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Phenotypic and functional characterization of the CD6-ALCAM T cell costimulatory pathway after allogeneic cell transplantation

Running Title: TARGETING CD6 FOR aGVHD TREATMENT

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B.R. designed research studies, conducted experiments, acquired and analyzed data, and wrote the manuscript; H.T.K. analyzed data, performed statistical analysis, and wrote the manuscript; C.R., M.M., M.H. and A.W. acquired data; Y.A. and T.A. help in the design of functional experiments; M.G., J.K., C.C., S.N., V.T.H., J.H.A., R.R., J.A., C.N., S.C. and R.J.S. analyzed data and edited the manuscript; J.R. designed research studies, analyzed data, and wrote the manuscript.

Conflict of Interest Disclosures

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Synopsis

High expression of CD6 on Tcon and ALCAM on DCs and monocytes is maintained during aGVHD, representing a suitable therapeutic target. Itolizumab, an anti-CD6 monoclonal antibody, without inducing T-cell depletion, abrogates ALCAM mediated T-cell proliferation upon TCR engagement.

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Abstract

CD6 is a co-stimulatory receptor expressed on T cells that binds activated leukocyte cell-adhesion molecule (ALCAM), expressed on antigen presenting cells, epithelial and endothelial tissues. The CD6-ALCAM pathway plays an integral role in modulating T cell activation, proliferation, and trafficking. In this study we examined expression of CD6 by reconstituting T cells in 95 patients after allogeneic cell transplantation and evaluated the effects of itolizumab, an anti-CD6 monoclonal antibody, on T cell activation. CD6 T cells reconstituted early after transplant with Treg expressing lower levels of CD6 compared to Tcon and CD8 T cells. After onset of acute graft versus host disease (aGVHD), CD6 expression was further reduced in Treg and CD8 T cells compared to healthy donors, while no difference was observed for Tcon. ALCAM expression was highest in plasmacytoid dendritic cells (pDC), lowest in myeloid dendritic cells (mDC) and intermediate in monocytes and was generally increased after aGVHD onset. Itolizumab inhibited CD4 and CD8 T cell activation and proliferation in preGVHD samples, but inhibition was less prominent in samples collected after aGVHD onset, especially for CD8 T cells. Functional studies showed that itolizumab did not mediate direct cytolytic activity or antibody-dependent cytotoxicity *in vitro*. However, itolizumab efficiently abrogated the costimulatory activity of ALCAM on T cell proliferation, activation and maturation. Our results identify the CD6-ALCAM pathway as a potential target for aGVHD control and a phase I/II study using itolizumab as first line treatment in combination with steroids for patients with aGVHD is currently ongoing (NCT03763318).

Introduction

Acute graft versus host disease (aGVHD) continues to be an important cause of morbidity and mortality after allogeneic hematopoietic cell transplantation (HCT).[1] Steroids provide effective treatment, but most patients with severe aGVHD do not achieve a complete response.[2,3]. Moreover, steroid treatment is often associated with severe toxicities. Novel therapeutic options are needed and different strategies to selectively modulate alloreactive T cells and APCs have been studied for the treatment of steroid refractory GVHD. Various approaches are currently being evaluated in clinical trials including 1) targeting key inflammatory mediators (IL-6, siltuximab or tocilizumab), 2) selective depletion of alloreactive T cells (ricin-conjugated anti-CD3/CD7, anti-CD30 brentuximab, post-transplant cyclophosphamide), 3) modulation of cytokine-driven signal transduction (ruxolitinib), 4) inhibition of target organ homing (anti-integrin$\alpha 4\beta 7$, vedolizumab) and 5) inhibition of costimulatory signals (abatacept).[4] Despite these advances, treatment of steroid refractory GVHD remains a challenge.[5]

The costimulatory receptor CD6 is a 105-130 kDa type I transmembrane glycoprotein belonging to the highly conserved scavenger receptor cysteine-rich superfamily (SRCR-SF).[6] CD6 is expressed on the majority of T cells and minor populations of B and NK cells.[7,8] CD166, activated leukocyte cell adhesion molecule (ALCAM) is the primary ligand for CD6.[9] ALCAM is expressed on APCs and various epithelial and endothelial cells.[10,11] Upon ligation, the CD6-ALCAM complex helps stabilize the immunological synapse between the T cell and the APC. In this context, CD6-ALCAM binding promotes T cell activation, proliferation, maturation, and trafficking from the intravascular space into tissues, including the central nervous

system.[12–17] Early studies by Soiffer and colleagues demonstrated that ex-vivo depletion of $CD6^+$ donor T cells prior to transplantation markedly decreased the incidence of aGVHD, highlighting the importance of $CD6^+$ T cells in pathogenesis of GVHD.[18–21]

Itolizumab, a humanized IgG1 anti-CD6 monoclonal antibody, has been shown to block CD6 signaling, leading to a reduction in T cell activation and proliferation.[22] Itolizumab therapy has been evaluated for treatment of different autoimmune disorders,[9] such as severe chronic plaque psoriasis[23] and COVID-19 cytokine-release syndrome with promising results.[24] However, it is not known whether blocking the CD6-ALCAM pathway with itolizumab can modulate T cell responses after allogeneic HCT in the setting of aGVHD.

In the present study, we characterized the expression of CD6 and ALCAM by reconstituting immune cells in a cohort of 95 patients who underwent allogeneic HCT and examined the effects of itolizumab on T cell responses *in vitro* in the setting of aGVHD. Our results show that T cells and dendritic cells (DCs) expressed CD6 and ALCAM, respectively early after HCT and surface expression of both structures is maintained during aGVHD. Then, we demonstrated the ability of itolizumab to inhibit *in vitro* T cell proliferation and activation in peripheral blood (PB) obtained from patients with aGVHD, in an ALCAM-dependent manner without causing T cell depletion. Our results provide new insights into the mechanisms of action of itolizumab and suggest that targeting the CD6-ALCAM costimulatory pathway represents a novel approach for prevention and treatment of aGVHD.

Methods

Patients and sample collection

This study included 95 patients who underwent allogeneic HCT at the Dana-Farber Cancer Institute and Brigham and Woman's Hospital (Boston, MA) between September 2018 and January 2020. Blood samples were obtained at 1, 2, 3 and 6 months after transplant for analysis of CD6 and ALCAM expression. Samples from 9 healthy donors (HD) were used as controls. Samples from 9 additional patients and 9 HD were used for *in vitro* functional assays (*Supplemental Table S1*). Written informed consent was obtained from all patients and HD prior to sample collection, in accordance with the Declaration of Helsinki. Protocol approval was obtained from the Human Subjects Protection Committee of the Dana-Farber/Harvard Cancer Center.

Monitoring CD6 and ALCAM immune reconstitution

Immune reconstitution was evaluated by flow cytometry using fresh whole blood samples. For analysis, both percentages of positive cells and median fluorescence intensity (MFI) were considered. Two panels of directly conjugated monoclonal antibodies (*Supplemental Table S2*) were used to define the expression of CD6 and ALCAM on functionally distinct T cell and APC subsets, respectively. After staining, cells were acquired on a Fortessa LSR flow cytometer (BD) and analyzed using FlowJo and Cytobank software. Cell gating strategy and markers used for cell subset definition are described in *Supplemental Figure S1 and S2*.

Functional activity of itolizumab *in vitro*

Frozen peripheral blood mononuclear cells (PBMC) obtained from patients who developed aGVHD after transplant (*Supplemental Table S1*) were stimulated using antiCD3/CD2/CD28 beads in the presence of itolizumab or isotype control cetuximab. T cell proliferation, activation and maturation were evaluated after 72 hours of culture using the flow cytometry panel in *Supplemental Table S3*. An example of the gating strategy used in this analysis is shown in *Supplemental Figure S3*. Detailed description of the protocol is provided in *Supplemental Methods*.

Complement dependent cytotoxicity (CDC), antibody direct cytotoxicity (ADC) and antibody dependent cellular cytotoxicity (ADCC) assays

The ability of itolizumab to induce CDC, ADC or ADCC was measured as the percentage of cell lysis in the presence of itolizumab compared to alemtuzumab as positive control or cetuximab as negative control. For ADCC evaluation we also measured the percent $CD107a^+$ cells on NK cells after 6 hours of culture.[25] Detailed descriptions of CDC, ADC and ADCC protocols are provided in *Supplemental Methods*.

T cell stimulation using ALCAM-Fc and anti-CD3 antibody

Recombinant Human ALCAM Fc Chimera (ALCAM-Fc) and anti-CD3 Ab were resuspended in PBS overnight at 4'C in flat bottom 96 well plate. The day after, HD $CD3^+$ T cells were added and cultured in media with itolizumab or cetuximab for 96 hours. Cells were analyzed using flow cytometry (*Supplemental Table S4*). Detailed description of the protocol is provided in *Supplemental Methods* and *Supplemental Figure S3*.

Statistical analysis

Statistical methods are provided in *Supplemental Methods*.

Results

Patient characteristics

To investigate the expression of CD6 and its ligand ALCAM during immune reconstitution after allogeneic HCT, we prospectively studied a homogeneous population of 95 adult patients with hematological malignancies. Clinical characteristics of these patients are summarized in Table 1. Median patient age at the time of transplant was 61 years (range, 19-76). All patients received pre-transplant conditioning with busulfan and fludarabine and GVHD prophylaxis with tacrolimus and methotrexate. Half of the patients received a myeloablative conditioning regimen (50.5%) and almost all patients received PB stem cell (PBSC) grafts (97.9%). A matched unrelated donor (MUD) was used in 63 patients (66.3%). Median follow-up among survivors was 12 months (range, 4-20). Median donor T cell chimerism was 76% 1 month after HCT and 96% at 6 months (Table 2). Acute GVHD occurred in 42 patients (44.2%) at a median of 50 days after transplant (range 20-294 days).

CD6 is expressed on T cells early after transplant but expression levels vary in different T cell populations

We first examined the percent of T cells expressing CD6 as well as the level of CD6 expression on different T cell populations measured by median fluorescence intensity (MFI). While almost all T cells expressed CD6, expression level was highest in conventional CD4 T cells (Tcon) and lowest in CD4 regulatory T cells (Treg) while CD8 T cells displayed intermediate levels of CD6 expression (Figure 1A and B). CD6 expression was maintained at all time points after HCT and both percentage of $CD6^+$

cells and CD6 MFI were comparable between HD and patients at the different time points analyzed. The only exception was a reduction of CD6 MFI in Treg cells after transplant compared to HD. We also divided T cells into 5 maturation stages based on the expression of CD45RA, CCR7 and CD95: Naïve ($CD45RA^+$, $CCR7^+$, $CD95^-$), Stem cell memory (SCM, $CD45RA^+$, $CCR7^+$, $CD95^+$), central memory (CM, $CD45RA^-$, $CCR7^+$), effector memory (EM, $CD45RA^-$, $CCR7^-$) and terminally differentiated effector memory (TEMRA, $CD45RA^+$, $CCR7^-$) (Figure 1C). As shown in the heat map in Figure 1D, Tcon subsets displayed the highest CD6 MFI. In both Tcon, Treg and CD8 T cells, CM T cells have the highest expression of CD6, while EM T cells express the lowest levels. CD6 expression was maintained after transplant at levels similar to HD, with the exception of lower CD6 MFI in CM and EM Treg in patients compared to HD. Finally, we examined whether CD6 was differentially expressed on PD-1 positive T cells, since PD-1 represents a marker of T cell activation and exhaustion that is upregulated after T cell receptor (TCR) engagement.[26] For all 3 T cell populations, we observed that PD-1 positive T cells expressed significantly lower levels of CD6 compared to PD-1 negative T cells in both HD and patients after transplant (Figure 1E).

ALCAM expression on monocytes and dendritic cells (DC) after HCT

To examine whether the CD6-ALCAM pathway represents a suitable target for aGVHD treatment, we also analyzed ALCAM expression on monocytes and DCs. DCs were divided into two major populations based on the expression of CD11c and CD123: myeloid DCs (mDCs, $CD11c^+$ and $CD123^-$) and plasmacytoid DCs (pDCs, $CD11c^-$ and $CD123^+$, Figure 2A). In HD, almost all monocytes and pDCs expressed ALCAM, but

significantly fewer mDCs expressed ALCAM. After transplant, significantly fewer monocytes and pDCs expressed ALCAM compared to HD but the magnitude of this difference was relatively small (Figure 2B). ALCAM expression (MFI) was highest on pDCs, relatively low on mDCs and intermediate on monocytes (Figure 2C). ALCAM MFI on pDCs after transplant was similar to HD controls whereas ALCAM MFI on monocytes was significantly elevated compared to HD in the first month after transplant. In contrast, ALCAM MFI was lower in mDCs after transplant compared to HD. We also examined whether ALCAM expression on mDCs and pDCs was correlated with expression of PD-L1. As shown in Figure 2D and E, there was a weak positive correlation between ALCAM and PD-L1 expression on mDCs, while no correlation was observed for pDCs, where almost all cells uniformly express high levels of ALCAM.

CD6 and ALCAM expression are maintained during aGVHD

To examine whether CD6 and ALCAM expression were affected by the development of aGVHD, we compared CD6 and ALCAM MFI in samples obtained before (preGVHD) and after aGVHD onset (postGVHD) with samples from HD and from patients that did not develop aGVHD (noGVHD). Based on the median time of aGVHD onset, preGVHD samples were compared to noGVHD samples collected at 1 and 2 months after transplant while postGVHD samples were compared with noGVHD samples collected at 3 and 6 months after transplant. As shown in Figure 3A, Tcon expressed high levels of CD6 in both pre- and postGVHD samples that were similar to HD and patients who did not develop aGVHD. CD6 expression by Treg and CD8 T cells in aGVHD samples was significantly decreased compared to HD but was similar to

noGVHD samples. When compared to HD, ALCAM expression was increased on CD14 monocytes preGVHD but there were no differences in expression by mDCs and pDCs (Figure 3B). Despite small differences, ALCAM expression was generally maintained at similar levels in monocytes, mDCs and pDCs in patients with and without aGVHD. We only observed a slightly increased ALCAM MFI on mDCs in postGVHD samples compared to noGVHD samples (Figure 3B).

We also compared samples from patients who developed aGVHD with patients who did not at specific time points after transplant. We observed a small decrease of CD6 MFI in Tcon and CD8 T cells in the GVHD cohort at 1 month, compared with the no-GVHD group. For ALCAM we observed a higher ALCAM MFI and percentage of ALCAM positive cells on mDCs in the GVHD cohort compared to noGVHD at 3 months after transplant (*Supplemental Figure S4 and S5*). To address the impact of systemic aGVHD treatment (steroids) on CD6 and ALCAM expression, we compared samples from patients with low grade aGVHD who only received topical therapy with patients with higher grade aGVHD who received systemic steroids. With the limitation of a low number of samples, we did not observe significant differences for CD6 expression on T cells and no clear trend was observed for ALCAM expression on monocytes and DCs (data not shown).

Itolizumab inhibits *in vitro* T cell proliferation, activation and maturation after TCR engagement

To test the ability of itolizumab to modulate T cell responses after TCR stimulation, cryopreserved PBMC obtained from 9 patients before (preGVHD, median

time between GVHD and sample collection -24, range -32; -7 days) and after GVHD onset (postGVHD, median time between GVHD and sample collection 20, range 0; 64 days) were stimulated with antiCD3/CD2/CD28 in the presence of itolizumab or isotype control antibody (cetuximab). Clinical characteristics of these patients are summarized in *Supplemental Table S1*. Of note, postGVHD samples were collected from patients who were receiving aGVHD treatment (steroids) and one patient was also receiving ruxolitinib at the time of sample collection. Only 1 sample was collected at aGVHD onset prior to starting treatment for aGVHD. T cell proliferation, activation and maturation were evaluated 72 hours after stimulation using flow cytometry. Prior to GVHD onset, itolizumab inhibited CD4 and CD8 T cell proliferation in a similar fashion to HD control. This effect was less prominent in CD8 T cells collected after GVHD onset (Figure 4A). Similar results were observed for CD25 expression, as a marker of T cell activation and CD45RO expression, as a marker of T cell maturation into memory subsets (Figure 4B and C). Itolizumab inhibited these other measures of T cell activation in both CD4 and CD8 T cells from healthy donors and from patients prior to aGVHD. However, increased expression of CD25 and CD45RO on T cells in postGVHD samples were no longer inhibited by itolizumab. After TCR stimulation, we also observed increased ALCAM expression on both CD4 and CD8 T cells from healthy donors and transplant patients. However, except for CD8 T cells collected preGVHD, no statistically significant inhibition was observed using itolizumab (Figure 4D).

Comparisons of inhibition achieved by itolizumab for each of these assays in CD4 and CD8 T cells from healthy donors and patients after transplant are shown in Figure 4E. In all assays, the level of inhibition induced by itolizumab in pre-GVHD T cells was

similar to that achieved in healthy donors. Itolizumab induced less inhibition of proliferation in both $CD4^+$ and $CD8^+$ T cells collected after GVHD onset. Less inhibition was also noted for CD25 expression in $CD8^+$ T cells post GVHD but there were no differences in the ability of itolizumab to inhibit CD45RO and ALCAM expression in both $CD4^+$ and $CD8^+$ T cells in any groups.

Itolizumab does not induce complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADC) or antibody direct cellular cytotoxicity (ADCC)

To test the ability of itolizumab to induce direct or indirect killing of target cells we evaluated the effects of itolizumab in CDC, ADC and ADCC assays. Cell lysis in the presence of itolizumab was compared to alemtuzumab as positive control or cetuximab as negative control. The gating strategy used to measure target cell lysis is shown in *Supplemental Figure S6*. Itolizumab did not mediate CDC or ADC at 6 or 24 hours. After 6 hours of incubation, itolizumab did not induce ADCC measured either by direct lysis of target cells or degranulation of CD107a on NK cells (Figure 5).

Itolizumab activity is dependent on the presence of ALCAM

To test whether the inhibitory activity of itolizumab was dependent on the presence of ALCAM, T cells from HD were stimulated with anti-CD3 antibody with or without ALCAM-Fc for 96 hours. Itolizumab or isotype control cetuximab were added at the start of each culture. As shown in Figure 6A, the addition of cetuximab or itolizumab did not induce proliferation of CD4 or CD8 T cells alone, and anti-CD3 induced proliferation of CD4 or CD8 T cells was not inhibited by the addition of either antibody.

The addition of ALCAM-Fc to anti-CD3 further enhanced CD4 and CD8 T cell proliferation. The addition of itolizumab significantly impaired this increased proliferation when compared to the addition of cetuximab. When T cells were monitored for activation (expression of CD25, Figure 6B) and maturation (expression of CD45RO, Figure 6C), itolizumab had similar effects on both CD4 and CD8 T cells and inhibitory activity was only detected in the presence of both anti-CD3 and ALCAM-Fc. As shown Figure 6D, activation of CD4 and CD8 T cells with anti-CD3 also induces expression of ALCAM and this is further enhanced in the presence of ALCAM-Fc. In this setting, the presence of itolizumab inhibits increased expression of ALCAM even if ALCAM-Fc is not present.

Discussion

Acute GVHD remains a frequent cause of non-relapse mortality and contributes to poor quality of life in patients who have undergone allogeneic HCT. Corticosteroids are administered as first line therapy, but steroid treatment fails to achieve complete responses in up to 50% of cases and is also associated with short and long term toxicities.[5] Ideally, new treatment approaches for GVHD should be aimed at specific modulation of donor T cell alloreactivity and induction of tolerance rather than nonspecific T cell depletion or broad inhibition of T cell functions which can increase risk of tumor immune escape as well as opportunistic infections.[27] In this study we demonstrate that blocking the CD6-ALCAM costimulatory pathway inhibits T cell activation and proliferation thus representing a new potential target for GVHD prevention and treatment. Indeed, CD6 is highly expressed on Tcon after HCT, and its expression is maintained at the onset of aGVHD. Moreover, expression of ALCAM, the primary ligand for CD6, is increased in DCs and monocytes in patients who develop aGVHD. In contrast, Treg express lower levels of CD6,[28–30] and targeting CD6 with itolizumab could preferentially affect Tcon while sparing Treg, thus promoting a more tolerogenic state. CD6 expression also varies as T cells undergo maturation.[31] Naïve and central memory T cells express high levels of CD6 compared to more mature effector memory T cells. Previous studies have suggested that GVHD is primarily mediated by naïve T cells and these cells may also be preferentially affected by itolizumab.[32–39] Similarly, we observed that PD-1 positive T cells, that are already exposed to TCR activation,[26] express lower levels of CD6 compared to PD-1 negative cells. Therefore, targeting the CD6-ALCAM pathway could potentially spare tolerogenic T cell subsets (Treg) and more mature (EM)

pathogen specific T cells, leading to a more favorable balance between GVHD control and maintenances of GVL and anti-infection capabilities.

This hypothesis is supported by previous clinical studies using T12, the first anti-CD6 monoclonal antibody used in the transplant setting. T12, an IgM antibody, induced profound *ex vivo* depletion of $CD6^+$ T cells when donor grafts were manipulated *ex vivo* in the presence of complement.[18] Patients in these trials received myeloablative conditioning and did not receive any prophylactic immune suppressive medications. $CD3^+CD6^-$ T cells recovered rapidly after transplantation with CD6-depleted allogeneic stem cell products with very low incidence of acute or chronic GVHD.[19] Functional immune recovery was not delayed and there was no increase in viral reactivation after transplant. There was also a very low incidence of graft failure and graft versus leukemia activity appeared to be preserved.[20,21,40] Taken together, these previous clinical trials suggested that *in vivo* blockade of CD6 function might also be an effective approach for selective modulation of alloreactive T cells without affecting other critical T cell functions.

As expected, in our cohort of HCT patients, we observed mixed donor-recipient T cell chimerism in the early post-transplant period with a median donor T cell chimerism of 76% at 1 month. We are not able to distinguish CD6 expression on individual donor versus recipient T cells, but we found that CD6 expression on T cells in patients with >95% T cell donor chimerism was similar to levels of expression in patients with <95% T cell donor chimerism. Similarly, no difference was observed for ALCAM expression on monocytes, mDCs and pDCs (*Supplemental Table S5*). This suggests that CD6 and ALCAM are equally expressed on recipient and donor cells after transplant.

Our studies also found that ALCAM expression was increased on both DCs and monocytes in patients with aGVHD compared to patients without aGVHD. In myeloid DC, ALCAM expression was positively correlated with PD-L1 expression. ALCAM is expressed at high levels on all pDCs, and this did not change when aGVHD developed. These observations support the concept that ALCAM is upregulated when DCs are activated.[41] ALCAM expression on DCs is important for the promotion and maintenance of the immunological synapse and this is one of the mechanisms whereby ALCAM promotes T cell activation.[13] Since immune reconstitution varies depending on the transplant setting, the expression of CD6 and ALCAM should also be explored in patients with haploidentical donors and with different GVHD prophylaxis regimens such as ATG or post-transplant cyclophosphamide.

Itolizumab, an IgG1 anti-CD6 monoclonal antibody has been evaluated in different autoimmune disorders,[9] including psoriasis,[23] rheumatoid arthritis,[42] Sjogren syndrome[43] and in Covid-19 infection.[24,44] Using PBMC from patients with aGVHD, itolizumab inhibited T cell proliferation, activation, and maturation of both CD4 and CD8 T cells. This was most evident with T cells obtained just prior to development of aGVHD and less inhibition was observed in post-GVHD T cells obtained from patients receiving immune suppressive medications. These findings suggest that itolizumab may be more effective in early stages of GVHD in combination with steroids or as GVHD prophylaxis. Even though these *ex vivo* experiments lack GVHD target tissues and do not perfectly recapitulate aGVHD *in vivo*, using whole PBMC, we were able to mimic the alloreactive activation of T cells in the presence of ALCAM expressed on circulating APCs (monocytes and B cells) and demonstrate the inhibitory activity of itolizumab.

Infusion of itolizumab in patients with autoimmune diseases has resulted in only transient reduction of $CD6^+$ circulating T cells, suggesting that this antibody does not induce profound T cell depletion.[45] This is consistent with our results demonstrating that itolizumab does not directly eliminate T cells *in vitro* in CDC, ADC or ADCC assays. Thus, the mechanism by which itolizumab inhibits T cell function *in vivo* is likely through its ability to prevent the interaction between CD6 on T cells and ALCAM on APCs.[22] This is consistent with our *in vitro* results showing that the addition of itolizumab to activated CD3 T cells in the absence of ALCAM did not inhibit T cell proliferation, activation, or maturation. However, in the presence of ALCAM, itolizumab efficiently abrogates the costimulatory effects of this interaction and is able to suppress T cell proliferation, activation, and maturation. In contrast to other pan-T cell antibodies that either activate T cells (anti-CD3) or deplete T cells (ATG, anti-CD52), itolizumab only inhibits T cell activation in the presence of ALCAM and has no direct or indirect depleting functions.

While ruxolitinib, a JAK1/2 inhibitor, is currently the only drug approved for the treatment of steroid refractory aGVHD,[46] several drugs that target different T cell co-stimulatory molecules are currently being tested in preclinical models and in patients with aGVHD.[4,27] In this setting, itolizumab may be a promising agent for either prevention or treatment of aGVHD, due to higher expression of CD6 on naïve and central memory Tcon cells, while sparing more tolerogenic Treg and effector memory T cells, important for control of both tumor cells and opportunistic infections after transplant.[37] Moreover, itolizumab may not inhibit responses to viral infections or reactivation. Indeed, different

NK and T cell populations are involved in responses to CMV infection ($NKG2C^+CD8^+$ T cells) and these cells have low expression of CD6.[47]

Recently, Ruth and colleagues demonstrated that targeting CD6 may also be a novel approach to enhance cancer immunotherapy.[48] This study utilized UMCD6, an anti-CD6 monoclonal antibody that binds the same CD6 domain (Domain 1) as itolizumab. UMCD6 upregulated the expression of the activating receptor NKG2D and down-regulated expression of the inhibitory receptor NKG2A on both NK cells and $CD8^+$ T cells, with concurrent increased expression of perforin and granzyme-B.[48] The combined capabilities of an anti-CD6 monoclonal antibody to control autoimmunity through effects on $CD4^+$ T cells and the enhanced killing of cancer cells through distinct effects on $CD8^+$ T cells and NK cells, may represent a new approach to induce tolerance without suppressing GVL activity after HCT.

In conclusion, our studies demonstrate that functional inhibition of the CD6-ALCAM costimulatory pathway may be a novel therapeutic strategy for prevention or treatment of aGVHD. On the basis of these biological properties, a phase I/II study using itolizumab as first line treatment in combination with steroids for patients with aGVHD is currently ongoing (NCT03763318). Future clinical studies may also test the feasibility and clinical efficacy of itolizumab for prevention of GVHD.

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	N (%)
Total	95 (100)
Patient Age at HCT, Median (Range), Years	61 (19-76)
Patient Sex, Male	55 (57.9)
Donor Age, Median (Range), Years	29 (18-67)
Donor Sex, Male	57 (60)
Donor type	
HLA Matched Related Donor	26 (27.4)
HLA Matched Unrelated Donor	63 (66.3)
Partially HLA Mismatched Unrelated Donor	6 (6.3)
Female Donor for Male Recipient	17 (18)
Patient or Donor CMV Serostatus, Positive	64 (67.4)
Disease	
ALL	6 (6.3)
AML	52 (54.7)
MDS	12 (12.6)
MPD	8 (8.4)
NHL	8 (8.4)
Others†	9 (9.6)
Disease Status at transplant	
Complete remission (CR)	66 (69.5)
Partial remission (PR)	2 (2.1)
Active Disease /Untreated	27 (28.4)
Cell Source	
Bone Marrow	2 (2.1)
Peripheral blood stem cells	93 (97.9)
Conditioning Intensity	
Myeloablative	48 (50.5)
Reduced intensity	47 (49.5)

Table 1. Patient characteristics. ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; NHL, non-Hodgkin lymphoma; CR, complete remission; PR, partial remission; †Chronic myeloid leukemia (CML, n=2); chronic lymphocytic leukemia (CLL, n=1); chronic myelomonocytic leukemia (CMML, n=3) and mixed myelodysplastic/ myeloproliferative neoplasm (Mixed MDS/MPN, n=3).

	N (%)
Engraftment	
ANC ≥ 500 cells/mm^3	95 (100)
% Donor T cell Chimerism - Median (Range)	
Day +30	76 (14-100)
Day +100	86 (24-100)
Day +180	96 (45-100)
Acute GVHD	42 (44.2)
Days to acute GVHD onset - Median (Range)	50 (20-294)
Maximum Grade	
1	16 (38.1)
2	17 (40.5)
3	3 (7.1)
4	6 (14.3)
Acute GVHD Sites	
Skin	31 (73.8)
Liver	6 (14.3)
Gut	17 (40.5%)
Acute GVHD patients treated systemically	34 (81%)
Steroid refractory acute GVHD	4 (11.8%)

Table 2. Engraftment, chimerism and acute GVHD outcomes.

Figure 1. CD6 expression on T cells after HCT. (A) Percentage of CD6 positive cells and (B) CD6 median fluorescence intensity (MFI) on regulatory CD4 T cells (Treg, red boxes), conventional CD4 T cells (Tcon, blue boxes) and CD8 T cells (CD8, green boxes) in healthy donors (HD) and in patients at 1, 2, 3 and 6 months after HCT. Box plots indicate median, Q1 and Q3 and min and max. (C) Representative gating strategy used to define T cell subsets, based on the expression of CD45RA, CCR7 and CD95 markers. (D) Heat map summarizes the median CD6 MFI values in the different T cell subsets in HD and in patients at 1, 2, 3 and 6 months after HCT. White stars show statistically significant differences between HD and samples after transplant (any $p<0.05$). (E) CD6 MFI on Treg, Tcon and CD8 T cells based on the expression of PD-1 in HD and in patients at 1, 2, 3 and 6 months after HCT.
Statistically significant differences are noted: **** P< 0.0001; *** P< 0.001; ** P< 0.01; * P< 0.05; Wilcoxon rank-sum test.

Figure 2. ALCAM expression on monocytes and DCs after HCT. (A) Representative gating strategy used to define DC subsets, based on the expression of CD11c and CD123 in flow cytometry. (B) Percentage of ALCAM positive cells and (C) levels of ALCAM expression (MFI) on $CD14^+$ monocytes (grey boxes), myeloid DC (mDCs, light-blue boxes) and plasmacytoid DC (pDCs, green boxes) in healthy donors (HD) and in patients at 1, 2, 3 and 6 months after HCT. Correlation of PD-L1 and ALCAM expression on (D) mDCs and (E) pDCs. Statistically significant differences are noted: **** P< 0.0001; *** P< 0.001; ** P< 0.01; * P< 0.05; Wilcoxon rank-sum test.

Figure 3. Expression of CD6 and ALCAM in patients after HCT with and without aGVHD. (A) Levels of CD6 expression (MFI) in Treg, Tcon and CD8 T cells. (B) Levels of ALCAM expression (MFI) in Treg, Tcon and CD8 T cells. PreGVHD samples (green boxes) and postGVHD samples (red boxes) from patients who developed acute GVHD are compared with noGVHD samples obtained at different times after transplant (grey boxes) and healthy donors (blue boxes). Statistically significant differences are noted: *** P< 0.001; ** P< 0.01; * P< 0.05; Wilcoxon rank-sum test. Healthy donor (HD, blue

boxes) n=9, preGVHD n=30, postGVHD n=20, noGVHD 1-month (1m) n=38, 2-months (2m) n=40, 3-months (3m) n=43 and 6-months (6m) n=21.

Figure 4. Inhibition of T cell proliferation, activation and differentiation by itolizumab. Cryopreserved peripheral blood mononuclear cells (PBMC) obtained from healthy donors (HD) and patients before or after aGVHD onset were stimulated with anti-CD3/CD2/CD28 beads in the presence of itolizumab or isotype control (cetuximab). (A) Proliferation of CD4 and CD8 T cells measured by CFSE dye dilution; (B) Activation of CD4 and CD8 T cells measured by expression of CD25; (C) maturation of CD4 and CD8 T cells measured by expression of CD45RO; (D) ALCAM expression is absent in resting T cells and is an additional marker of T cell activation. (E) Percent inhibition induced by itolizumab, comparing activity against HD, pre- and postGVHD samples in CD4 and CD8 T cells. Percentage inhibition was calculated using the following formula: (%cells in isotype control - %cells in itolizumab)/%cells in isotype control. If no difference was observed between isotype control and itolizumab the percentage of itolizumab inhibition equals 0%. Statistically significant differences are noted: ** $P< 0.01$; * $P< 0.05$; Wilcoxon rank-sum test. HD, n=9, preGVHD, n=7, postGVHD, n=8.

Figure 5. Testing itolizumab for complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADC) and antibody direct cellular cytotoxicity (ADCC). For CDC and ADC, $CD3^+$ T cells were isolated from cryopreserved peripheral blood mononuclear cells (PBMC) from healthy donors (HD) and cultured in the presence of medium + antibody + 25% of human serum (HS) and medium + antibody, respectively. Percentage cell lysis was calculated by combining the percentage of positive cells for 7-AAD or Annexin V or both. CDC activity was calculated by subtracting the values obtained in medium + antibody from the values obtained in the culture with medium + antibody + HS. ADC activity was calculated by subtracting the values obtained in the culture with medium alone from the values obtained in the culture with medium + antibody. CDC and ADC were assessed after 6 and 24 hours of culture. For ADCC PBMC from HD were cultured in the presence of antibody for 6 hours. Both percentage cell lysis and CD107a expression on NK cells were evaluated after 6 hours of

culture. The effects of itolizumab (red boxes) were compared to alemtuzumab (green boxes- positive control) and cetuximab (blue boxes - negative control). Values are expressed as mean and SD, paired t test, 2 tails was used. Statistically significant differences are noted: **** P< 0.0001; *** P< 0.001; ** P< 0.01; * P< 0.05; paired t test. HD, n=5.

Figure 6. Itolizumab activity is dependent on the presence of ALCAM. T cells from HD were stimulated with anti-CD3 antibody with or without ALCAM-Fc for 96 hours. Itolizumab or isotype control cetuximab were added at the start of each culture. (A) Proliferation of CD4 and CD8 T cells; (B) Expression of CD25; (C) Expression of CD45RO; (D) Expression of ALCAM after stimulation with anti-CD3 antibody alone or in combination with ALCAM-Fc, in the presence of cetuximab (blue boxes) or itolizumab (red boxes). Statistically significant differences are noted: ** P< 0.01; * P< 0.05; Wilcoxon rank-sum test. HD, n=10.

Figure 1

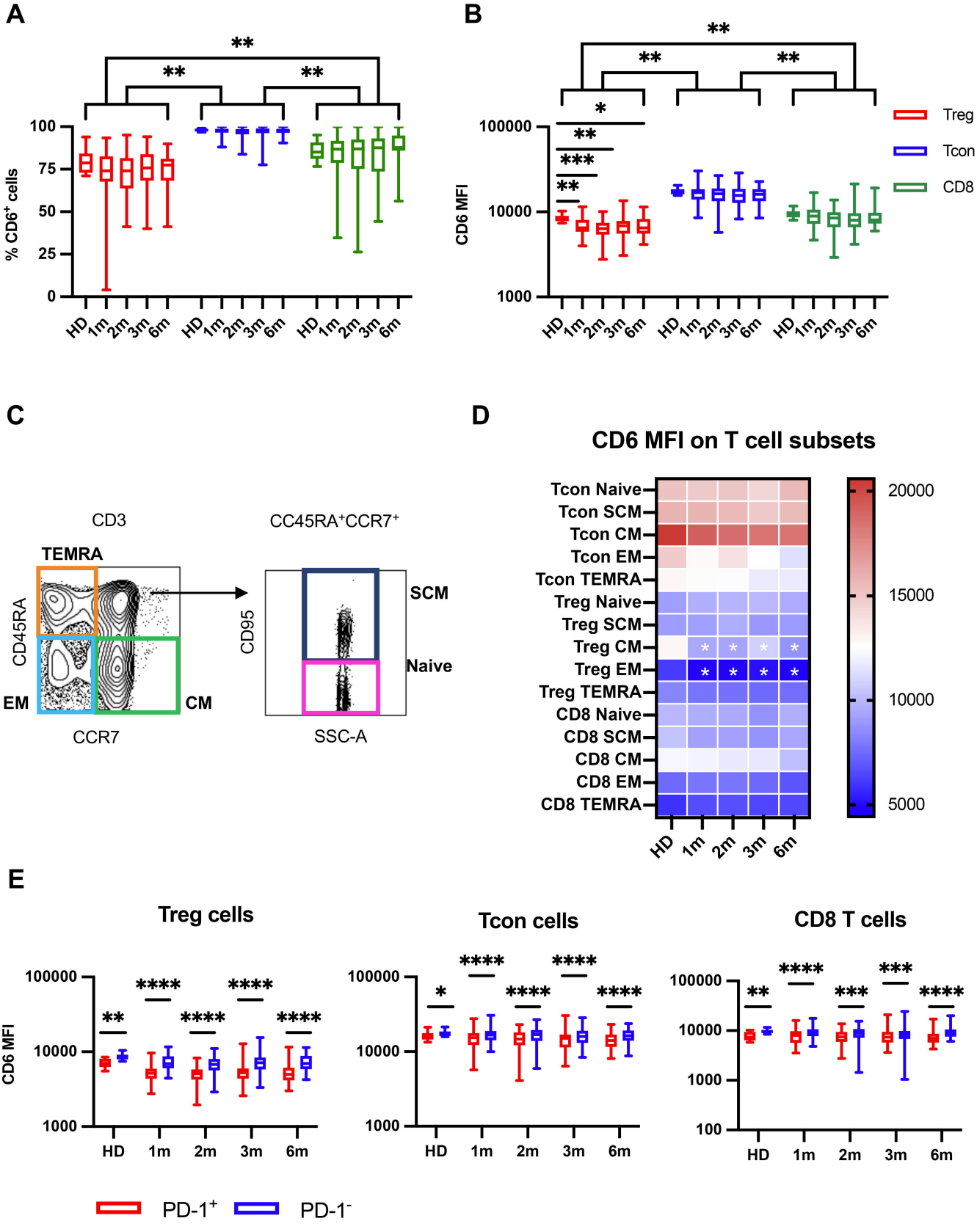

A
B
C
D
E
% CD6+ cells
CD6 MFI
Treg
Tcon
CD8
HD
1m
2m
3m
6m
CD3
CC45RA+CCR7+
TEMRA
CD45RA
EM
CM
CCR7
CD95
SCM
Naive
SSC-A
CD6 MFI on T cell subsets
Tcon Naive
Tcon SCM
Tcon CM
Tcon EM
Tcon TEMRA
Treg Naive
Treg SCM
Treg CM
Treg EM
Treg TEMRA
CD8 Naive
CD8 SCM
CD8 CM
CD8 EM
CD8 TEMRA
20000
15000
10000
5000
Treg cells
Tcon cells
CD8 T cells
PD-1+
PD-1-

Figure 2

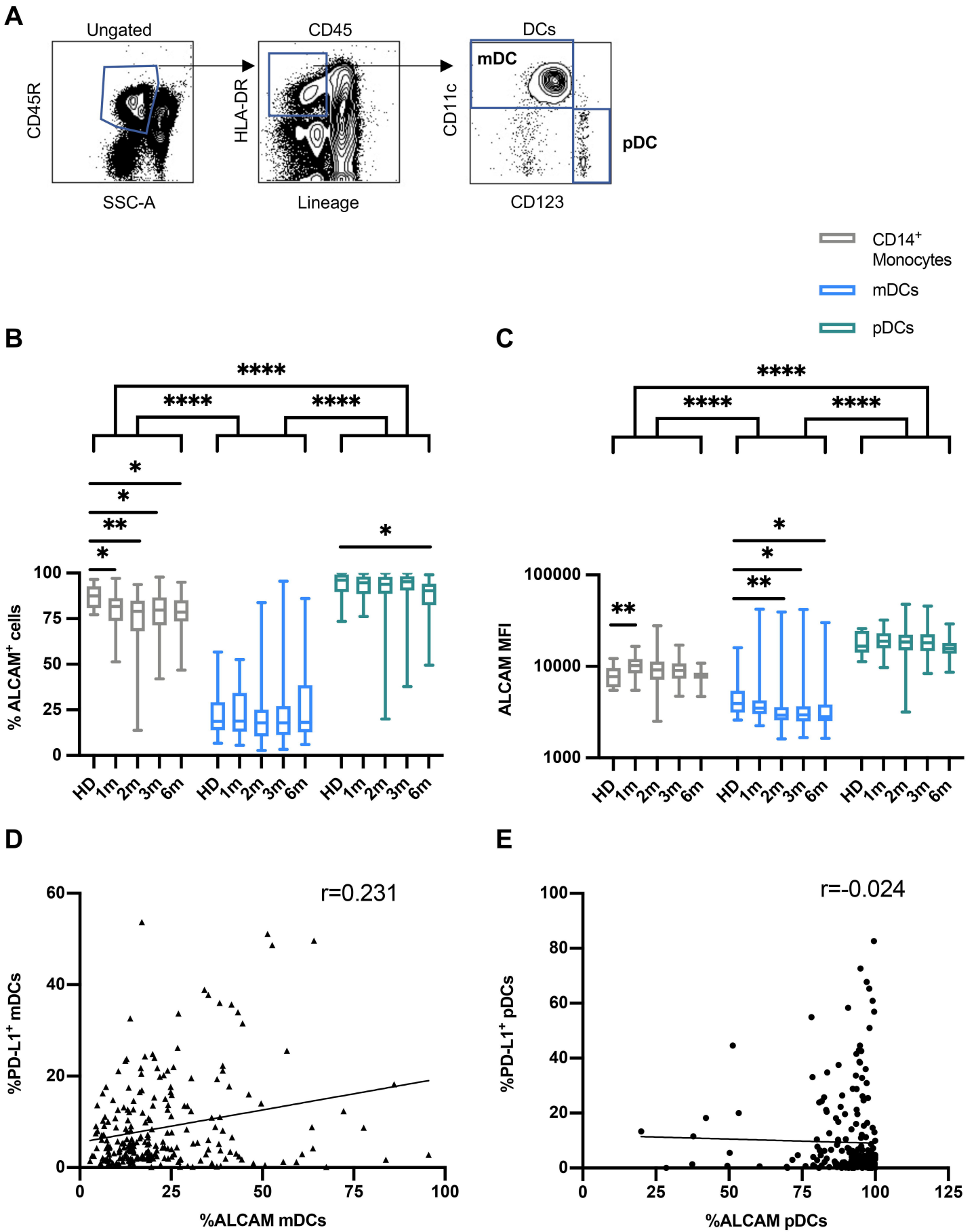

A
Ungated
CD45
DCs
mDC
pDC
CD45R
SSC-A
HLA-DR
Lineage
CD11c
CD123
CD14+ Monocytes
mDCs
pDCs
B
% ALCAM+ cells
HD
1m
2m
3m
6m
C
ALCAM MFI
D
%PD-L1+ mDCs
r=0.231
%ALCAM mDCs
E
%PD-L1+ pDCs
r=-0.024
%ALCAM pDCs

Figure 3

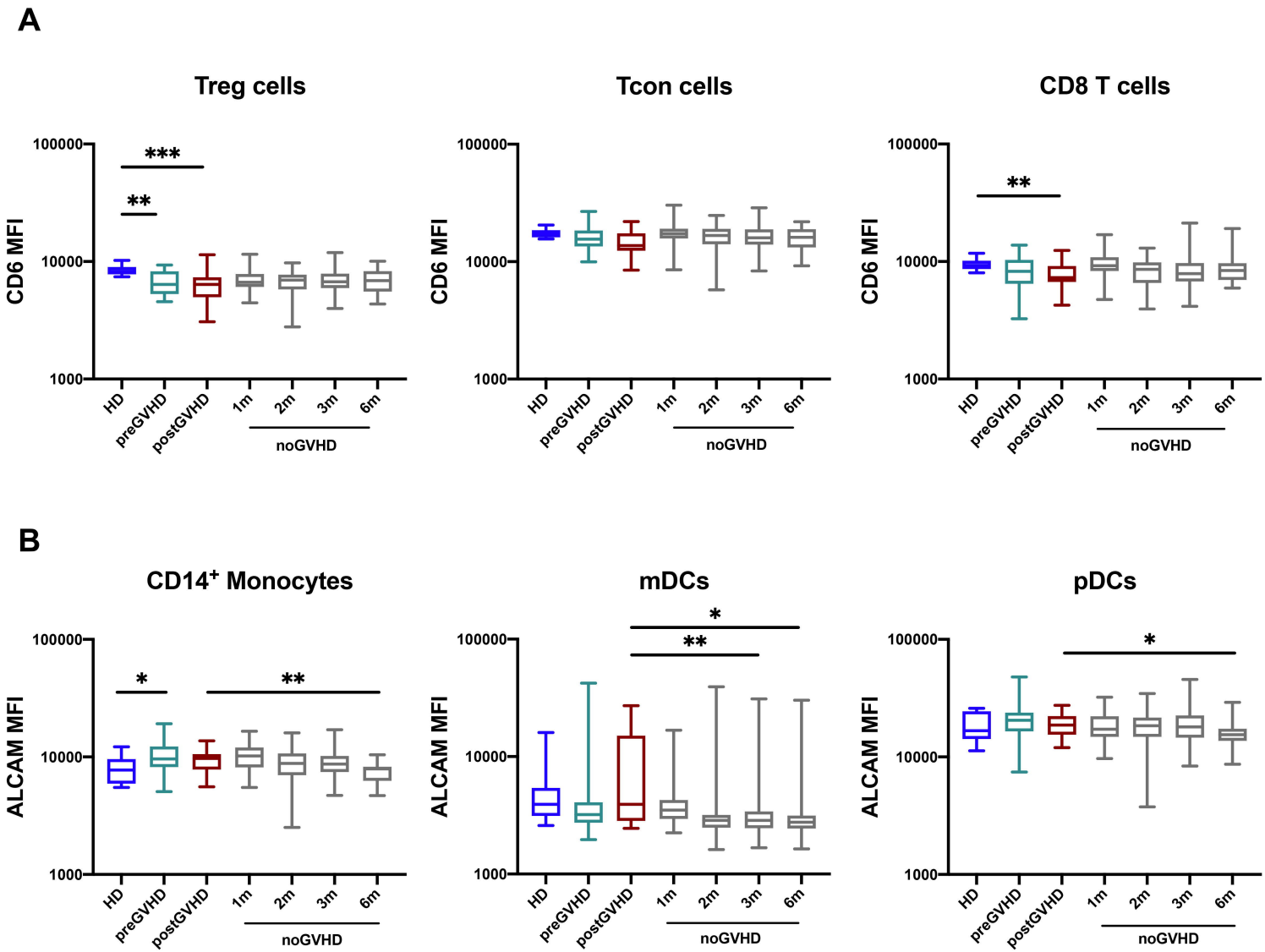

A
Treg cells
Tcon cells
CD8 T cells
CD6 MFI
100000
10000
1000
***
**
HD
preGVHD
postGVHD
1m
2m
3m
6m
noGVHD
B
CD14+ Monocytes
mDCs
pDCs
ALCAM MFI
*

Figure 4

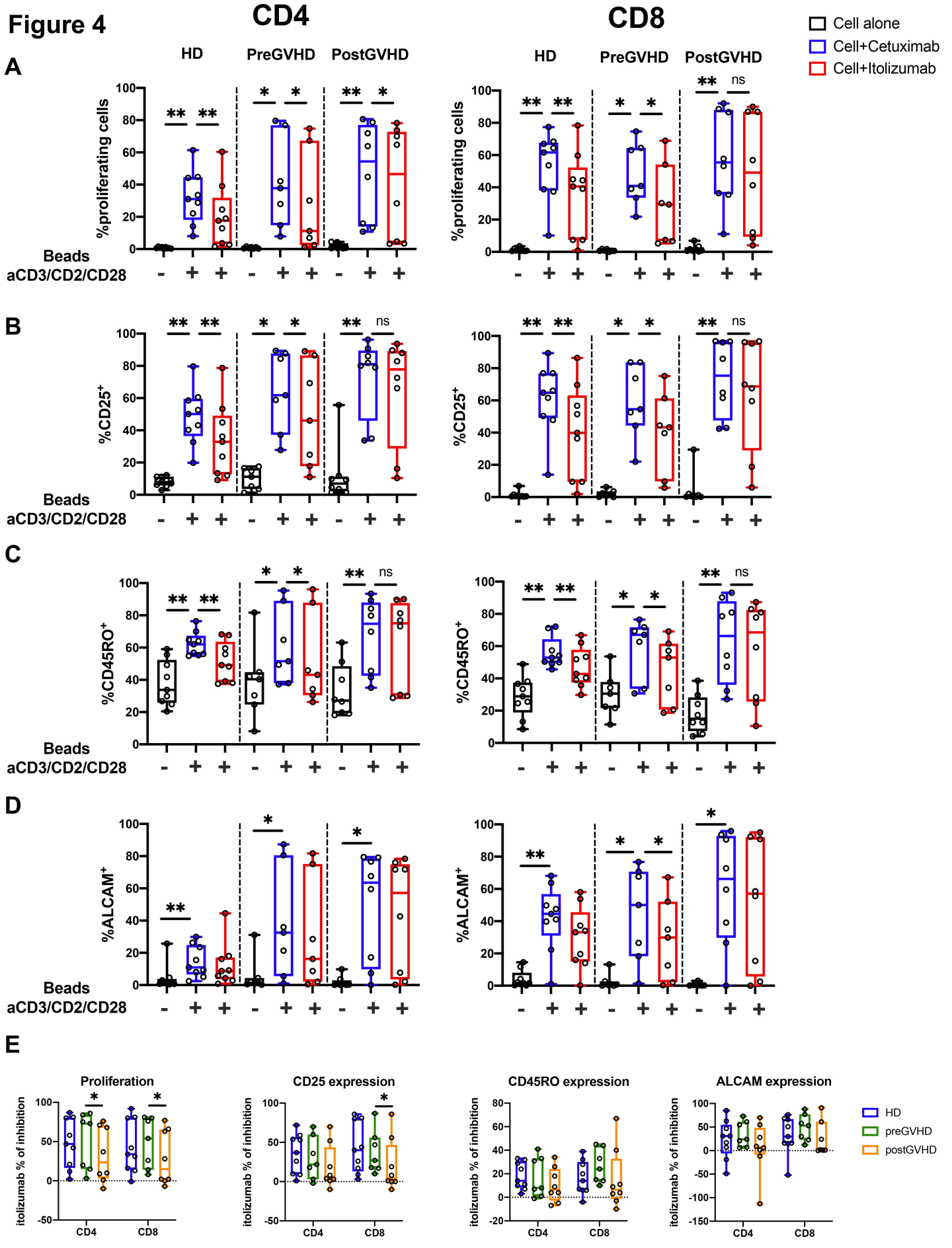
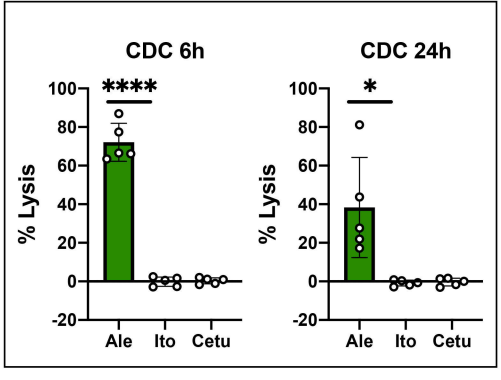
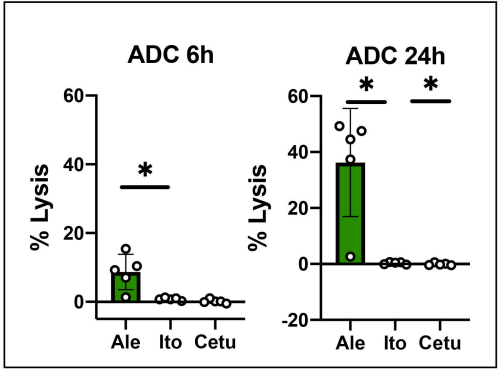

CD4
CD8
Cell alone
Cell+Cetuximab
Cell+Itolizumab
HD
PreGVHD
PostGVHD
A
%proliferating cells
B
%CD25+
C
%CD45RO+
D
%ALCAM+
Beads
aCD3/CD2/CD28
E
Proliferation
CD25 expression
CD45RO expression
ALCAM expression
itolizumab % of inhibition
CD4
CD8
HD
preGVHD
postGVHD

Figure 5


CDC 6h
CDC 24h
% Lysis
****
*
Ale
Ito
Cetu


ADC 6h
ADC 24h
% Lysis
*
Ale
Ito
Cetu

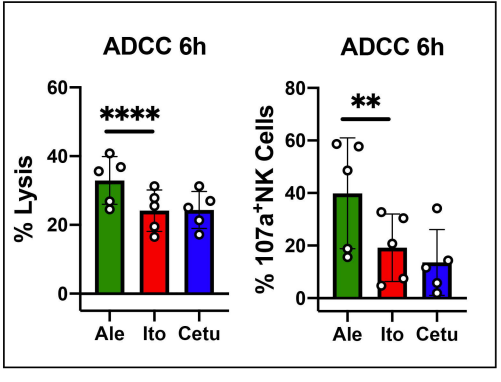
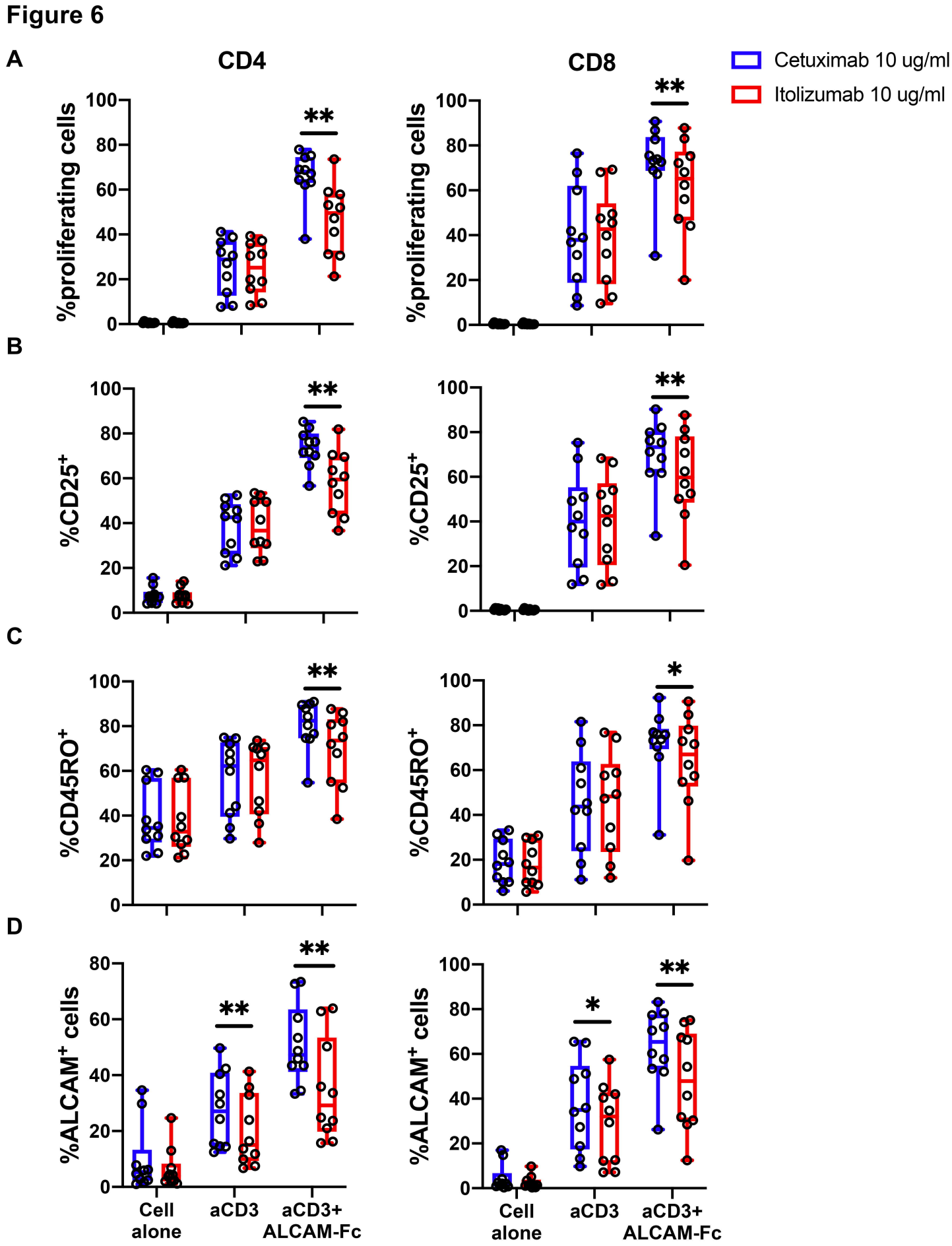

ADCC 6h
ADCC 6h
% Lysis
% 107a+NK Cells
****
**
Ale
Ito
Cetu

Figure 6


A
CD4
CD8
Cetuximab 10 ug/ml
Itolizumab 10 ug/ml
%proliferating cells
B
%CD25+
C
%CD45RO+
D
%ALCAM+ cells
**
*
Cell alone
aCD3
aCD3+ ALCAM-Fc

Supplemental Materials for

Phenotypic and functional characterization of the CD6-ALCAM T cell costimulatory pathway after allogeneic cell transplantation

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SUPPLEMENTAL METHODS

Cell processing for *in vitro* functional assays

For functional *in vitro* assay, PBMC were isolated from freshly drawn blood samples by density gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare) and cryopreserved in RPMI 10% DMSO before being utilized. For *in vitro* culture of PBMC or T cells complete medium (CM) was used. CM was made using DMEM (Cat 11995-065 Gibco), 10% FBS (Heat Inactivated, Gibco 10437-028), 1% Penicillin-Streptomycin (Gibco, 100X, 15140122), HEPES 10mM (Gibco, 1M, 15630080), 1% MEM Non-ess A.A. (Gibco, 100X, 11140050) and 2-Mercaptoethanol 0.05mM (Sigma-Aldrich, 14.3M, M3148).

Functional activity of itolizumab *in vitro*

Frozen PBMC obtained from patients who developed aGVHD after transplant (Supplemental Table S1) were stimulated using a combination of antiCD3/CD2/CD28 beads (Treg Suppression Inspector human 2.5 ml 5x10'7 Anti-Biotin MACSiBead particles preloaded with biotinylated CD2/CD3/CD28 antibodies, Miltenyi 130-092-909) at a bead:cell ratio of 1:2 in the presence of itolizumab 10 μg/ml (Biocon Ltd, Bangalore, India) or isotype control 10 μg/ml (cetuximab Erbitux NDC 66733-948-23, LOT c1800010). To detect T cell proliferation, PBMC were stained with CFSE (Invitrogen Thermo Fisher Scientific) as per product instruction. T cell proliferation, activation and maturation were evaluated after 72 hours of culture using the flow cytometry panel in Supplemental Table S3 and analyzed using FlowJo software. An example of the gating strategy used in this analysis is shown in Supplemental Figure S3.

Complement dependent cytotoxicity (CDC), antibody direct cytotoxicity (ADC)

After thawing HD PBMC, $CD3^+$ T cells were isolated using EasySep Human T-Cell isolation Kit (STEMCELL Cat 17951 Lot 17F79887) as per manufacturer's instructions. Purity of isolated $CD3^+$ T cells was assessed using flow cytometry with FITC-conjugated anti-CD3 antibody (Ab) (BioLegend, clone UCHT1, cat 300406). After T cell isolation, we obtained a median percentage of 96.9%, $CD3^+$ cells (range 90.5-98.1%). After 15 minutes pre-incubation with itolizumab (Biocon Ltd, Bangalore, India), positive control alemtuzumab (10 μg/ml, Campath-1H, anti-CD52, human IgG1, BioVision A1105-200, Lot 4M13A11050) or negative control cetuximab (10 μg/ml, Erbitux NDC 66733-948-23, LOT c1800010), isolated $CD3^+$ T cells were cultured at a concentration of 1 x 10^6/ml in the presence of CM with (for CDC) or without (for ADC) 25% human serum. Cells were incubated at 37'C, 5% CO2 for 6 or 24 hours. At the end of cell culture, the cell plate was kept on ice to prevent

further complement activity. Harvested cells were stained with 7-AAD (BD, 51-68981E' sold as 559925) and PE-conjugated Annexin V (BD, 51-65875X, sold as 556422) as per manufacturer's instructions and acquired on Fortessa LSR flow cytometer (BD). Each sample was acquired at medium velocity for 180 seconds. Analysis was performed using FlowJo software. An example of gating strategy is shown in Supplemental Figure 6. Percentage lysis was calculated by the average of % of 7-AAD and/or Annexin V positive cells. To detect complement-mediated cell death (CDC), percent specific lysis was calculated by subtracting the value obtained in conditions with Ab alone from the value obtained in the presence of AB+ human serum (HS). ADC activity was calculated by subtracting the values obtained in culture with medium alone from the values obtained in cultures with medium + antibody. CDC and ADC were assessed after 6 and 24 hours of culture.

Antibody dependent cellular cytotoxicity (ADCC) assay

Thawed HD PBMC were cultured at a final concentration of 1 x 10^6/ml in the presence of CM and specific ab at a concentration of 10 μg/ml. Cells were incubated at 37'C, 5% CO2 for 6 hours. Harvested cells were stained with FITC-conjugated anti-CD3 Ab (BioLegend, clone UCHT1, cat 300406), BV510-conjugated anti-CD56 Ab (BioLegend, clone HCD56 NCAM, cat 318340) and APC-conjugated anti-CD107a LAMP-1 (BioLegend, clone H4A3, cat 328620) and incubated for 30 minutes. Cells were washed two times and then stained with 7-AAD (BD, 51-68981E' sold as 559925) and PE-conjugated Annexin V (BD, 51-65875X, sold as 556422) as per manufacturer's instructions and acquired on Fortessa LSR flow cytometer (BD). Each sample was acquired at medium velocity for 180 seconds. Analysis was performed using FlowJo software. To set the positive threshold for CD107a expression an APC-conjugated isotype mouse IgG1 (BioLegend, clone MOPC-21, cat 400122) was used. An example of gating strategy is shown in Supplemental Figure 6.

T cell stimulation assay using ALCAM-Fc and anti-CD3 Ab

Recombinant Human ALCAM Fc Chimera (ALCAM-Fc, R&D Systems, cat 7187-AL-100) 10 μg/ml and anti-CD3 Ab 5 μg/ml (Invitrogen Thermo Fisher Scientific, clone OKT3, cat 16-0037-85) were resuspend in PBS (Gibco 10010-023, ph7.4) overnight at 4'C in flat bottom 96 well plate. Plate was washed 2 times with PBS before adding cells. After thawing HD PBMC, $CD3^+$ T cells were isolated through negative selection using the EasySep Human T-Cell isolation Kit (STEMCELL Cat 17951 Lot 17F79887) as per manufacturer's instructions. Purity of isolated CD3 T cells was assessed using flowcytometry with FITC-conjugated anti-CD3 antibody (Ab) (BioLegend, clone UCHT1, cat 300406), median $CD3^+$ cells of 98.15%, range 94.7-99.2%. Isolated T cells were cultured in complete medium (CM) with itolizumab (10 μg/ml) or cetuximab (10 μg/ml) at 37'C, 5% CO2 for 96 hours.

Cells were analyzed using flow cytometry (supplemental Table 4) and analyzed using FlowJo software. An example of gating strategy used is shown in Supplemental Figure 3.

Statistical analysis

Data analysis was primarily descriptive using the Wilcoxon rank-sum test for unpaired group comparison and the Wilcoxon signed-rank test for paired comparison. All tests were 2-sided at the significance level of 0.05 and multiple comparisons were not considered. Statistical analysis was performed using SAS version 9.2 (SAS Institute, Inc., Cary, NC) and R version 3.3.2 (the CRAN project) and Prism software (GraphPad). All graphs were made using Prism software (GraphPad). Heatmap was generated using GENE-E (http://www.broadinstitute.org/ cancer/software/GENE-E).

EXP	SEX	AGE	DX	DONOR	GVHD PROPHY	GVHD GRADE	SKIN	LIVER	GUT	AGVHD TX
1	F	37	NHL	MUD	Siro/Tac/MTX	3	3	0	0	Pred/ECP
2	M	65	NHL	MUD	Siro/Tac/MTX	2	3	0	1	Pred
3	M	42	NHL	MRD	Siro/Tac/MTX	2	3	0	0	Pred
4	F	52	MM	MUD	Siro/Tac/MTX	2	0	1	1	Pred
5	F	49	NHL	MUD	Tac/MTX	1	2	0	0	Pred
6	M	71	MF	MUD	Tac/MTX	2	0	0	1	Pred
7	F	52	NHL	MUD	Tac/MTX	2	3	0	0	Pred
8	M	71	MDS	MUD	Tac/MTX	4	0	2	4	Pred /Ruxolitinib
9	M	61	AML	MUD	Tac/MTX	2	3	0	0	Pred

Table S1. Clinical characteristics of patients with aGVHD used for *in vitro* functional assays. EXP, experiment number; DX, diagnosis; GVHD PROPHY, Graft versus host disease prophylaxis; aGVHD TX, acute GVHD treatment; NHL, non-Hodgkin lymphoma; MM, multiple myeloma, MF, Myelofibrosis; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; MUD, matched unrelated donor; MRD, matched related donor; Siro, sirolimus; Tac, tacrolimus; MTX, methotrexate; Pred, prednisone > 1 mg/Kg; ECP, extracorporeal photopheresis. All patients received a reduced intensity conditioning based on busulfan and fludarabine combination and a peripheral blood stem cell graft.

TUBE	TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
T CELL TUBE	CD45RA	BV605	HL100	BioLegend	304133
	CD279 PD-1	PE	J105	Invitrogen	12-2799-42
	CD127	APC	eBioRDR5	Invitrogen	17-1278-42
	CD25	FITC	m-A251	BioLegend	356105
	CCR7	BV711	G043-H7	BioLegend	353227
	CD4	BV510	RPA-T4	BioLegend	300545
	CD3	BV786	UCHT1	BioLegend	300472
	CD8	Alexa 700	RPA-T8	BioLegend	301027
	CD95	PeCF594 (PE Dazzle)	DX2	BioLegend	305633
	CD6	BV650	M-T605	BD	743448
APC TUBE	CD45	APC-Cy7	2D1	BD	560178
	LINEAGE:	PE-Cy7			
	CD3		SK7	BD	557851
	CD19		SJ25C1	BD	341093
	CD56		B159	BD	557747
	CD14		M5E2	BD	557742
	HLA-DR	BV510	G46-6	BD	563083
	CD11c	PE-Cy5	B-LY6	BD	551077
	CD123	FITC	AC145	Miltenyi Biotec	130-090-897
	CD166 ALCAM	BV421	3A6	BD	562936
	PD-L1	BV786	MIH1	BD	563739
	CD14	BV605	M5E2	BioLegend	301833

Table S2. Conjugated monoclonal antibodies in the flow cytometry immune monitoring panel. One tube was used for the characterization of CD6 expression on T cells (T CELL TUBE) and another tube for the characterization of ALCAM expression on monocytes and antigen presenting cells (APC TUBE).

TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
CD3	AF700	UCHT1	BD	557943
CD4	BV510	SK3	BD	562970
CD8	PerCP/Cy5.5	RPA-T8	BioLegend	301032
CD25	PE-CF594	M-A251	BD	562403
CD45RO	BV786	UCHL1	BD	564290
CD14	PE-Cy7	M5E2	BioLegend	301814
CD166	BV421	3A6	BD	562936
ANNEXIN V	PE	/	BD	556422
CFSE	CFSE	/	Invitrogen Thermo Fisher Scientific	C34554
ZOMBIE NIR	APC-Cy7	/	BioLegend	423105

Table S3. Conjugated monoclonal antibodies used in the T cell activation assay (Figure 4)

TARGET	FLUOROCHROME	CLONE	COMPANY	PRODUCT
CD4	BV510	SK3	BD	562970
CD8	PerCP/Cy5.5	RPA-T8	BioLegend	301032
CD25	PE-CF594	M-A251	BD	562403
CD45RO	BV786	UCHL1	BD	564290
CD166	BV421	3A6	BD	562936
CFSE	CFSE	/	Invitrogen Thermo Fisher Scientific	C34554
ZOMBIE NIR	APC-Cy7	/	BioLegend	423105

Table S4 Conjugated monoclonal antibodies used for ALCAM and T cell activation assay (Figure 6).

	Day 30 T cell chimerism at 1 month after HCT								
	<95				95-100				
	N	**Median**	**Q1**	**Q3**	**N**	**Median**	**Q1**	**Q3**	**p-value**
%CD6$^+$ Treg	56	74.25	68.11	82.68	11	69.9	58.83	80.45	0.26
%CD6$^+$ Tcon	56	98.08	97.07	98.84	11	97.42	95.98	98.1	0.25
%CD6$^+$ CD8$^+$ T cells	56	87.04	82.92	91.7	11	76	70.91	94.49	0.62
%ALCAM in CD14$^+$ monocytes	75	80.44	72.83	85.79	12	83.78	81.16	85.91	0.16
%ALCAM$^+$ mDC	75	18.74	13.51	28.61	12	16.73	10.01	21.91	0.37
%ALCAM$^+$ pDC	75	95	92.05	97.97	12	94.11	88	99.13	0.71
CD6 MFI in CD8 + T cells	56	8886	7225.8	10488	11	9149.6	6234.4	10784	0.77
CD6 MFI in Treg	56	6507.4	5936	7969.9	10	6508.4	4721.9	8022.3	0.49
CD6 MFI in Tcon	56	17057	14328	18599	11	16107	13399	17949	0.44
ALCAM MFI in CD14+ monocytes	75	10131	8379.5	11385	12	12141	8668.4	13496	0.22
ALCAM MFI in mDCs	75	3609.5	3043.6	4314.3	12	3209.7	2914.9	4103.2	0.37
ALCAM MFI in pDCs	75	18307	15962	22602	12	21176	16139	25528	0.30

Table S5. Expression of CD6 and ALCAM in patients with different levels of donor chimerism.

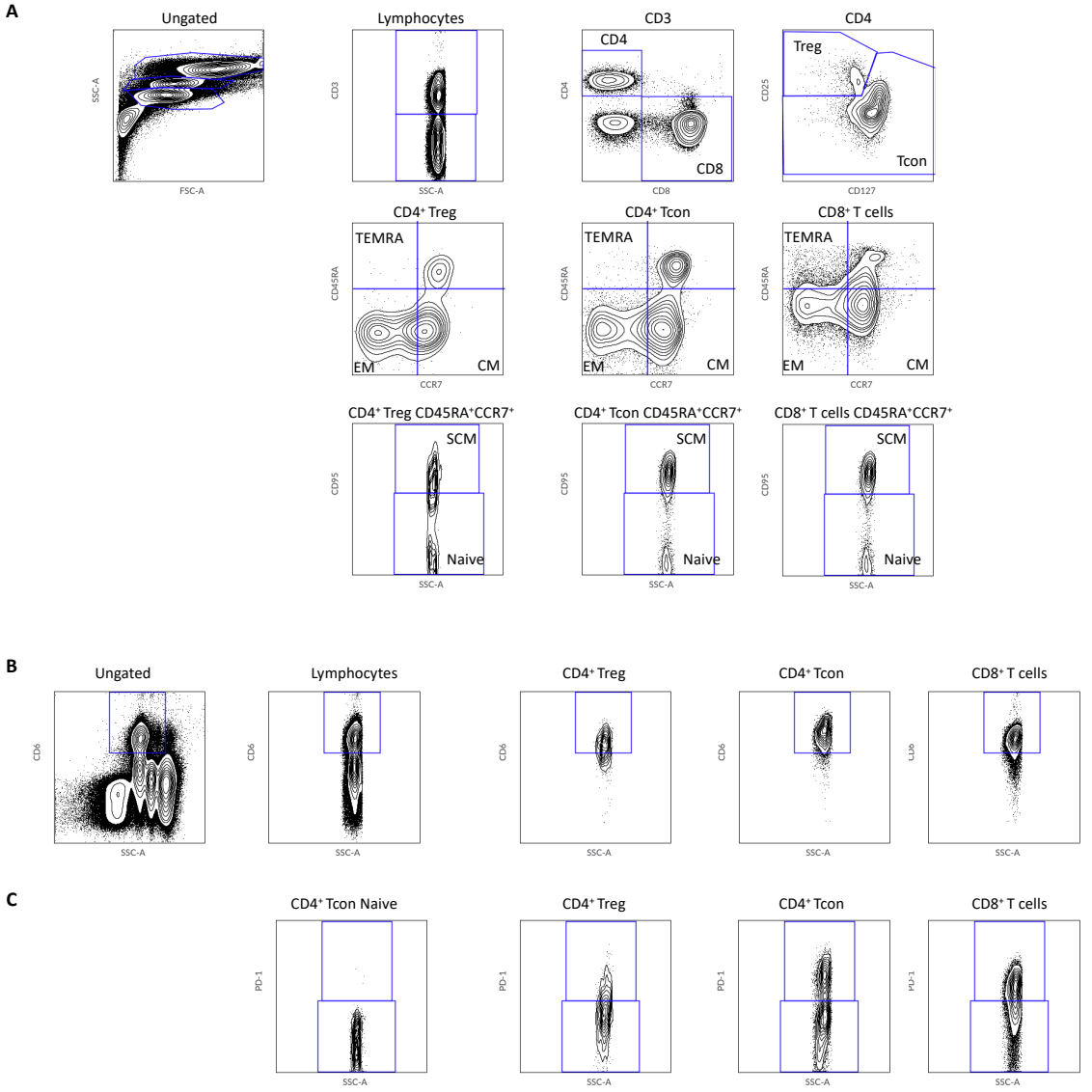


Figure S1. **Gating strategy for T cell subset analysis**. (A) Three major T cell populations, CD4Treg, CD4Tcon, and CD8 T cells, were defined as $CD3^+CD4^+CD8^-CD25^+CD127^-$, $CD3^+CD4^+CD8^-$ non-Treg cells and $CD3^+CD4^-CD8^+$, respectively. Within each T cell population, subsets were defined as follows: central memory (CM, $CD45RA^-CCR7^+$), effector memory (EM, $CD45RA^-CCR7^-$), terminally differentiated effector memory (TEMRA, $CD45RA^+CCR7^-$), T cells naïve ($CD45RA^+CCR7^+$ $CD95^-$) and stem cell memory (SCM, $CD45RA^+CCR7^+$ $CD95^+$). (B) The threshold of CD6 gate on T cell subsets was made using monocytes as internal negative control. (C) PD-1 expression was monitored on each T cell subset, using Tcon naïve cells as internal negative control. 265 peripheral blood samples were tested with this panel: n=9 HD; n=67 at 1 mo; n=76 at 2 mo; n=78 at 3 mo; n=35 at 6mo.

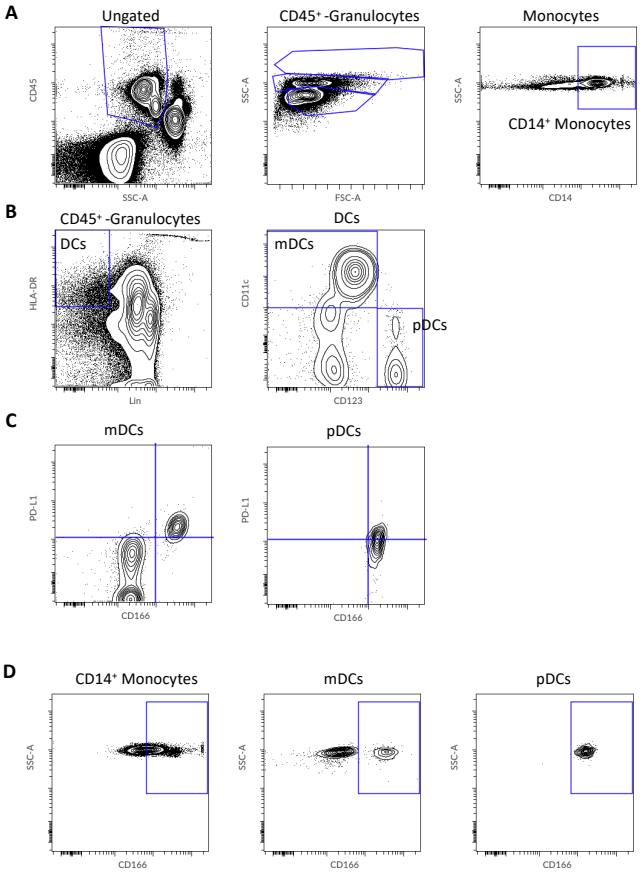


Figure S2. **Gating strategy for dendritic cell subsets and monocyte analysis**. (A) Monocyte were defined within the CD45$^+$ cells, after excluding granulocytes, based on the SSC-A, FSC-A and CD14 expression. (B) Dendritic cells (DCs) were defined within the CD45+ cells, after excluding granulocytes, based on the positivity of HLA-DR and negativity of lineage markers (CD14, CD3, CD19 and CD56). DC subsets were defined as follows: myeloid DCs (mDCs, CD11c$^+$CD123$^-$) and plasmacytoid DCs (pDCs, CD11c$^-$CD123$^+$). (C) PD-L1 and CD166 (ALCAM) expression on both mDCs and pDCs. (D) CD166 (ALCAM) expression on CD14$^+$ monocytes, mDCs and pDCs. Samples with less than 1000 DC events were excluded from the analysis. 236 peripheral blood samples were tested with this panel: n=9 HD; n=62 at 1 mo; n=66 at 2 mo; n=69 at 3 mo; n=30 at 6mo.

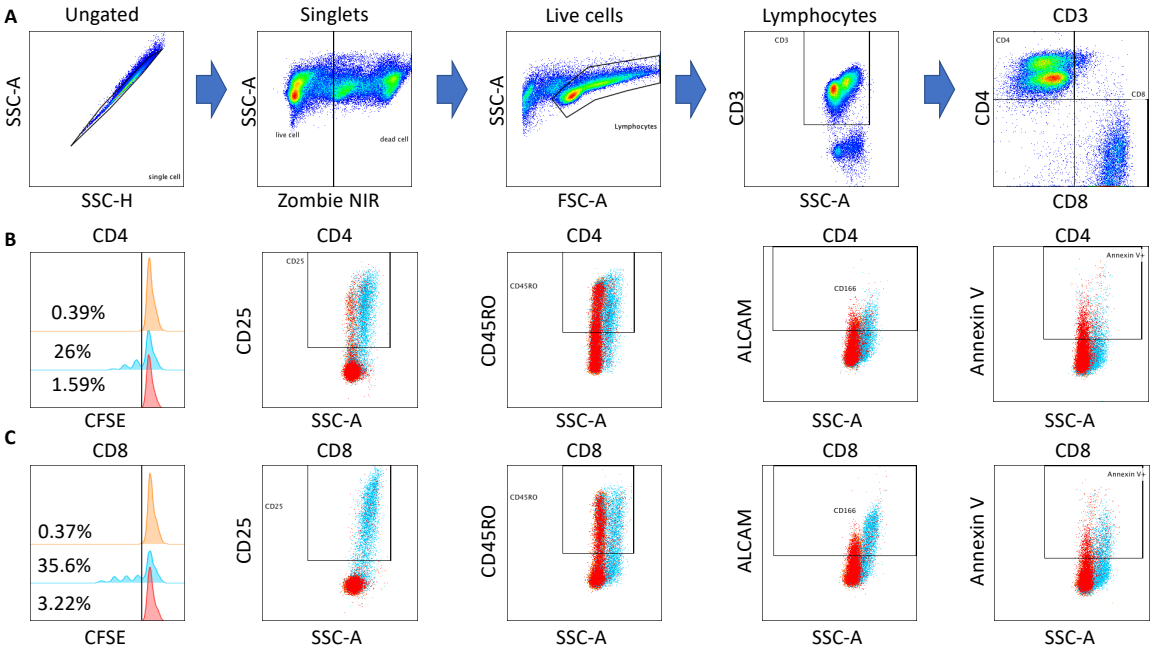


Figure S3. **Gating strategy for *in vitro* functional assays**. (A) Example of the gating strategy of $CD4^+$ and $CD8^+$ T cells, after exclusion of doublets and dead cells. Gating strategy on CD4+ T cells (B) and CD8+ T cells (C) for CFSE dilution (T cell proliferation), CD25 (T cell activation), CD45RO (T cell maturation), ALCAM and Annexin V (T cell apoptosis) expression. Cell alone (orange), cells+beads+cetuximab (blue), cells+beads+itolizumab (red).

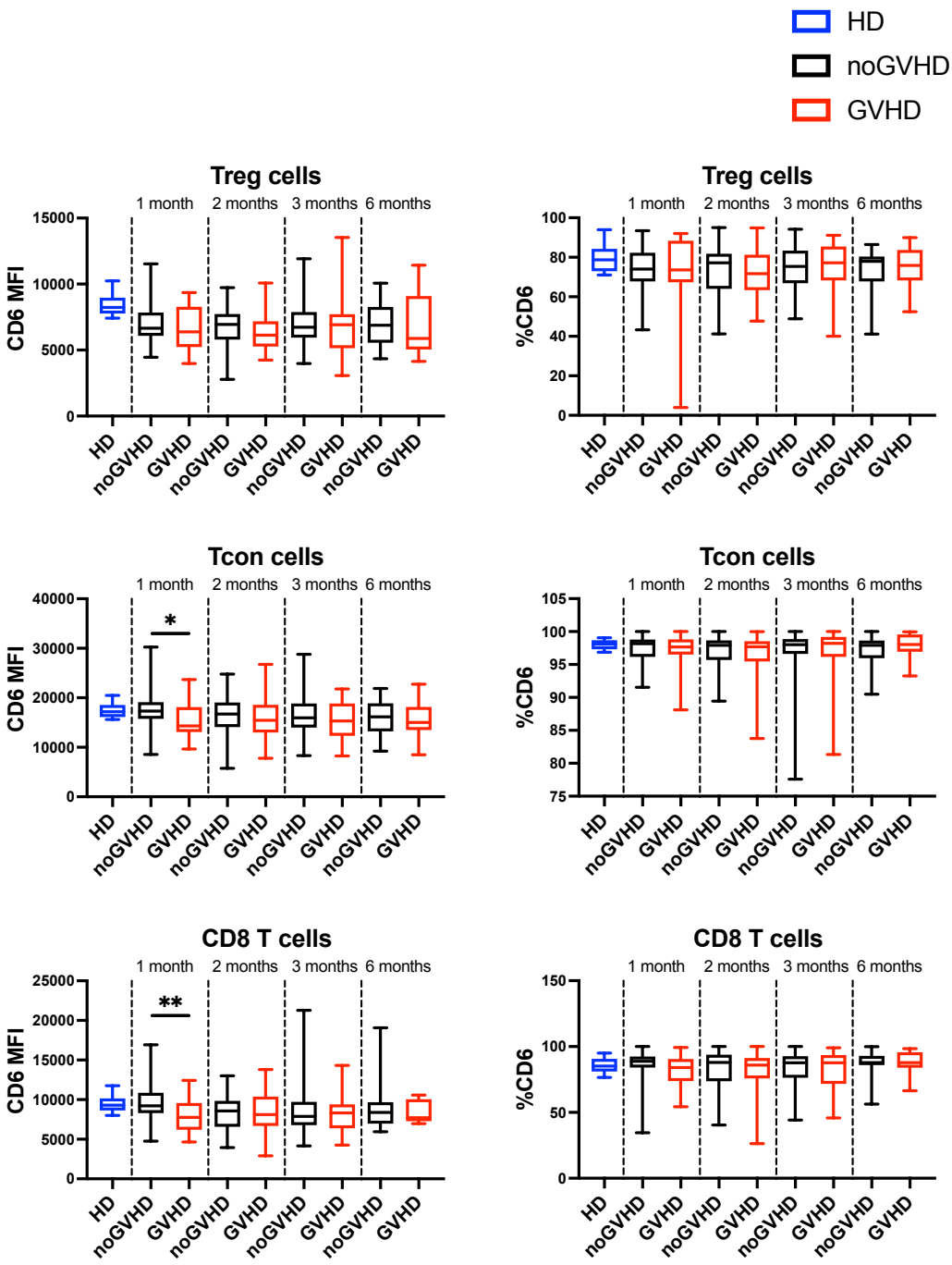


Figure S4. Comparison of CD6 expression on Treg, Tcon and CD8 T cells in patients with and without GVHD at specific time points after transplant. CD6 MFI was lower in GVHD group compared noGVHD group at 1 month after transplant in Tcon and CD8 T cells. Statistically significant differences are noted: * P< 0.05; ** P< 0.01. Wilcoxon rank-sum test. HD, n=9; noGVHD month 1, n=41; GVHD month 1, n=26; noGVHD month 2, n=40; GVHD month 2, n=36; noGVHD month 3, n=45; GVHD month 3, n=33; noGVHD month 6, n=23; GVHD month 6, n=12.

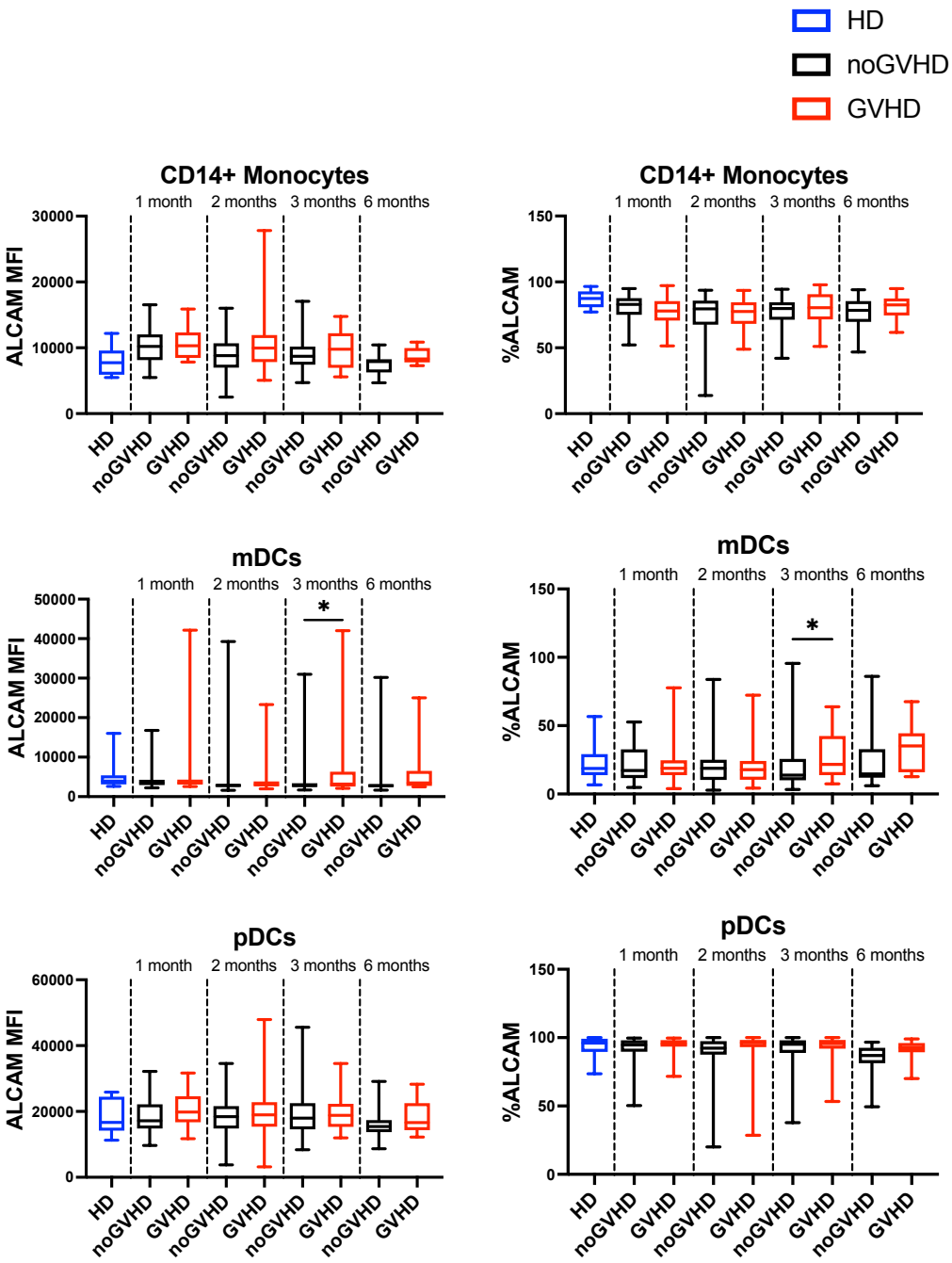


Figure S5. Comparison of ALCAM expression on CD14+ Monocytes, mDCs and pDCs in patients with and without GVHD at specific time points after transplant. ALCAM MFI and % of ALCAM positive cells on mDCs was higher in the GVHD cohort compared to noGVHD at 3 months after transplant. Statistically significant differences are noted: * P< 0.05; Wilcoxon rank-sum test. HD, n=9; noGVHD month 1, n=41; GVHD month 1, n=26; noGVHD month 2, n=40; GVHD month 2, n=36; noGVHD month 3, n=45; GVHD month 3, n=33; noGVHD month 6, n=23; GVHD month 6, n=12.

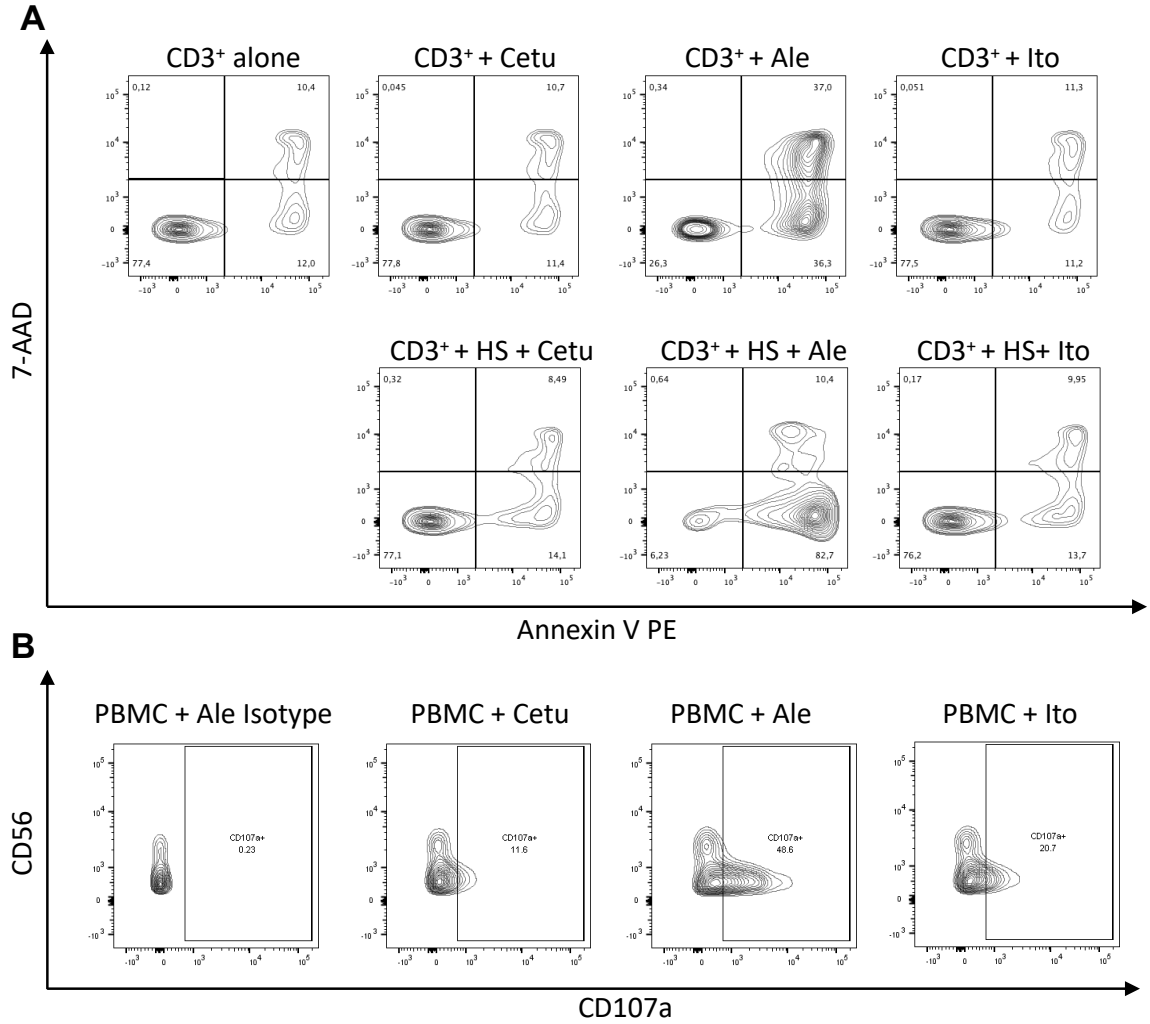


Figure S6. **Gating strategy to test itolizumab complement dependent cytotoxicity (CDC), antibody dependent cytotoxicity (ADC) and antibody direct cellular cytotoxicity (ADCC) analysis.**

(A) Representative example of percentage of cell lyses, based on the expression of 7-AAD and annexin V. (B) Representative example of CD107a expression on NK cells, as marker of ADCC. An APC-conjugated mouse isotype was used to design the gate for CD107a. Percentage of cell lysis was calculated by adding the percentage of positive cells for 7-AAD or Annexin V or both. CDC activity was calculated by subtracting the values obtained in the medium + antibody from the values obtained in the culture with medium + antibody + human serum (HS). ADC activity was calculated by subtracting the values obtained in the culture with medium alone from the values obtained in the culture with medium + antibody. For ADCC PBMC form HD were cultured in the presence of antibody for 6 hours. Both percentage of cell lyses and CD107a expression on NK cells were evaluated after 6 hours of culture.