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## **Expansion of natural CD8<sup>+</sup>Tregs for cell therapy**

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## BACKGROUND

Tregs play a critical role in the control of immune responses and tolerance induction; however our understanding of CD8<sup>+</sup> Tregs is limited while they are particularly promising for therapeutic application. We previously reported that CD8<sup>+</sup>CD45RC<sup>low</sup> Tregs use IFN $\gamma$  and IL-34 to suppress donor responses in a rat model of allotransplantation (Guillonéau et al, JCI, 2007; Bezie et al, JCI, 2015). Here, we aim to characterize human CD8<sup>+</sup> Tregs to assess their potential for cell therapy in transplantation.

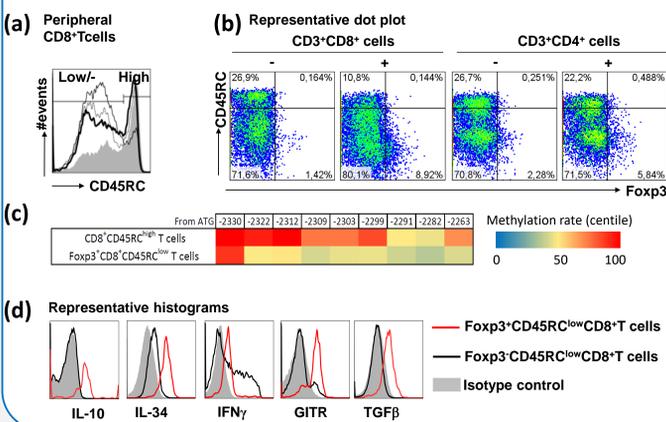
## MATERIALS AND METHODS

- All cells were isolated from blood of healthy volunteers.
- Expansion process:** CD8<sup>+</sup>CD45RC<sup>low</sup> Tregs and CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs were sorted by FACS Aria and plated with allogeneic APCs (Tregs:APC ratio 1:4) or in presence of anti-CD3 (OKT3 clone, coated, 1 $\mu$ g/ml) and anti-CD28 (CD28.2 clone, soluble, 1 $\mu$ g/ml) mAbs in RPMI 1640 medium supplemented with AB serum 10%, IL-2 (1000U/ml) and IL-15 (10ng/ml). Cells were stimulated again with mAbs at day 7, and cytokines were freshly added at day 7, 10 and 12. IS were added at day 0 and day 7 when indicated.
- Proteomic analysis:** T cells were stimulated 7h with PMA and ionomycin including 4h with BFA before cytokines staining and were analyzed by flow cytometry.
- Transcriptomic analysis:** Tregs were sorted, expanded or not for 14 days, and analyzed by 3<sup>rd</sup> DGE RNA-sequencing as compared to non-expanded freshly sorted Tregs.
- Suppression assay:** CD4<sup>+</sup>CD25<sup>+</sup> T cells were sorted by FACS Aria, labeled with CFSE and stimulated by allogeneic APCs, or cDCs, or pDCs, in presence or absence of CD8<sup>+</sup>CD45RC<sup>low</sup> or high T cells, or CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs. 50 $\mu$ g/ml of blocking Abs or isotypic controls were added at day 0 when indicated. 0.4 $\mu$ m pore size Transwell membrane were used to separate Tregs from Teff. Proliferation was quantified by CFSE dilution in DAPI CD4<sup>+</sup>T cells after 5 days co-culture.
- Cytotoxicity assay:** Apoptosis was assessed by DAPI/Annexin V staining after 15h culture of Tregs with target cells.
- Skin transplantation model:** NSG mice were grafted with 1cm<sup>2</sup> human skin and injected 4 to 6 weeks later with 5x10<sup>6</sup> human PBMCs  $\pm$  a range of expanded CD8<sup>+</sup>Tregs. Allogeneic rejection was assessed by macroscopic observation.
- GVHD model:** NSG mice were 1,5Gy-irradiated and injected with 1.5x10<sup>7</sup> PBMCs  $\pm$  a range of expanded CD8<sup>+</sup>Tregs.

## CD8<sup>+</sup>CD45RC<sup>LOW</sup> T CELLS ARE NATURAL TREGS ACTING THROUGH IFN $\gamma$ , IL-34, TGF $\beta$ and IL-10 SECRETION

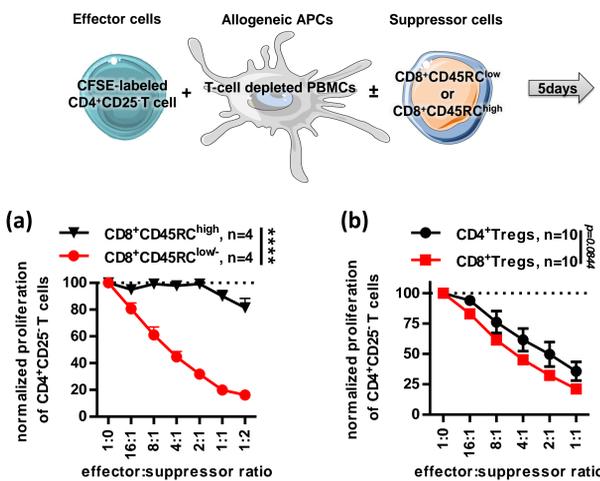
### 1. Identification

CD45RC marker is differentially represented in blood CD8<sup>+</sup>T cells in healthy volunteers (a, overlay of 5 individuals). CD45RC<sup>low</sup> cells include most of Foxp3<sup>+</sup> cells (b). Low methylation of CpG sites suggest a stable expression of Foxp3 (c). Foxp3<sup>+</sup>CD8<sup>+</sup>CD45RC<sup>low</sup> T cells exclusively express IL-10, IL-34, GITR and TGF $\beta$  and low level of IFN $\gamma$  (d).



### 2. Function

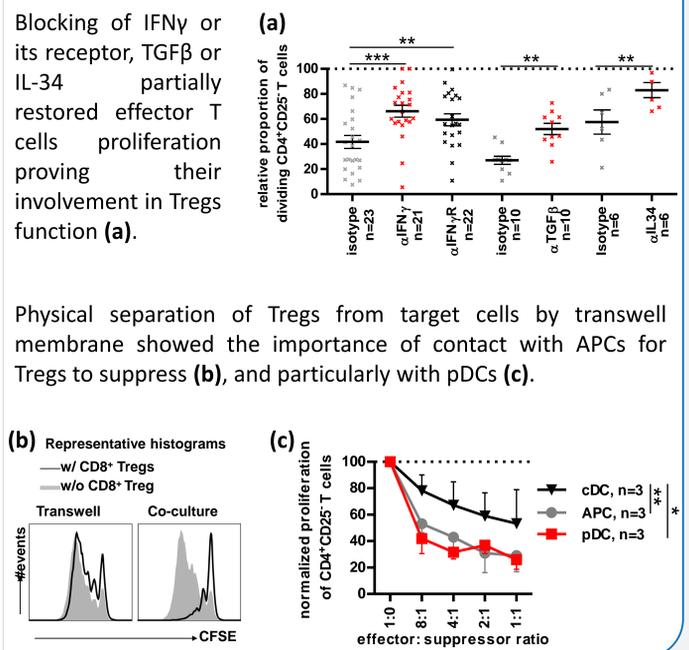
In contrast to CD8<sup>+</sup>CD45RC<sup>high</sup> T cells, CD8<sup>+</sup>CD45RC<sup>low</sup> T cells efficiently inhibited responder T cells proliferation in response to allogeneic APCs (a) as efficiently as classical CD4<sup>+</sup>Tregs (b).



### 3. Mechanisms

Blocking of IFN $\gamma$  or its receptor, TGF $\beta$  or IL-34 partially restored effector T cells proliferation proving their involvement in Tregs function (a).

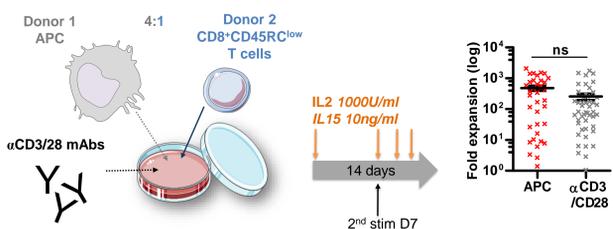
Physical separation of Tregs from target cells by transwell membrane showed the importance of contact with APCs for Tregs to suppress (b), and particularly with pDCs (c).



## CELL THERAPY USING EXPANDED CD8<sup>+</sup>TREGS INHIBITS TRANSPLANT REJECTION

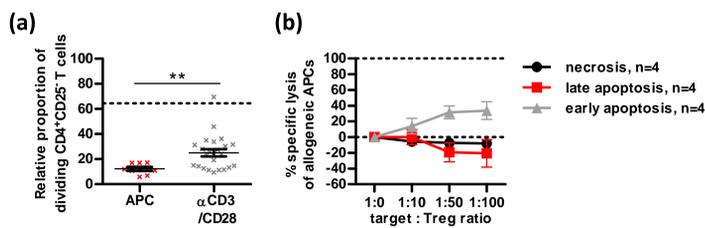
### 1. Expansion process

CD8<sup>+</sup>CD45RC<sup>low</sup>Tregs were expanded up to 2000 fold in 14 days in presence of allogeneic APCs or anti-CD3/28 mAbs, high dose of IL-2 and IL-15.

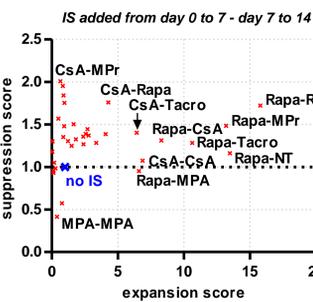


### 2. Function

Expansion process increased suppressive activity (a) but not cytotoxicity (b) of Tregs.



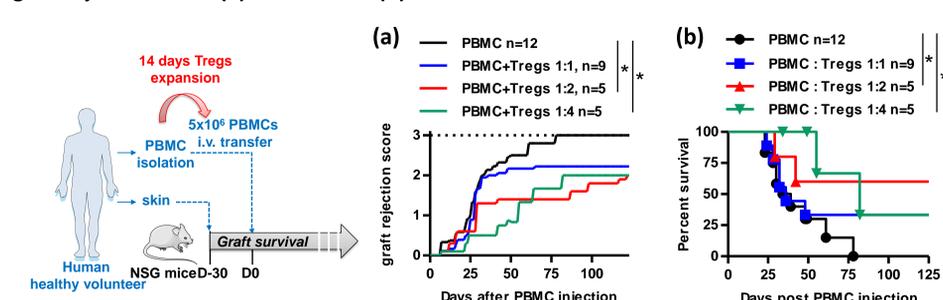
### 3. IS supplementation



Rapamycin addition in culture medium for at least the first 7 days improved Tregs yield and function.

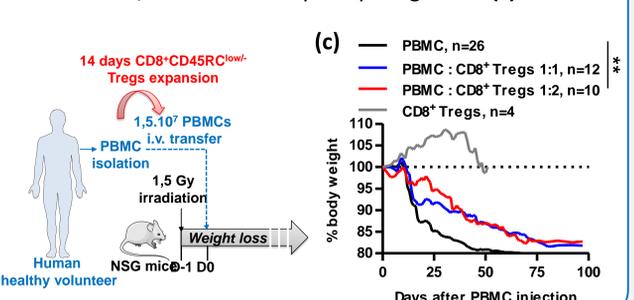
### 5. SOT

Transfer of expanded CD8<sup>+</sup>Tregs significantly inhibited rejection of human skin graft induced by human allogeneic PBMCs injection in NSG mice, in a dose dependent manner, as monitored by graft rejection score (a) and survival (b).



### 6. GVHD

Transfer of expanded CD8<sup>+</sup>Tregs significantly inhibited GVHD development induced by human allogeneic PBMCs injection in NSG mice, as monitored by body weight loss (c).



## CONCLUSIONS

We report here existence of highly suppressive human CD8<sup>+</sup>CD45RC<sup>low</sup>Tregs expressing Foxp3 and producing IFN $\gamma$ , IL-10, IL-34 and TGF $\beta$  to mediate their suppressive activity. We have shown that CD8<sup>+</sup>CD45RC<sup>low</sup>Tregs were as efficient as canonical CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>Tregs for suppression of allogeneic immune responses *in vitro*. Robustly expanded CD8<sup>+</sup>Tregs displayed a specific gene signature and were able to delay human skin rejection and GVHD development in humanized mice. These results open new possibilities in cell therapy in transplantation and by extension in other diseases.