Anti-CD6 Monoclonal Antibody Itolizumab Efficiently Inhibits T Cell Proliferation After *In Vitro* TCR Stimulation in the Setting of Acute Graft Versus Host Disease

Equillium

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I. Introduction and Aims

- CD6 is a co-stimulatory receptor expressed on T cells that binds activated leukocyte cell adhesion molecule (ALCAM, CD166), a ligand expressed on antigen presenting cells and various epithelial and endothelial tissues. The CD6-ALCAM pathway plays an integral role in modulating T cell activation, proliferation and trafficking and is central to inflammation.
- Early studies by Soiffer et al. demonstrated that ex vivo depletion of CD6+ donor cells prior to hematopoietic cell transplantation (HCT) decreased the incidence of acute graft versus host disease (aGVHD), highlighting the importance of CD6+ cells in GVHD pathogenesis.
- Itolizumab, a humanized IgG1 anti-CD6 monoclonal antibody, has been shown to modulate T cell activation and proliferation.

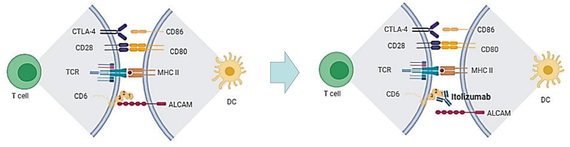

Figure 1. Itolizumab abrogates CD6/ALCAM interaction.

The aims of this study were to characterize:
- Expression of CD6 and ALCAM in patients early after HCT
- *In vitro* activity of itolizumab on T cell responses in peripheral blood from HCT patients pre- and post-aGvHD.

II. Patients and Methods

We analyzed immune reconstitution in 31 adult patients who underwent HLA matched donor HCT for hematological malignancies (Table 1). Patients received peripheral blood stem cell grafts and GVHD prophylaxis with tacrolimus and methotrexate. Twelve of 31 patients developed aGVHD at a median of 58 days, range 27-208, after HCT and systemic treatment was started in 83% of these cases. aGVHD grade severity was 25%, 58.3% and 16.7% of grade I, II and IV, respectively. Patient samples were collected at 1, 2 and 3 months after HCT and analyzed using multi-color flow cytometry. Nine healthy donors (HD) were analyzed as controls.

Table 1. Patient clinical characteristics

Clinical characteristics	N (%)
N	31 (100)
Age at HSCT, median (range)	63 (35-76)
Pt sex, Male	19 (61)
Disease	
AL	18 (58)
MDS	5 (16)
Lymphoma	6 (19)
Others	2 (7)
MUD	23 (74)
MRD	4 (13)
MMRD	2 (6.5)
MMUD	2 (6.5)
RIC	23 (72)

Suppressive activity of itolizumab was tested using peripheral blood mononuclear cells (PBMC) obtained from HD and patients before (preGVHD) and after (postGVHD) aGvHD onset (within 30 days). PBMC were stimulated with antiCD3/CD2/CD28 coated beads in the presence of itolizumab or isotype control (cetuximab) for 72 hours (Fig 2). T cell proliferation was measured by CFSE dilution, while T cell activation and maturation was measured by expression of CD25 and CD45RO, respectively. For statistical analysis, non-parametric unpaired (Mann-Whitney) or paired (Wilcoxon matched-pairs signed rank) test were used.

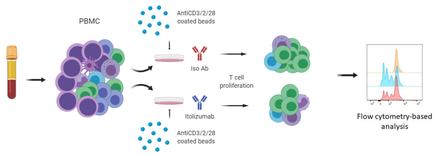

Figure 2. In vitro model of T cell inhibition using antiCD6 Itolizumab.

III. Early CD6+ T cell reconstitution after HCT (Tcon>CD8>Treg)

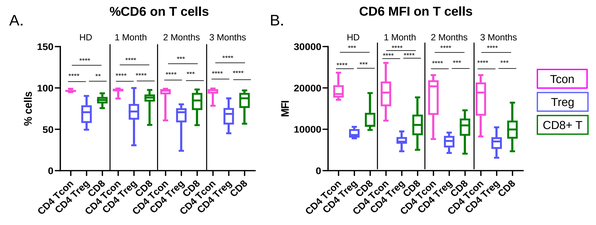

Figure 3. (A) %CD6+ Tcon (non-Treg CD3+CD4+), **Treg** (CD3+CD4+CD25+CD127-) and **CD8+ T cells** (CD3+CD8+) in peripheral blood mononuclear cells from healthy donor (HD) and patients after hematopoietic cell transplantation (HCT). **(B) CD6 median fluorescence intensity (MFI) in Tcon, Treg and CD8 T cells.** HD (n=9); 1 month (n=21); 2 months (n=20); 3 month (n=21). Box and whiskers show median and range. Non-parametric unpaired (Mann-Whitney) test was used for statistical analysis. **p<0.01; ***p<0.001; ****p<0.0001.

CD6+ T cells reconstitute early after transplant, accounting for 95% of CD3 T cells, range 57-100% at 1 month. Similar to HD PBMC, expression of CD6 is highest in CD4 Tcon while CD4 Treg had a lower CD6 expression compared to both CD4 Tcon and CD8 T cells.

IV. Reduced CD6 expression in CD8 Naïve and CM Treg after HCT

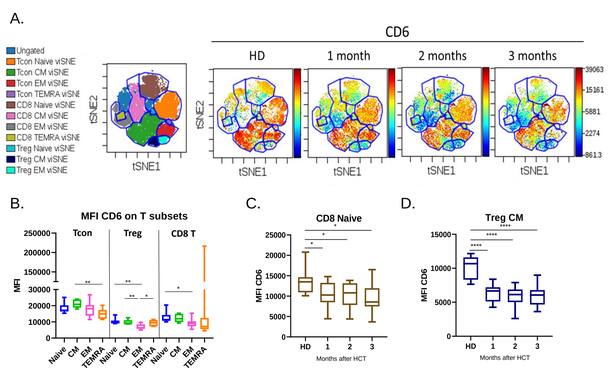

Figure 4. (A) To characterize the expression of CD6 on different T cell subsets, a t-Distributed Stochastic Neighbor Embedding (t-SNE) algorithm was used and the data were visualized using a viSNE map. An example of a viSNE map of CD6 expression on different T cell subsets in a HD and in one patient after HCT at different time points. T cell subsets were divided based on the expression of CD45RA and CCR7: CD45RA-CCR7+ Central memory (**CM**), CD45RA-CCR7- effector memory (**EM**), CD45RA+CCR7- terminally differentiated effector memory (**TEMRA**) and CD45RA+CCR7+ **Naïve** T cells. **(B)** Summary of CD6 MFI distribution in HD within the different T cell subsets. Comparison of CD6 MFI in Naïve CD8+ T cells **(C)** and CM Treg **(D)** in HD and patients after HCT. Box and whiskers show median and range. Non-parametric unpaired (Mann-Whitney) test were used for statistical analysis. *p<0.05; ****p<0.0001.

CD6 expression on Tcon subsets was similar in HD and patients at all 3 time points after HCT. When compared to HD, CD6 expression was reduced in naïve CD8 T cells and CM Treg at all time points after transplant.

V. ALCAM (CD166) is expressed by DCs early after HCT

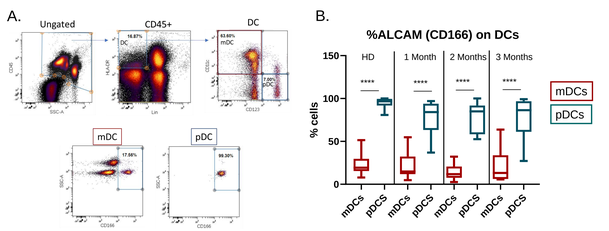

Figure 5. Distribution of ALCAM (CD166+) on myeloid DCs (CD11c+ CD123-) (**mDCs)** and plasmacytoid DCs (CD11c-CD123+) (**pDCs**), **(A)** dot plot example; **(B)** data summary from healthy donor (HD) (n=9) and from patients after hematopoietic cell transplantation (HCT) at 1 month (n=18), 2 months (n=14) and 3 month (n=13). Box and whiskers show median and range. Non-parametric unpaired (Mann-Whitney) test were used for statistical analysis. ****p<0.0001.

ALCAM was expressed by the majority of pDCs and only in a subgroup of mDCs both in HD and in patients after HCT. Moreover, in HD, ALCAM (CD166) expression was detected in 35% of CD14+ monocytes, 23% of CD19+ B cells.

VI. Itolizumab inhibits T cell proliferation, activation and maturation

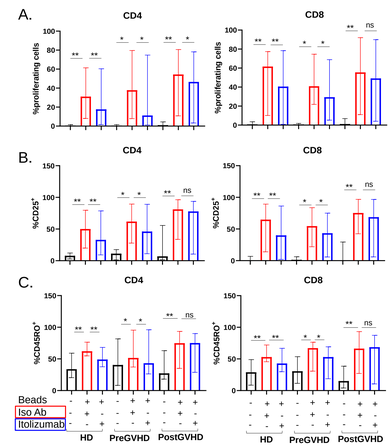

Figure 6. Itolizumab activity on CD4+ and CD8+ T cells after stimulation with antiCD3/CD2/CD28 coated beads in the presence of Isotype control antibody (Iso Ab, cetuximab, red bars) or Itolizumab (blue bars). **(A) T cell proliferation. (B) T cell activation assessed by CD25 expression.** Healthy donor (HD), n=9; preGVHD, n=7; postGVHD, n=8. Bar graphs show median and range. Non-parametric paired (Wilcoxon matched-pairs signed rank) test were used for statistical analysis. *p<0.05; **p<0.01.

Itolizumab inhibited CD4 and CD8 T cell proliferation, activation and maturation in preGVHD samples, similar to HD controls. This effect was less prominent in samples collected from patients who had developed GVHD and were already receiving immunosuppressive medications, potentially confounding the ability to assess the effect of itolizumab in this assay.

VII. Itolizumab does not induce Annexin V and reduces ALCAM expression on T cells

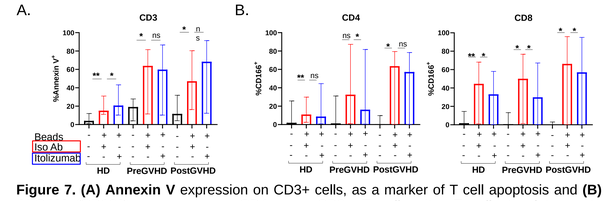

Figure 7. (A) Annexin V expression on CD3+ cells, as a marker of T cell apoptosis and **(B) ALCAM (CD166)** expression on CD4+ and CD8+ T cells after T cell stimulation with antiCD3/CD2/CD28 coated beads in the presence of Isotype control antibody (red bars) or Itolizumab (blue bars). Healthy donor (HD), n=9; preGVHD, n=7; postGVHD, n=8. Iso Ab: Isotype antibody (Cetuximab). Bar graphs show median and range. Non-parametric paired (Wilcoxon matched-pairs signed rank) test was used for statistical analysis. *p<0.05; **p<0.01.

Itolizumab did not increase rates of cell death in HCT patient T cells, suggesting that itolizumab-mediated T cell inhibition was not due to increased T cell apoptosis. In HD, there was a slight increase in Annexin V expression in the itolizumab group vs isotype control. Finally, increased expression of ALCAM (CD166) on both CD4 and CD8 T cells after bead stimulation was partially abrogated in the presence of Itolizumab.

VIII. Conclusions

- CD6+ T cells reconstitute rapidly in peripheral blood after HCT and CD6 expression is highest in Tcon while lowest in Treg (Tcon>CD8>Treg)
- After HCT, the expression of CD6 in each T cell subsets remains stable compared to HD, with the exception of reduced CD6 expression in both CD8 Naïve and Treg CM subsets
- After HCT, ALCAM (CD166) is expressed by a subgroup of mDCs and by the majority of pDCs
- ALCAM (CD166) is expressed on activated T cells and expression is partially abrogated by Itolizumab
- Itolizumab efficiently inhibits T cell proliferation and activation, without increasing apoptotosis after in vitro TCR stimulation of PBMC from both HD and patients after HCT who developed aGvHD, thus representing a potential therapeutic for treating aGvHD
- A phase I/II study using itolizumab as first line treatment in combination with steroids for patients with aGVHD is currently ongoing (NCT03763318)

Affiliations and Disclosure

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