POSTER 532

CD6 – A Costimulatory Receptor at the Nexus of Teff/Treg Development

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Introduction

- The imbalance of proinflammatory effector T cells (T_{eff}) and anti-inflammatory regulatory T cells (T_{reg}) is associated with autoimmune and inflammatory diseases.
- T_{reg} cells are a critical component of maintaining this balance. (Fig 1.)
- CD6 is a costimulatory receptor expressed on T cells with its greatest expression on pathogenic T_{eff} cells, while Tregs are $CD6^{low}$[1,2]. Notably T cells from CD6knockout mice fail to differentiate to Th17 cells but were seen to skew to a Treg phenotype more rapidly.[1]
- Although the role of CD6 in T_{eff} development is well characterized, little is understood about how CD6 signaling affects T_{reg} development or function.
- Itolizumab is an anti-human CD6 monoclonal antibody that causes cleavage of cell surface CD6 and can be used as a tool to create human T cell populations with different levels of cell surface CD6 expression.[3]
- Itolizumab is currently being evaluated in a phase 3 clinical study (EQUATOR) for the first-line treatment of patients with aGVHD (NCT05263999)
- Using itolizumab, we sought to **investigate the role of CD6 in the development of T_{reg} cells.**



Figure 1. When challenged with foreign antigen, responses are initiated that are aimed at clearing the antigen and restoring homeostasis. Regulatory T cells are a critical component of maintaining this balance. Under some circumstances, the immune response overreacts, and balance is lost leading to autoimmunity or pathology.

Methods

- **Generation of $CD6^{low}$ and $CD6^{high}$ T cells:** Frozen PBMCs (peripheral blood mononuclear cells) were thawed gently in complete media. Total PBMCs were treated with isotype control or itolizumab for 24hrs at 37°C. After 24hrs cells were collected for confirmation of loss of CD6 on CD4 T cells and enrichment of naïve T cells. (Fig 2.)
- **Differentiation of T_{regs} from naïve T cells:** Those PBMCs that were treated with isotype or itolizumab were enriched for naïve T cells with a STEMCELL kit. To differentiate T_{regs} from the $CD6^{low}$ and $CD6^{high}$ naïve T cells, CD3/CD28 activator was added in combination with a T_{reg} differentiation cocktail from STEMCELL for a total of 7 days. (Fig 2).
- **Confirmation of T_{reg} phenotype:** After 7 days under T_{reg} differentiation conditions, $CD6^{low}$ and $CD6^{high}$ were collected for intracellular staining and acquired by a flow cytometer. Cells were confirmed by Foxp3 and Helios co-expression.

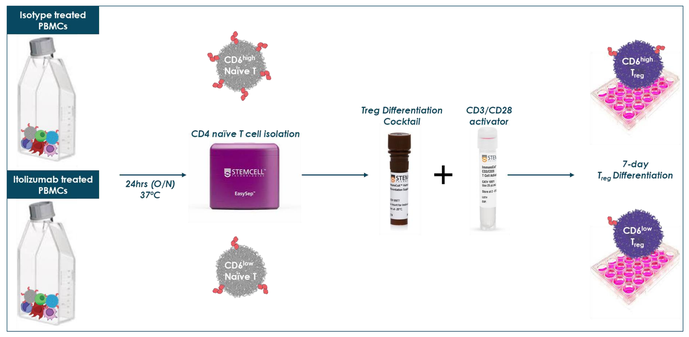


Figure 2. Schematic of the generation of $CD6^{low}$ and $CD6^{high}$ naïve T cells and the differentiation of T_{regs}.

Suppression assay

- Preparation of T_{regs}: At day 7 of differentiation, $CD6^{low}$ and $CD6^{high}$ T_{regs} were collected. T_{regs} were counted and seeded at 100k, 50k and 25k to generate three ratios 1:1, 1:2 and 1:4.
- Generation of T responder cells: Frozen PBMCs from the same donor were gently thawed. PBMCs were enriched for CD4+ T cells and CD25-, a total of two kits were combined to generate these cells. Both kits were from STEMCELL. Once these cells were isolated, cells were labeled with Cell Trace Violet.
- Stimulation of T responders and co-culture with T_{regs}: T responders were added to the wells with T_{regs}. Conditions tested were unstimulated and varying levels of stimuli. Suppression assay was cultured for a total of 4 days at 37°C.
- Calculating suppression of Tresponders: Tresponders that were cultured alone with varying levels of stimuli were measured for their proliferation of Cell Trace Violet. Conventional gating method approach was used to calculate suppression of the Tresponders proliferation.

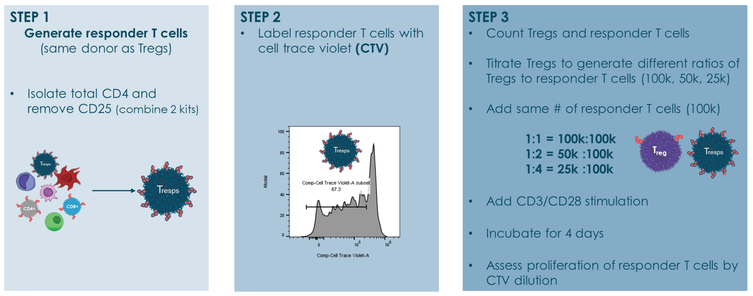
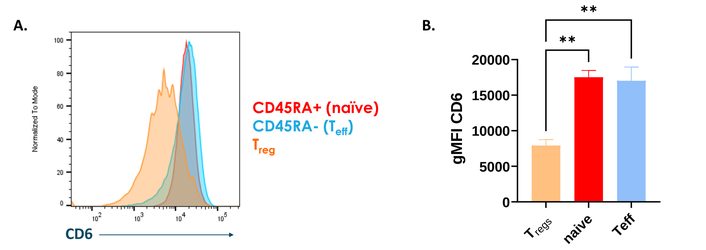


Figure 3. Schematic of the suppression assay

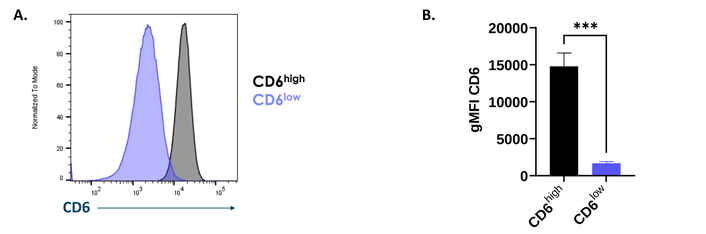
Results

Figure 4. Endogenous T_{regs} express low levels of CD6



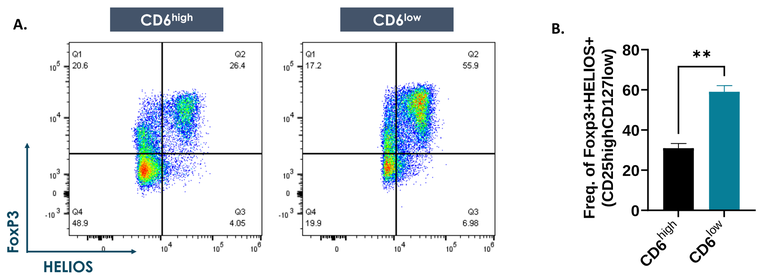
T_{regs} from PBMCs express lower levels of CD6 compared to naïve and effector T cells. Frozen PBMCs were gently thawed and immunophenotyped by CD4+CD45RA+ for naïve T cells, CD4+CD45RA- for effector T cells and CD4+CD45RA-CD25hiCD127low for T_{regs}. Cells were stained for expression of CD6 by a non-competing CD6 detection antibody. Endogenously, T_{regs} express lower levels of surface CD6 compared to T_{eff}. A) histogram of CD6 expression and B) bar graph of geo MFI of CD6 expression. Ordinary one-way ANOVA. P value summary 0.0012 and 0.0017 p value summary **

Figure 5. Enriched naïve T cells treated with itolizumab express lower levels of CD6



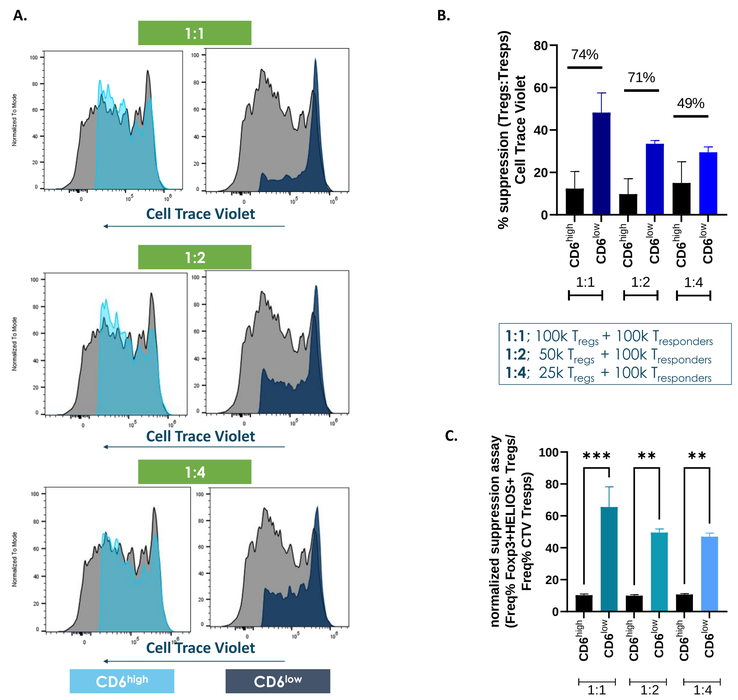
Naïve CD4+ T cells (CD3+CD4+CD45RA+CD45RO-) isolated from total PBMCs previously treated with isotype or itolizumab overnight to generate **$CD6^{high}$ or $CD6^{low}$ naïve T cells**, respectively. Cells were stained for expression of CD6 by a non-competing CD6 detection antibody A) Histogram of CD6 expression and B) Bar graph of geo MFI of CD6. Unpaired t test p value 0.0004 p value summary ****

Figure 6. Naïve T cells enriched from PBMCs treated with itolizumab differentiated into T_{regs} with greater Foxp3 and HELIOS co-expression



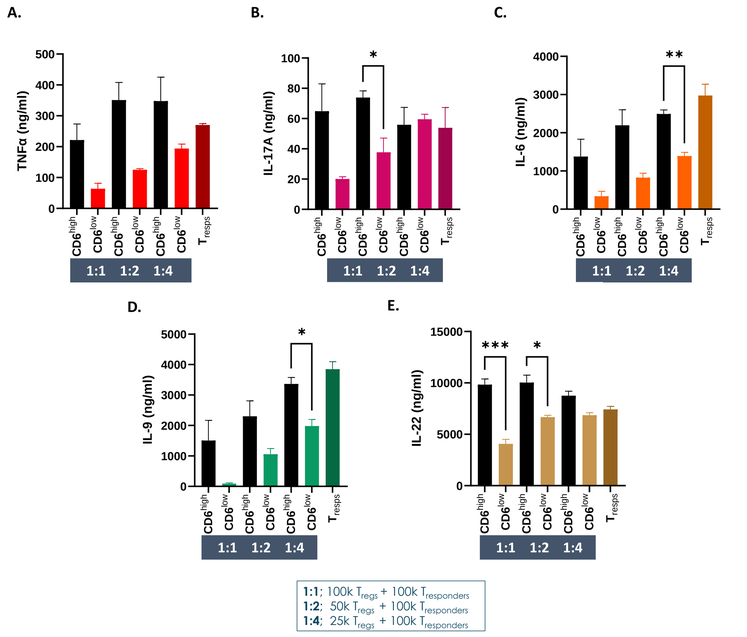
T_{regs} were differentiated for a total of 7 days. A) At takedown T_{regs} were immunophenotyped for Foxp3 and HELIOS expression. These results have been observed across several runs and donors. B) Bar graph of co-expression of FoxP3 and HELIOS. Loss of CD6 on naïve T cells leads to T_{regs} that express higher levels of Foxp3 co-expressed with HELIOS, suggesting a more stable and suppressive T_{reg}[4,5]. Unpaired t test p value 0.0053 p value summary **

Figure 7. Naïve T cells enriched from PBMCs treated with itolizumab differentiated into T_{regs} with greater suppression activity measured by proliferation



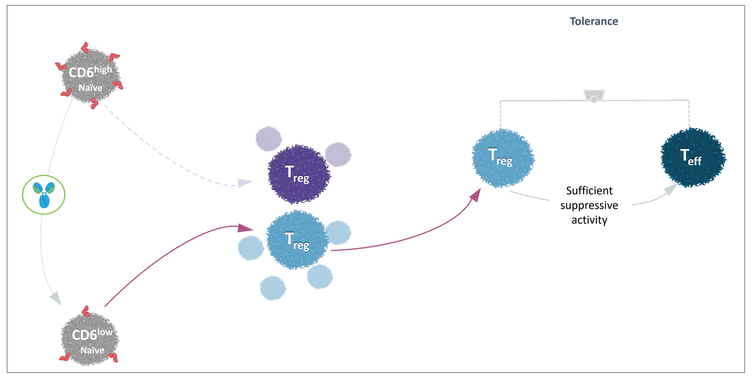
T_{regs} generated from $CD6^{low}$ naïve T cells have greater suppressive activity at all T_{reg} to responder T cell ratios. Similar observations across 3 different donors. A) suppression measured by T_{resps} labeled with Cell Trace Violet. Greatest suppression is observed at a 1:1, T_{reg}:$T_{responder}$ ratio. B) Calculating suppression activity using the conventional gating method. C) Normalizing suppression activity by taking percent of double positive Foxp3+HELIOS+ population and dividing by percent frequency of Cell Trace Violet Tresponders. Ordinary one-way ANOVA p value 0.0002 p value summary ***,**

Figure 8. Naïve T cells enriched from PBMCs treated with itolizumab differentiated into T_{regs} with greater suppression activity measured by release of pro-inflammatory cytokines by $T_{responders}$



T_{regs} generated from $CD6^{low}$ naïve T cells have greater suppressive activity at all T_{reg} to responder T cell ratios. Similar observations across 3 different donors. Legendplex™ Hu Th cytokine panel (12-plex) was used to measure the release of pro-inflammatory cytokines released by Tresponders alone (last bar on each graph) or T conditions. Those T_{regs} generated from $CD6^{low}$ naïve T cells confirm suppression activity not only by proliferation (Fig. 7) but by blockade of pro-inflammatory cytokines such as A) TNFα, B) IL-17A, C) IL-6, D) IL-9 and E) IL-22. Unpaired t test *, **, ***

Figure 9. Itolizumab Treatment Restores Immune Balance Between T_{eff} and T_{reg}



Schematic of the restoration of the immune balance by itolizumab. Itolizumab treatment generates naïve T cells that have lower levels of surface CD6. Differentiation of $CD6^{low}$ naïve cells into Tregs yields Tregs that have higher suppressive capabilities which contributes to the restoration of immune balance & tolerance.

Conclusions

- This is the first study to directly characterize the role of CD6 in the development and activity of T_{reg} cells.
- T_{regs} derived from $CD6^{low}$ cells had greater co-expression of FOXP3 and HELIOS (~2-fold) vs. T_{regs} derived from isotype-treated $CD6^{high}$.
- These T_{regs} possessed greater suppressive function, with increased inhibition of proliferation and cytokine release by T responder cells by at least 50% more than T_{regs} derived from $CD6^{high}$ cells. Similarly, $CD6^{low}$ Tregs were able to suppress T responder's production of proinflammatory cytokines by 60-90% compared to $CD6^{high}$ Tregs.
- These data suggest that reduced levels of cell surface CD6 facilitate the development of T_{reg} cells with greater stability and suppressive activity. Therefore, modulating the levels of CD6 may increase the Treg/Teff ratio in patients with autoimmune and inflammatory diseases which contributes to restoring balance to the immune system.

References

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Disclosures

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