Modulating levels of cell surface CD6 is a novel mechanism for regulating T cell activity

POSTER 300

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Introduction

- CD6 is a co-receptor primarily expressed on the surface of CD4+ T cells that promotes immune synapse formation, T cell activation, and migration into tissues by engaging its ligand, activated leukocyte cell adhesion molecule, or ALCAM.
- T cells expressing higher levels of CD6 ($CD6^{high}$) have been described to be more pathogenic relative to cells with lower levels of CD6 ($CD6^{low}$). $CD6^{high}$ effector T cells are more proliferative and secrete higher levels of inflammatory cytokines[1]. Furthermore, CD6 knockout mice have decreased proliferation and inflammatory responses[2].
- Early studies have shown that ex-vivo depletion of CD6+ donor cells prior to hematopoietic stem cell transplantation (HSCT) decreases the incidence of acute Graft-versus-Host-Disease (aGVHD)[3,4].
- Collectively, the data suggests that modulating surface levels of CD6, and hence, co-stimulation through the CD6-ALCAM pathway, may be a promising therapeutic that can have favorable outcomes for autoimmune/ inflammatory conditions.
- We recently demonstrated that the anti-CD6 mAb, itolizumab, induces a decrease in surface levels of CD6 (Figure 1)[5].

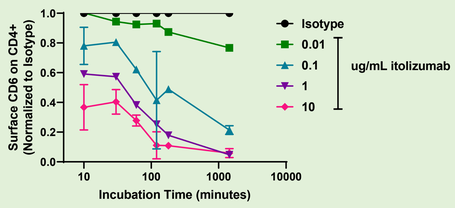


Figure 1. Itolizumab reduces the levels of CD6 from the surface of CD4+ T cells in a dose- and time-dependent manner (CD6 geometric mean fluorescence intensity, gMFI, of itolizumab treated cells normalized to isotype treated cells).

The objectives of the work shown here is to characterize the mechanism of CD6 cleavage and the downstream functional consequences of reduced cell surface CD6.

Methods

Understanding CD6 antigenic modulation on T cells by itolizumab

Commercially available human PBMCs (peripheral blood mononuclear cells) from healthy subjects were thawed and incubated with itolizumab or isotype (hIgG1κ) at 37°C. T cells, monocytes, NK cells, or B cells were enriched from PBMCs and granulocytes from whole blood by magnetic negative selection (STEMCELL Technologies). Enriched T cells were incubated with monocytes, NK cells, B cells, or granulocytes in the presence of isotype or itolizumab. Itolizumab F(ab) and $F(ab)_2$ fragments were generated by papain and pepsin digestion, respectively, to test functional Fc receptor binding on CD6 cleavage. N-glycans were removed from itolizumab, and the efficiency of CD6 cleavage was tested on PBMCs. To further evaluate events that precede the itolizumab-induced cleavage event, PBMCs were pretreated with Fc receptor blocking antibodies targeting FcγRI (CD64), FcγRII (CD32), or FcγRIII (CD16) prior to itolizumab treatment. Surface levels of CD6 was measured by flow cytometry using an anti-CD6 detection antibody that does not compete with itolizumab binding. Soluble levels of CD6 in the supernatant was quantitated using a proprietary electrochemiluminescence assay.

Assessing effects of surface levels of CD6 on T cell stimulation

48-well plates are coated with anti-CD3 (clone: UCHT1) and ALCAM-Fc in PBS for 2 hours at 37°C. After coating for 2 hours, wells were washed with PBS, and blocked with 5% BSA for 30 minutes at 37°C. At the end of the blocking step, wells were aspirated and washed with PBS. PBMCs were thawed, plated, and incubated with itolizumab at 37°C for up to 72 hours.

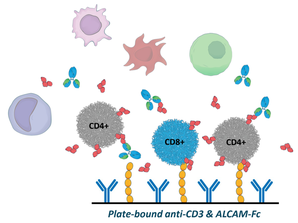


Figure 2. Schematic showing *in vitro* stimulation of PBMCs using plate-bound anti-CD3 and ALCAM-Fc in the presence of itolizumab.

Evaluating effects of surface CD6 on responder cells in a one-way MLR

Responder PBMCs were pretreated with itolizumab or isotype for 24 hours to generate $CD6^{low}$ and $CD6^{high}$ responder cells, respectively. The next day, alloreactive stimulator PBMCs were treated with mitomycin C followed by a series of washes. Responder and stimulator PBMCs were mixed at a 1:1 ratio and incubated for up to 7 days. Cytokine production was assessed using a multiplex bead-based immunoassay (BioLegend).

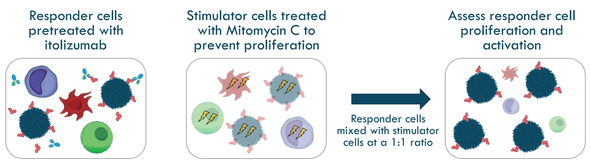
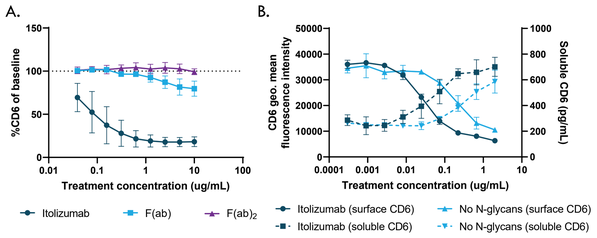


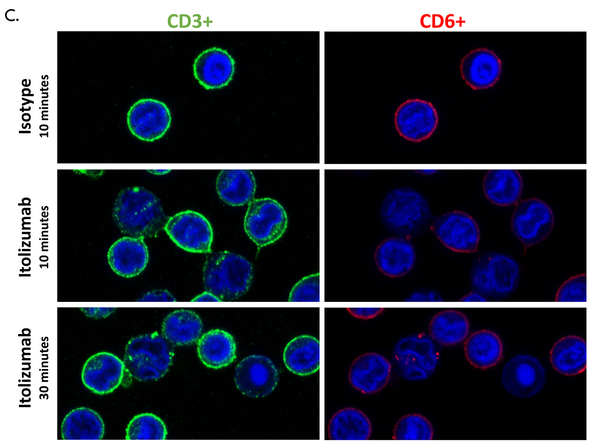
Figure 3. Schematic showing generation of $CD6^{low}$ and $CD6^{high}$ responder cells followed by a one-way mixed lymphocyte reaction setup with allogeneic stimulator cells in the absence of itolizumab.

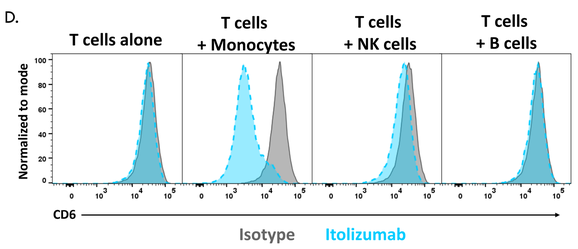
Results

Itolizumab-induced cleavage of CD6 from the surface of T cells requires binding of itolizumab to Fc receptors on monocytes

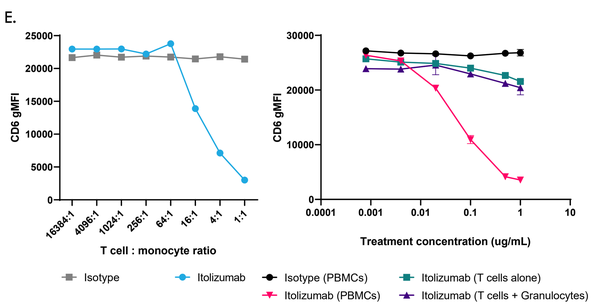


(A) PBMCs were treated with itolizumab, itolizumab F(ab), or $F(ab)_2$ for 2 hours. Reduction in CD6 from the surface of CD4+ T cells was observed with itolizumab treatment, but not itolizumab F(ab) or $F(ab)_2$. Data represented as %CD6 of baseline (no treatment). (B) PBMCs treated with itolizumab or itolizumab without N-glycans. A form of itolizumab without N-glycans was generated to reduce Fc receptor binding efficiency. Itolizumab without N-glycans is less efficient at cleaving CD6 (EC_{50}=0.1932 ug/mL) from the surface of T cells compared to the fully glycosylated form (EC_{50}=0.02935 ug/mL). **This suggests that the Fc region of itolizumab is important for mediating CD6 cleavage.**



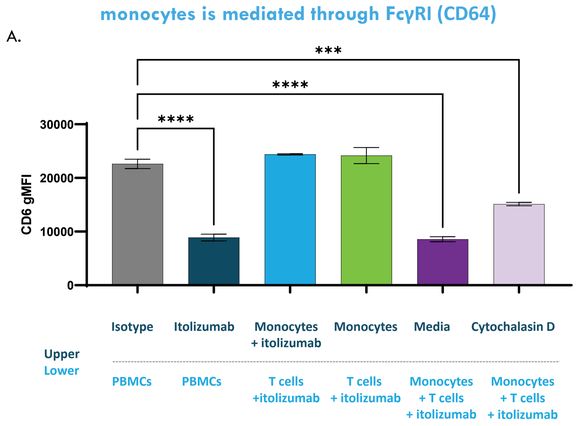


(C) Immunofluorescence of PBMCs show rapid cell-to-cell contact between CD3+ T cells and CD3- cells following itolizumab treatment. Within 10 minutes of treatment with itolizumab, CD3+ T cells come into direct contact with CD3-CD6- cells. (D) To investigate this further, T cells, monocytes, NK cells and B cells were isolated from PBMCs by magnetic negative selection. T cells and monocytes, NK cells, or B cells were incubated together in the presence of 10 ug/mL isotype or itolizumab at a 1:1, 2:1 and 1:1 ratio, respectively, for 2 hours. Significant itolizumab-induced cleavage of CD6 from the surface of CD4+ T cells is only observed in the presence of monocytes. **Itolizumab does not induce significant loss of surface CD6 on T cells alone, T cells + NK cells, or T cells + B cells.**

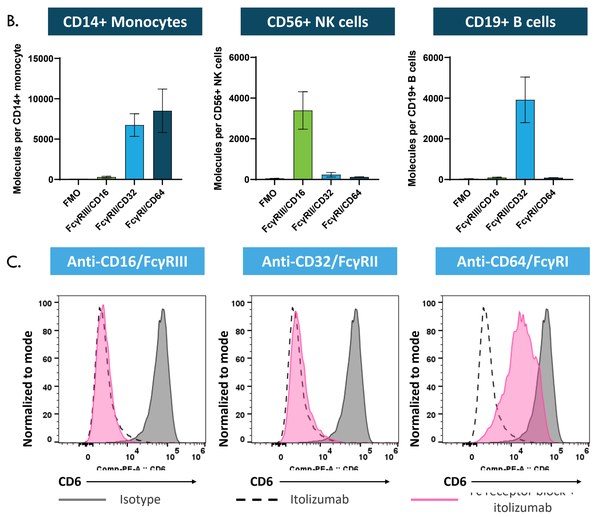


(E) To further study the contribution of other immune cell subsets to itolizumab-induced cleavage of CD6, T cells were treated with isotype or itolizumab for 24 hours in the presence of different T cell to monocyte ratios. Greater loss of CD6 from the surface of CD45RA-CCR7-CD4+ T cells is observed as the ratio of T cells and monocytes approach 1:1 (left). In a separate experiment, granulocytes were tested for induction of CD6 cleavage in the presence of itolizumab. CD6 loss from the surface of CD4+ T cells is only observed when PBMCs were treated with itolizumab. Granulocytes mixed with T cells at a 1:1 ratio do not induce loss of CD6 from the surface of CD4+ T cells in the presence of itolizumab (right). **This suggests that itolizumab-induced cleavage of CD6 is mediated through cell-to-cell contact between T cells and monocytes.**

Itolizumab-induced cell-to-cell contact between T cells and monocytes is mediated through FcγRI (CD64)

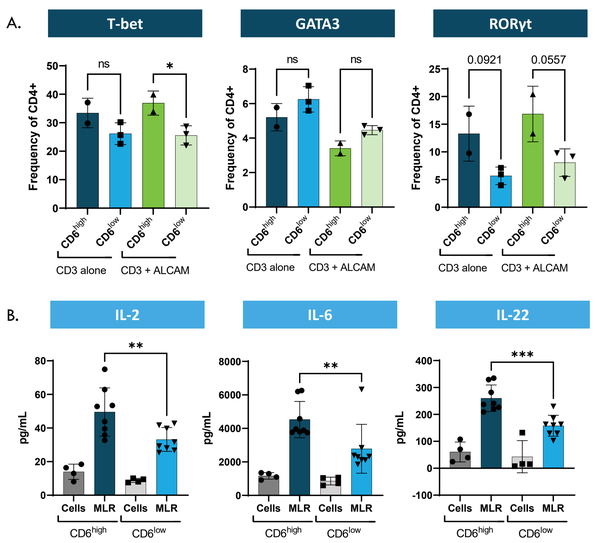


(A) Direct cell-to-cell contact between monocytes and T cells is required for itolizumab-induced cleavage of CD6 from the surface of T cells. Loss of surface CD6 on CD4+ effector memory cells (CD4+CCR7-CD45RA-) is not observed in the presence of itolizumab when monocytes (upper chamber) and T cells (lower chamber) are physically separated. However, a reduction in surface levels of CD6 is observed when monocytes and T cells are treated with itolizumab together in the lower chamber. *** p<0.001, **** p<0.0001



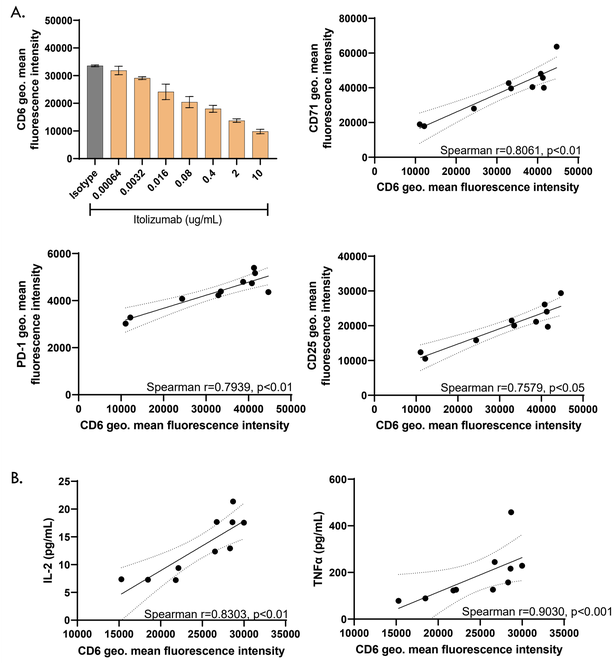
(B) Surface expression of FcγRIII (CD16), FcγRII (CD32), and FcγRI (CD64) was evaluated on three unstimulated PBMC donors. CD14+ monocytes express high levels of FcγRI and FcγRII, but not FcγRIII. CD56+ NK cells express high levels of FcγRIII, but not FcγRI or FcγRII. CD19+ B cells express high levels of FcγRII, but not FcγRI or FcγRIII. (C) To study the involvement of specific Fc receptors in itolizumab-induced cleavage of CD6, PBMCs were pretreated with blocking antibodies against FcγRIII, FcγRII, or FcγRI for 30 minutes prior to itolizumab treatment for 2 hours. Pretreating PBMCs with an anti-CD64/FcγRI antibody significantly reduced the cleavage of CD6 that is induced by itolizumab. **This suggests that the interaction between T cells and monocytes induced by itolizumab is mediated through binding of itolizumab to CD6 on T cells and FcγRI on monocytes.**

$CD6^{low}$ cells have decreased T_{eff} function and are less alloreactive



(A) To assess the function of cells with decreased levels of surface CD6, $CD6^{low}$ (previously treated with itolizumab) and $CD6^{high}$ (previously treated with isotype) cells were stimulated with anti-CD3 and ALCAM-Fc for 48 hours. Following stimulation, $CD6^{low}CD4+$ cells express lower levels of T-bet ($p<0.05$) and RORγt ($p=0.0557$) while no differences in GATA3 expression was observed between $CD6^{low}$ and $CD6^{high}$ cells. (B) In a one-way MLR, $CD6^{low}$ responder cells are less alloreactive and produce less cytokines compared to $CD6^{high}$ responder cells.

Surface CD6 correlates with T cell activation and proliferation



(A) PBMCs stimulated with anti-CD3 (clone UCHT1) and ALCAM-Fc for 72 hours in the presence of a dose-titration of itolizumab resulted in CD4+ T cells with varying levels of surface CD6. Surface markers of activation and proliferation, CD71, PD-1, and CD25 significantly correlated with surface levels of CD6 on CD4+ T cells following 72 hours of stimulation. (B) Furthermore, IL-2 and TNFα production significantly correlated with surface levels of CD6. **This suggests that using itolizumab to alter the surface levels of CD6 is an effective method for modulating T cell activity during stimulation.**

Conclusions

- $CD6^{high}$ cells are highly pathogenic and levels of CD6 have been implicated in numerous autoimmune and inflammatory disease including multiple sclerosis, inflammatory bowel disease, Crohn's disease, ulcerative colitis, and aGVHD.
- Itolizumab-induced CD6 cleavage from the surface of T cells occurs through a transcellular event between a CD6+ T cell and a CD14+ monocyte. This cleavage event is mediated by Fc receptor binding of itolizumab to FcγRI.
- The loss of the extracellular domains of CD6 from the surface results in T cells that are hyporesponsive and less alloreactive to T cell stimulation.

These results further support targeting CD6 to modulate pathogenic T cell activity in the treatment of autoimmune and inflammatory diseases.

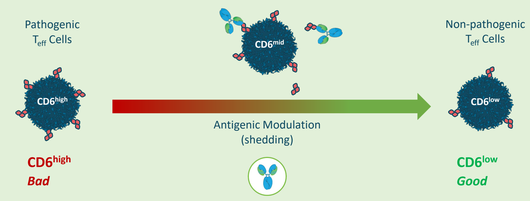


Figure 4. Schematic showing conversion of a pathogenic $CD6^{high}$ cell into a non-pathogenic $CD6^{low}$ cell using itolizumab. The resulting $CD6^{low}$ cell is hyporesponsive to T cell stimulation and less alloreactive.

Acknowledgments

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Disclosures

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Dalena Chu, Phoi Tiet, Valeria Marrocco, Jeanette Ampudia, Maya Rieder, and Cherie Ng are currently employees and stockholders of Equillium

Stephen Connelly is currently an employee, stockholder, and officer of Equillium

References

1. Ma C et al., Journal of Crohn's and Colitis 2019.
2. Li Y et al., PNAS 2017.
3. Soiffer RJ et al., Journal of Clinical Oncology 1992.
4. Soiffer RJ et al., Bone Marrow Transplantation 1998.
5. Chu D et al., American Society of Hematology 2021.

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