the $CD4^+$ $\Delta NGFR^+$ population. **A.** One representative donor out of 6 tested is shown Numbers indicate the MFI. **B.** Mean ± SEM of 9 donors tested is shown. *P,* Mann Whitney test for comparison between $CD4^{IL\text{-}10}$ and $CD4^{\Delta NGFR}$ cells, unpaired T cells for comparison between $CD4^{IL\text{-}10}$ or $CD4^{\Delta NGFR}$ and freshly isolated $CD4^+$ T cells.

A.



B.

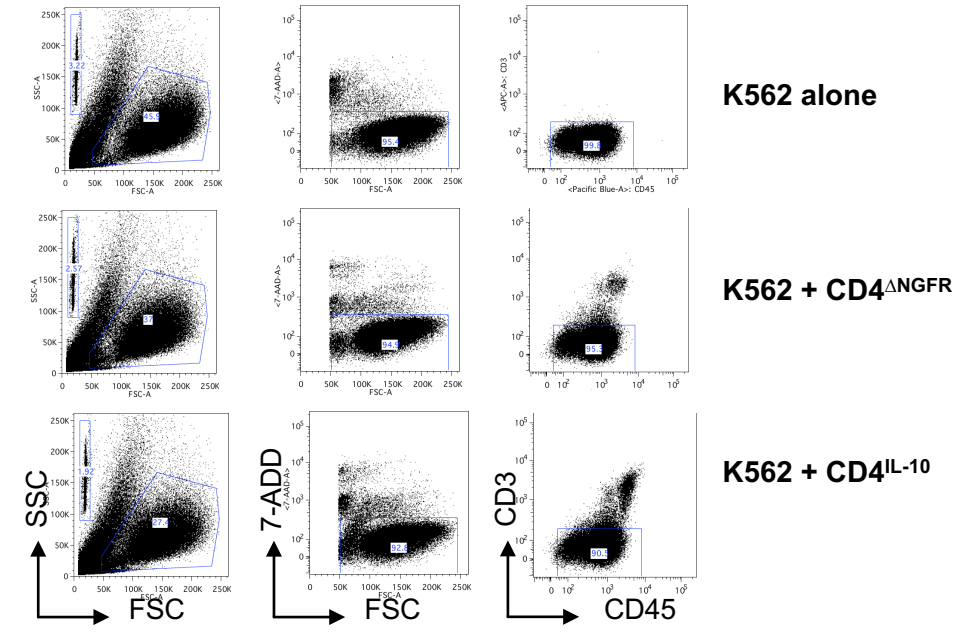


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Figure S3. Gating strategy to define residual leukemic cell lines after co-culture experiments. $CD4^{IL\text{-}10}$ and $CD4^{\Delta NGFR}$ cells were co-cultured with ALL-CM (**A**) or K562 cell lines (**B**) at 1:1 ratio for 3 days. Residual leukemic cell lines ($CD45^{low}CD3^{-}$) were analyzed and counted by FACS according to the gating strategy depicted. One representative donor is shown.

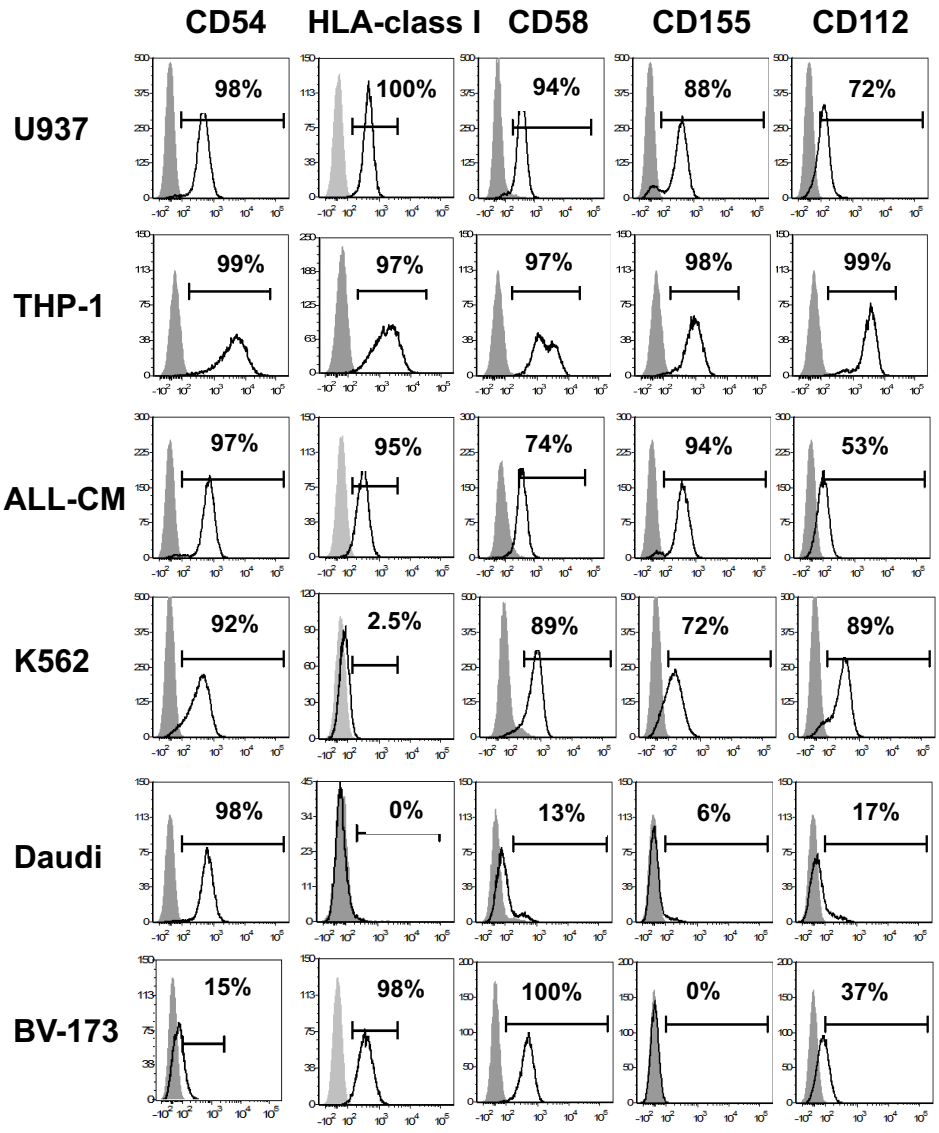

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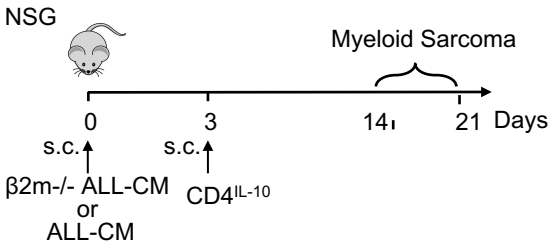
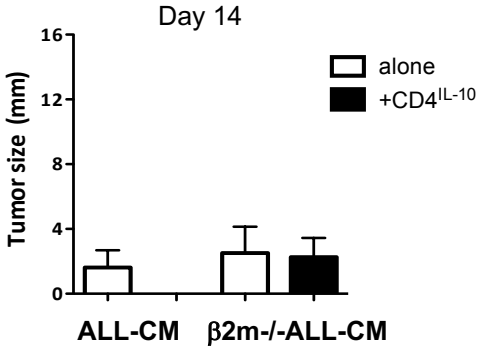
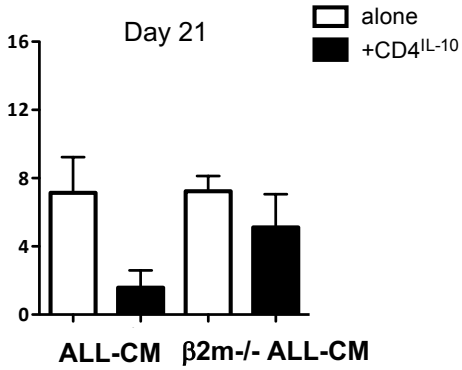
Figure S4. $CD4^{IL\text{-}10}$ cells specifically kill *in vitro* leukemic cell lines expressing CD54, HLA-class I, CD58, CD155, and CD112. The expression of the CD54, HLA-class I, CD58, CD155, and CD112 on U937, THP-1, ALL-CM, K562, Daudi and BV-173 cell lines was analyzed by FACS. Numbers indicate the percentages of positive cells (black solid line) according to isotype control (filled light gray histogram). Data representative of at least three independent experiments are shown.



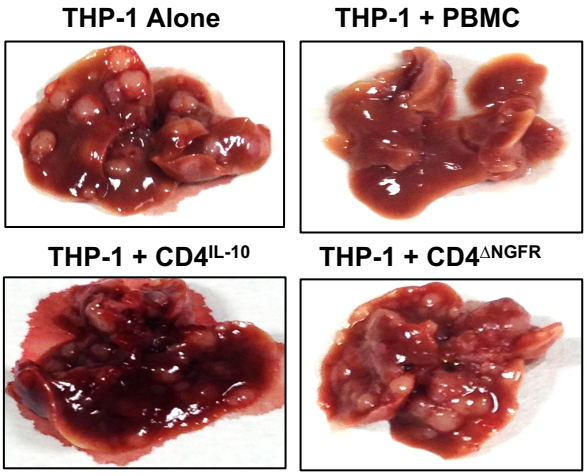




Locafaro G. and Andolfi G. et al., Figure S5

Figure S5. $CD4^{IL\text{-}10}$ cells do not kill HLA-class I deficient cells *in vivo*. NSG mice were sub-cutaneously injected with $\beta 2m^{-/-}$ ALL-CM or ALL-CM ($2x10^6$ cells/mouse) and on day 3, mice received allogeneic $CD4^{IL\text{-}10}$ cells ($1x10^6$ cells/mouse). Tumor growth was measured at the indicated time points. Data obtained from one experiments are presented.

A.



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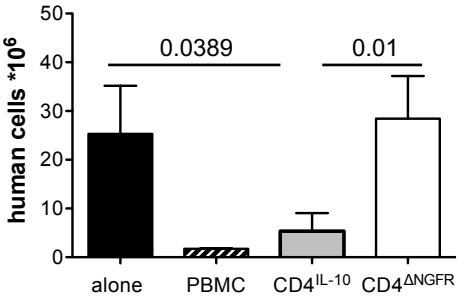

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Figure S6. $CD4^{IL\text{-}10}$ cells mediate anti-tumor activity in a model of THP-1 extra-medullary tumor. NSG mice were intravenously injected with THP-1 leukemia cells ($2x10^6$ cells/mouse) and 14 days later mice received allogeneic PBMC ($2x10^6$ cells/mouse), $CD4^{IL\text{-}10}$ or $CD4^{\Delta NGFR}$ cells ($1x10^6$ cells/mouse). A. Liver cistis in control untreated and PBMC-, $CD4^{IL\text{-}10}$- and $CD4^{\Delta NGFR}$-treated mice. One representative liver of n=12 mice for THP-1, n=3 mice THP-1+PBMC, n=8 mice for THP-1+$CD4^{IL\text{-}10}$ cells, and n=7 mice for THP-1+$CD4^{\Delta NGFR}$ cells. B. Absolute number of human THP-1 cells infiltrating the liver of mice. Mean±SEM of n=9 mice for THP-1, n=3 mice THP-1+PBMC, n=7 mice for THP-1+$CD4^{IL\text{-}10}$ cells, and n=7 mice for THP-1+$CD4^{\Delta NGFR}$ cells. Data obtained from two independent experiments are presented; *P*; two-sided Mann Whitney test.

Table S1. Polyclonal $CD4^{IL\text{-}10}$ cells recovery

	$CD4^{\Delta NGFR}$	$CD4^{IL\text{-}10}$
% of transduction (n=23)	44.5±12.8	51.3±12.5
% of recovery after beads selection (n=8)	44.8±15	57±19
Fold increase after 1 feeder (n=6)	3.7±0.7	2.3±0.6
Fold increase after 2 feeders (n=3)	5.2±0.7	3.0±0.7

Mean±StD are presented

Table S2. Allo-CD4^{IL-10} cells recovery

	CD4$^{\Delta NGFR}$	CD4^{IL-10}
% of transduction (n=24)	46±11	52.5±13.8
% of recovery after beads selection (n=10)	52.2±18	45±9.9
Fold increase after 1 feeder (n=6)	5±4.3	2.1±1
Fold increase after 2 feeders (n=6)	3.9±2	3.8±1.9

Mean±StD are presented