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## Expanded human CD8<sup>+</sup>CD45RClowFoxp3<sup>+</sup> Tregs secreting IFN $\gamma$ , IL-10, IL-34 and TGF $\beta$ inhibit human transplant rejection

Séverine Bézie, Dimitri Meistermann, Laetitia Boucault, Stephanie Kilens, Johanna Zoppi, Elodie Autrusseau, Audrey Donnart, Véronique Daguin, Frédérique Bellier-Waast, Eric Charpentier, et al.

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Séverine Bézie<sup>1,2</sup>, Dimitri Meistermann<sup>1,2</sup>, Laetitia Boucault<sup>1,2</sup>, Stéphanie Kilens<sup>1,2</sup>, Johanna Zoppi<sup>1,2</sup>, Elodie Autrusseau<sup>1,2</sup>, Audrey Donnart<sup>4</sup>, Véronique Daguin<sup>1,2</sup>, Frédérique Bellier-Waast<sup>3</sup>, Eric Charpentier<sup>4</sup>, Franck Duteille<sup>3</sup>, Laurent David<sup>1,2</sup>, Ignacio Anegón<sup>1,2</sup> and Carole Guillonau<sup>1,2</sup>

<sup>1</sup> Centre de Recherche en Transplantation et Immunologie UMR1064, INSERM, Université de Nantes, Nantes, France

<sup>2</sup> Institut de Transplantation Urologie Néphrologie (ITUN), CHU Nantes, Nantes, France

<sup>3</sup> Chirurgie Plastique Reconstructrice et Esthétique, CHU Nantes, Nantes, France

<sup>4</sup> INSERM UMR1087, CNRS UMR6291, Université de Nantes, l'institut du thorax, Nantes, F-44000 France;

## 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 (Guillonau 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 in comparison to CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs.

## MATERIALS AND METHODS

- All cells were isolated from blood of healthy volunteers.
- Culture:** 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.
- Proteomic analysis:** T cells were stimulated 7h with PMA and ionomycin including 4h with BFA before cytokines staining and were analyzed by flow cytometry. Apoptosis was assessed by DAPI/Annexin V staining after 15h culture.
- Transcriptomic analysis:** Tregs were sorted, expanded or not for 14 days, and analyzed by 3' DGE RNA-sequencing as compared to non-expanded freshly sorted Tregs.
- Suppression assay:** CD4<sup>+</sup>CD25<sup>high</sup> T cells were sorted by FACS Aria, labeled with CFSE and stimulated by allogeneic APCs in presence or absence of CD8<sup>+</sup>CD45RC<sup>low</sup> or <sup>high</sup> T cells, or CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup> Tregs. Secretion assay kits from Miltenyi were used to sort CD8<sup>+</sup>CD45RC<sup>low</sup> Tregs on IFN $\gamma$  and IL-10 expression. 50 $\mu$ g/ml of blocking Abs or isotypic controls were added at day 0 when indicated. Proliferation was quantified by CFSE dilution in DAPI<sup>+</sup>CD4<sup>+</sup>T cells after 5 days co-culture.
- 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.

## 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. Definition**  
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).

**2. Phenotype**  
Foxp3<sup>+</sup>CD8<sup>+</sup>CD45RC<sup>low</sup> T cells exclusively express IL-10, IL-34, and TGF $\beta$  and low level of IFN $\gamma$ .

**3. 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) in a dose dependant manner (b).

**4. 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.

**5. Relevance**  
CD8<sup>+</sup>CD45RC<sup>low</sup>Tregs secreting IL-10 and IFN $\gamma$  were significantly more efficient to inhibit T cell allo-response than CD4<sup>+</sup>Tregs.

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

**1. Expansion protocol**  
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. Phenotype**  
Expanded CD8<sup>+</sup>Tregs showed increased expression of Foxp3 and Tregs associated molecules at proteomic (a) and transcriptomic (b) levels.

**4. 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).

**5. Relevance**  
Expanded CD8<sup>+</sup>Tregs were significantly more efficient than expanded CD4<sup>+</sup>Tregs to inhibit rejection of human skin graft induced by human allogeneic PBMCs injection in NSG mice, as monitored by graft rejection score (a) and survival (b).

## 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 show that CD8<sup>+</sup>CD45RC<sup>low</sup>Tregs are superior to canonical CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>low</sup>Tregs for suppression of allogeneic immune responses in vitro and in vivo to delay human skin rejection in humanized mice. Robustly expanded CD8<sup>+</sup>Tregs displayed a specific gene signature. These results open new possibilities in cell therapy in transplantation and by extension in other diseases.