The CD6-ALCAM pathway promotes effector T cell migration

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POSTER 362

Introduction

- CD6 is a co-stimulatory receptor on T cells that **promotes alloreactive T cell proliferation and trafficking** via its ligand activated leukocyte cell adhesion molecule (ALCAM)[1].
- During inflammation, **ALCAM** is **overexpressed** on various tissues including the skin and gastrointestinal (GI) tract[2,3].
- Pathogenic effector T cells (T_{eff}), which express high CD6 levels, are enriched in inflamed tissues and play **a central role in the onset of autoimmune and inflammatory diseases, including Graft versus Host disease (GVHD)**[4].
- CD6 has previously been shown to be a clinically validated target in the prevention of GVHD, with high expression on reconstituting T cells soon after Hematopoietic Stem Cell Transplantation (HSCT)[5].

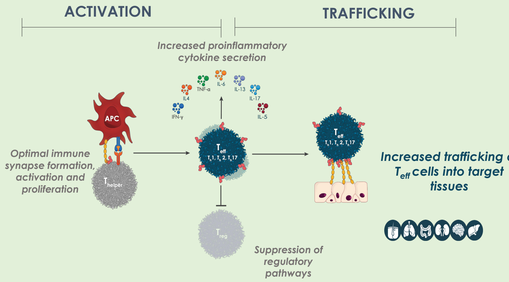
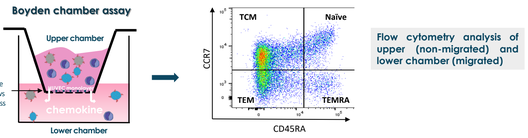


Fig.1 CD6 promotes T cell activation, cell proliferation and trafficking.

Objectives

- **Investigate the role of the CD6-ALCAM pathway in the migration of T_{eff} cells in response to chemokines important in GVHD pathology.**
- **Assess the effect of inhibiting the CD6-ALCAM pathway using EQ001 (itolizumab), a humanized anti-CD6 monoclonal antibody that causes cleavage of CD6 from the cell surface generating CD6^{low} cells[6].**

Methods



Flow cytometry analysis of upper (non-migrated) and lower chamber (migrated)

- The migration of T_{eff} cells was tested using a Boyden chamber assay that incorporated human umbilical vein endothelial cells (HUVEC) which express high levels of ALCAM (Fig2).
- HUVEC cells were plated on a PET insert (3µm) in growth media for 48h to form a monolayer.
- Before entering the transmigration assay, PBMCs were pre-treated with EQ001 or an isotype control. In some experiments, PBMCs were stimulated with soluble anti-CD3 5 µg/ml and anti-CD28 1µg/ml overnight.
- 500k PBMCs were introduced in the migration assay for 24h.
- To generate CD6 low cells, total PBMCs were incubated with EQ001 overnight (O/N) and washed twice to remove the antibody, then introduced in the migration assay.
- 2 donors were used for unstimulated and activated PBMCs.

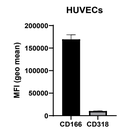
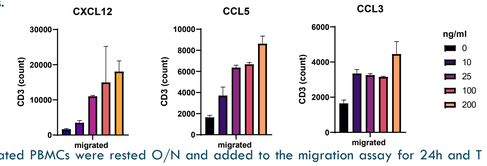


Fig. 2 ALCAM and CD318 (the two main CD6 ligands) expression levels on HUVECs

Results

- **T cells migrate in a dose-dependent manner to CXCL12 and CCL5, but not CCL3**

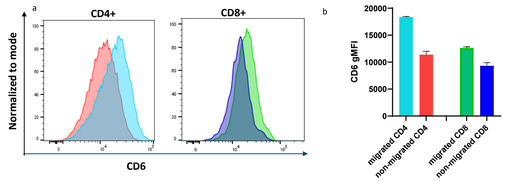
The effect of some of the chemokines expressed in GVHD was tested in vitro to study migration of T cells.



Unstimulated PBMCs were rested O/N and added to the migration assay for 24h and T cells migration in response to increased concentration of chemokines was analyzed by counting CD3+ cells in the lower chamber. CXCL10 was also tested but did not show any T cell specific migration in vitro.

- **T cells that migrate in response to CXCL12 preferentially express high levels of CD6**

We next investigated CD6 expression in migrated and non-migrated T cells

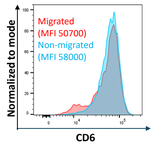


a. Histogram levels of CD6 on non-migrated CD4+ or CD8+ vs migrated T cells.

b. Geometric mean of CD6 MFI on non-migrated vs migrated CD4+ and CD8+ T cells (mean ± SD, p<0.0001, calculated with 2-way ANOVA test).

- **T cells that migrate in response to CCL5 do not express higher levels of CD6**

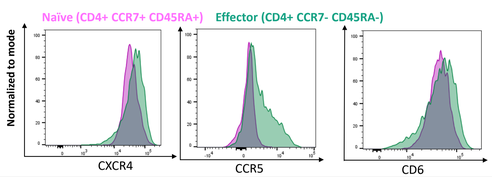
Histogram of CD6 levels on non-migrated CD4+ (blue) vs migrated CD4+ T cells (red) and relative CD6 MFI values.



Analysis of the levels of CD6 in T cells migrated in response to CCL5 showed similar CD6 values suggesting that there is not a preferential migration of T cells expressing high CD6 in response to CCL5, like it is observed for CXCL12.

- **Chemokine receptors expression on effectors vs. naïve CD4+ T cells**

To further understand the differences between CCL5 and CXCL12 on CD6^{high} T cells migration, we investigated the chemokine receptors in naïve and effectors populations that are known to express different levels of CD6. We focused on CD4+ T cells that express higher CD6 levels.

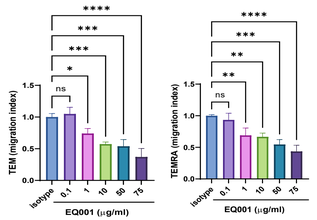


No direct correlation between CCR5, CXCR4 and CD6 was observed. For the study, we focused on CXCL12 because of the preferential migration of CD6^{high} cells. Further studies are in progress to analyze the role of CD6 in the migration induced by CCL5.

- **Migration of CD4+ TEM cells (CCR7- CD45RA-) and TEMRA (CCR7- CD45RA+) in response to CXCL12 is prevented by treatment with EQ001**

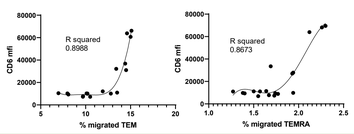
To assess the role of CD6 in the migration of effector T cells, total PBMCs were treated with different concentration of EQ001, generating a gradient of CD6 levels.

UNSTIMULATED PBMCs



TEM (CD4+CCR7-CD45RA-) and TEMRA (CD4+ CCR7- CD45RA+) migration index, calculated by dividing the number of migrated cells in the treatment by the number of migrated cells in the control (mean ± SD, p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****, calculated with One way ANOVA test).

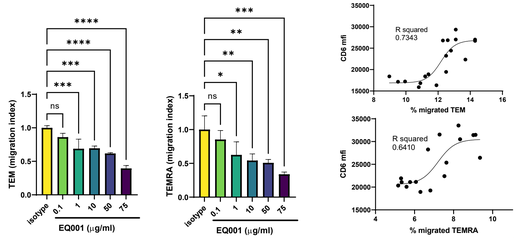
- **Reduced migration of CD4+ TEM and TEMRA correlates with CD6 levels**



Correlation between CD6 levels on TEM CD4+ cells and their % in the migrated cell (left panel).
Correlation between CD6 levels on TEMRA CD4+ cells and their % in the migrated cell (right panel).

ACTIVATED PBMCs

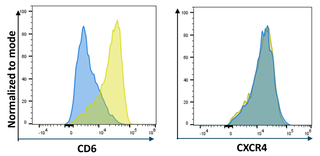
To boost the activity of T cells, total PBMCs were activated with soluble anti-CD3 and anti-CD28 overnight, pre-treated with EQ001 for 1h and introduced in the migration assay.



Migration index of TEM and TEMRA in activated PBMCs treated with isotype or EQ001 at different concentrations (left) and corresponding correlation with CD6 levels (right), (mean ± SD, p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****, calculated with One way ANOVA test).

CXCR4 analysis

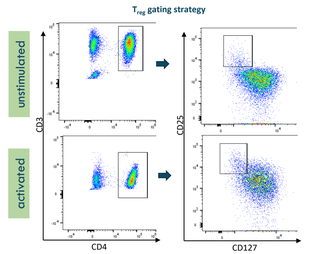
CXCR4 levels in migrated effector cells treated with isotype or EQ001 were investigated in order to show that the effect on migration is specific to CD6

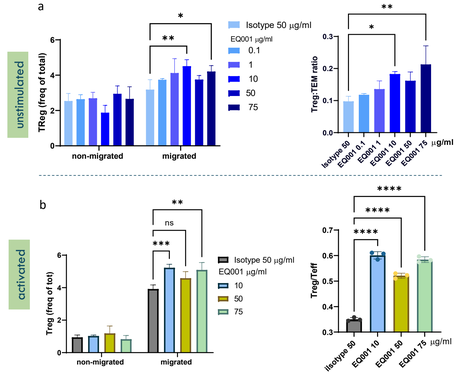


Histograms of CD6 and CXCR4 levels on migrated CD4+ TEM treated with isotype (yellow) or EQ001 50µg/ml (blue).

Blocking the CD6-ALCAM pathway in unstimulated and activated PBMCs reduces the migration of CD4+ TEM and TEMRA cells by ~60% and this correlates with CD6 expression levels on these cells, while it is not due to a reduction in CXCR4 expression.

- **Migration of T_{reg} cells are not affected by EQ001 treatment**

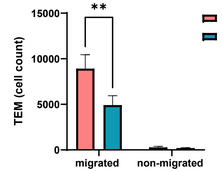




Frequency of T_{reg} in the migrated and non-migrated population for unstimulated (a) and activated (b) PBMCs. Data are shown as frequency of total cells and Teff/Treg ratio. (mean ± SD, p<0.05 *, p<0.01 **, p<0.001 ***, p<0.0001 ****, calculated with two-way ANOVA test).

Blocking the CD6-ALCAM pathway does not affect the migration of T_{reg} through the endothelial layer and, thus, increases the ratio of Treg to effector CD4+ T cells.

- **CD6^{low} T Cells migration is reduced compared CD6^{high} T cells migration**



To discriminate between the effect of blocking CD6 vs. the loss of CD6 due to the cleavage, PBMCs were pre-treated O/N with EQ001 and washed to remove the antibody (to generate CD6^{low} cells) (mean ± SD, p<0.01 **, calculated with two-way ANOVA test).

Loss of CD6 affects the migration of T_{eff} cells to migrate through the endothelial layer.

Conclusions

- **CD4+ and CD8+ T cells that migrate in response to CXCL12 express higher levels of CD6 and that the amount of migration correlates with levels of CD6 expression, suggesting that CD6 is engaged during T cell migration across the endothelial monolayer.**
- **Decreasing CD6 expression led to decreased migration of TEM and TEMRA cells while migration of T_{reg} cells was unaffected, thus suggesting targeting CD6 in aGVHD would decrease infiltration of pathogenic T cells while still permitting modulation of the immune response by T_{reg} cells.**
- **Altogether, this data suggests that CD6-ALCAM pathway is involved in the movement of T cells through the endothelial tissues and confirms CD6 as a target to prevent pathogenic T cell recruitment into inflamed organs**

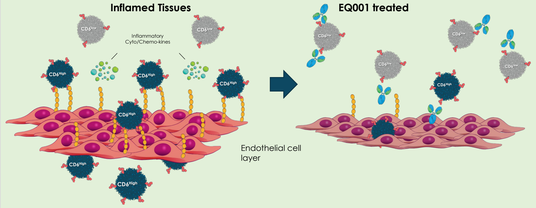


Fig. 3 Blocking the CD6-ALCAM pathway prevents the migration of pathogenic CD6^{high} T cells.

Acknowledgments

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Disclosures

This study was funded by Equillium, Inc. Valeria Marrocco, Jeanette Ampudia, 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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