Antigenic Modulation of CD6 by Itolizumab is a Novel Mechanism for Effector T Cell Inhibition

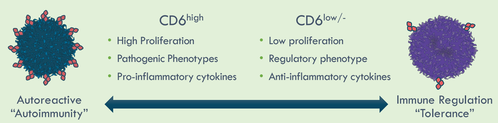
POSTER 995

Dalena Chu[1], Valeria Marrocco[1], Phoi Tiet[1], Jeanette Ampudia[1], Stephen Connelly[1], Cherie Ng[1]

[1]Equillium Inc., San Diego, CA

Introduction

Acute Graft-Versus-Host Disease (aGVHD) is a serious complication of hematopoietic stem cell transplantation (HSCT) that is primarily driven by alloreactive T cells. CD6 is a costimulatory receptor primarily expressed on T cells and promotes synapse formation, T cell activation, and migration into tissues by engaging its ligand, activated leukocyte cell adhesion molecule (ALCAM). CD6 is expressed on reconstituting T cells soon after HSCT (Rambaldi et al., 2019). Early studies have shown that ex-vivo depletion of $CD6^+$ donor cells prior to HSCT decreases the incidence of aGVHD (Soiffer et al., 1992; Soiffer et al., 1998). The reduced levels of aGVHD were attributed to an increased prevalence of $CD6^-$ T cells that were less alloreactive (Rasmussen et al., 1994). Therefore, modulating activity of the CD6-ALCAM pathway may ameliorate aGVHD.



Itolizumab is a humanized anti-CD6 monoclonal antibody that was previously described to block the engagement of ALCAM, thereby inhibiting T cell activity and trafficking. Here, we highlight a novel mechanism of itolizumab in which antibody-mediated loss of CD6 from the cell surface results in $CD6^{low}$ T cells that are hyporesponsive to T cell stimulation.

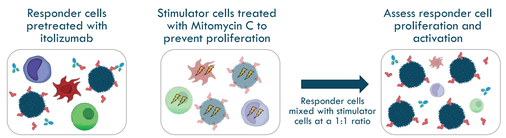
Methods

CD6 antigenic modulation by itolizumab

PBMCs (peripheral blood mononuclear cells) were thawed and incubated with itolizumab or isotype (hIgG1κ) at 37°C. Surface levels of CD6 was measured by flow cytometry using an anti-CD6 detection antibody that does not compete with itolizumab binding. PBMCs were treated with selected protease inhibitors in the presence of itolizumab to assess the mechanism of cleavage. CD6 protein levels were measured by western blot from total cell lysate of PBMCs treated with either itolizumab or isotype using a polyclonal anti-CD6 antibody. Soluble levels of CD6 in the supernatant was quantitated by electrochemiluminescence. The soluble form of CD6 was further immunoprecipitated from the cell supernatant of itolizumab treated PBMCs and detected by western blot.

Contribution of monocytes, NK cells, and B cells to CD6 antigenic modulation

T cells, monocytes, NK cells, or B cells were enriched from PBMCs by magnetic negative selection. Enriched T cells were incubated with monocytes, NK cells, or B cells in the presence of isotype or itolizumab. To evaluate cell-to-cell contact requirements, T cells and monocytes were separated using a transwell membrane with a 0.4μm pore to allow for soluble factors to move across while preventing direct cell-to-cell contact. To assess whether functional binding of itolizumab to Fc receptors (FcRs) preceded the CD6 cleavage event, PBMCs were pretreated with FcR blocking antibodies targeting FcγRI (CD64), FcγRII (CD32), or FcγRIII (CD16) alone or in combination prior to itolizumab treatment.

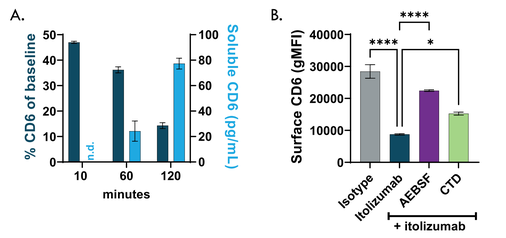


Assessing effects of itolizumab in a one-way mixed lymphocyte reaction

Responder PBMCs were pretreated with itolizumab or isotype for 2 hours prior to the addition of stimulator PBMCs. 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. Proliferation and activation of responder cells were assessed by flow cytometry.

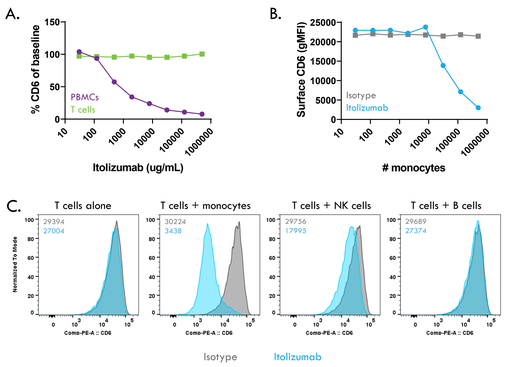
Results

Itolizumab induces cleavage of surface CD6



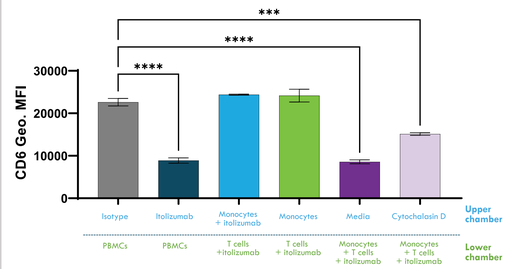
Concomitant decrease in surface CD6 and increase in soluble levels of CD6 is observed following itolizumab treatment. (A) Surface levels of CD6 on effector memory CD4+ cells (CD4+CCR7-CD45RA-) decreased while soluble CD6 levels detected in the cell supernatant increased in a time dependent manner following treatment with 10ug/mL itolizumab. Levels of CD6 is expressed as percentage of CD6 on cells treated with isotype. (B) Itolizumab-induced loss of surface CD6 is inhibited in the presence of AEBSF (4-benzenesulfonyl fluoride hydrochloride), a serine protease inhibitor, and cytochalasin D (CTD), an inhibitor of actin polymerization. (C) CD6 protein levels is reduced in total cell lysate following treatment with itolizumab. However, treatment with AEBSF inhibits loss of cellular CD6 induced by itolizumab. (D) Soluble CD6 is only detected in the supernatant of itolizumab treated PBMCs in the absence of AEBSF. This suggests that itolizumab induces cleavage of CD6 from the surface of T cells and that this event is mediated by a serine protease that requires cell membrane mobility.

Itolizumab-induced modulation of CD6 is not observed on T cells in the absence of Monocytes



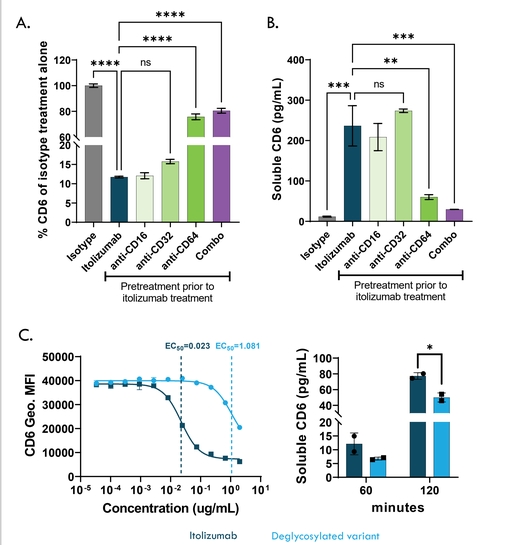
Itolizumab-induced cleavage of CD6 on T cells is only observed in the presence of monocytes and to a lesser extent, NK cells. (A) Enriched T cells and PBMCs were treated with increasing concentrations of itolizumab and loss of surface CD6 was not observed on enriched T cells treated with itolizumab. (B) Enriched T cells were treated with either isotype or itolizumab in the presence of increasing numbers of autologous monocytes. (C) Histograms showing CD6 levels on T cells.

Cell-to-cell contact mediates cleavage of CD6



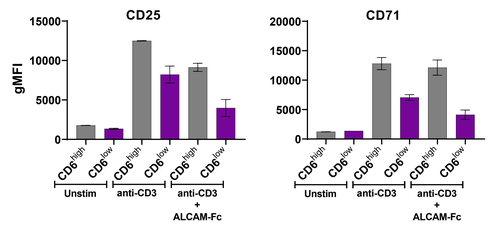
Cell-to-cell contact between monocytes and T cells is required for itolizumab-induced cleavage of CD6 from the surface of T cells. Loss of surface CD6 on CD4+ effector memory cells (CD4+CCR7-CD45RA-) is not observed in the presence of itolizumab when monocytes (upper chamber) and T cells (lower chamber) 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

Itolizumab binding to Fc receptors is a prerequisite for cleavage of CD6 from the surface of T cells



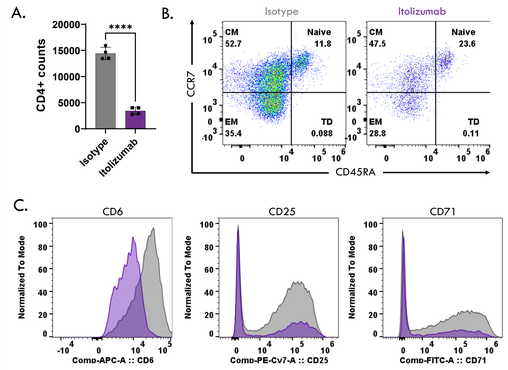
Functional binding of itolizumab to FcγRI (CD64) precedes the CD6 cleavage event. (A) PBMCs were pretreated with Fc receptor blocking antibodies for 30 minutes prior to addition of itolizumab for 2 hours. (A) Surface levels of CD6 on CD4+ effector memory cells and (B) soluble levels of CD6 detected in the cell supernatant. Pretreatment with anti-CD64 significantly reduces itolizumab-induced cleavage of CD6 compared to itolizumab treatment alone and suggests that binding of itolizumab to FcγRI precedes the CD6 cleavage event. Furthermore, (C) the deglycosylated variant of itolizumab is less effective in inducing cleavage of CD6 from the surface of T cells.

$CD6^{low}$ cells are hyporesponsive to stimulation



Cell surface levels of CD25 and CD71 as detected by flow cytometry on CD4 effector T cells (CD45RA-) following activation in the absence of itolizumab. Following stimulation by anti-CD3 or anti-CD3 + ALCAM-Fc, $CD6^{low}$ cells (purple) express lower levels of activation markers compared to $CD6^{high}$ cells (grey). Similar results with PD-1 (data not shown). ** p<0.01, *** p<0.001

$CD6^{low}$ cells are less alloreactive than $CD6^{high}$ cells



A mixed-lymphocyte reaction using alloreactive PBMC donors show that responder cells treated with itolizumab are less alloreactive compared to isotype treated cells. (A) The number of CD4+ counts is significantly reduced with itolizumab treatment following 168 hours. (B) The frequencies of naïve CD4+ increased while that of effector memory (EM) cells decreased with itolizumab treatment. (C) Surface levels of CD6, CD25, and CD71 are reduced with itolizumab treatment (purple) compared to isotype (grey).

Conclusions

Our results reveal a novel mechanism of antigenic modulation by itolizumab in which CD6 is cleaved from the T cell surface and released in a soluble form. Cleavage of CD6 occurs via a membrane-bound serine protease and appears to be dependent upon the engagement of itolizumab with Fc receptor(s) present on monocytes and NK cells. The loss of cell surface CD6 results in T cells with reduced responses to TCR-mediated stimulation and alloreactivity. aGVHD is a serious complication of HSCT that is primarily driven by alloreactive T cells. Here we show that *in vitro*, itolizumab generates T cells that are hyporesponsive and less alloreactive to stimulation by a mismatched unrelated donor. This further validates the potential for CD6 to be a therapeutic target for treating aGVHD and for itolizumab to be an effective treatment for alleviating disease pathology.

Acknowledgments

We would like to acknowledge Jerome Ritz, MD, who is the sponsor of this poster.

Disclosures

This study was funded by Equillium, Inc.

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

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

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Additional Information

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Presented at the American Society of Hematology (ASH) Annual Meeting – December 11-14, 2021