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Detection of alloreactive T cells from cryopreserved human peripheral blood mononuclear cells

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Abstract

Precise analyses of alloreactive T cell phenotype and function can inform both the nature and intensity of adaptive responses to transplant antigens. However, alloreactive T cells are sparse and difficult to detect, particularly in cryopreserved peripheral blood mononuclear cells (PBMCs) and from hypo-responsive individuals. An assay to identify and phenotype alloreactive cells would be particularly valuable, especially for multi-center clinical trials that often store frozen samples for batch analysis. Herein we demonstrate consistent and reproducible alloreactive T cell detection in cryopreserved PBMC following a short-term mixed lymphocyte reaction (MLR). The inherent background expression levels of activation markers on responder T cells were minimized by including a resting period prior to the assay. Stimulator cells were activated before inclusion in the MLR by addition of CD40L and IL-4. The time frame and markers to identify and phenotype alloreactive T cells following stimulation were optimized using short term co-cultures. We defined subsets of CD4⁺ and CD8⁺ T cells co-expressing CD69 and either CD154 or CD137 following allostimulation as alloreactive, and further phenotyped these cells with a variety of surface markers such as PD-1, LAG-3, and TIM-3. This assay may allow for the monitoring of donor-specific T cells in transplant recipients with longitudinally collected and cryopreserved PBMCs and provide a useful tool to identify biomarkers associated with tolerance. These biomarkers may add to mechanistic insights in immune recognition of transplanted tissues and/or cells.

Keywords

Alloreactive T cells; Transplantation; Peripheral blood mononuclear cell; CD154; CD137

1. Introduction

Recipient T cells are critical contributors to graft fate following solid organ transplantation. Detecting and phenotyping graft-reactive T cells following transplant could yield valuable mechanistic insight and potentially inform therapies to promote transplant tolerance and prevent graft rejection. Although major histocompatibility complexes (MHC)-multimer is

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excellent material to detect specific T clones, the allo-repertoire is too diverse to be assessed by MHC-multimer as a clinical assay. On the other hand, mixed lymphocyte reaction (MLR) is a classic, but practical system to observe allo-response. However, MLR has several limitations depending on its readout. The readout such as highly sensitive with low background and rapid detection before activation-induced alteration is desired for phenotyping alloreactive T cells. Proliferation requires certain incubation period that lead activation-induced alteration of phenotype. Cytokine detection may be more rapid, but sensitivity could be incertitude by selection of cytokines (Assenmacher et al., 1994). Among the activation markers, antigen-induced expression of CD154 (CD40 Ligand) and CD137 (4-1BB) have been reported as a highly sensitive and specific early marker for detecting antigen-specific T cells (Chattopadhyay et al., 2005; Frentsch et al., 2005; Wolfl et al., 2007). CD154 and CD137 are costimulatory-related molecules belonging to the tumor necrosis factor (TNF) and TNF receptor family, respectively. CD154 has been shown the rapid upregulation on antigen-specific CD4⁺ T cell with diverse cytokine profiles and memory phenotypes (Chattopadhyay et al., 2005; Ashok-kumar et al., 2009). In pediatric liver transplant recipients, the ratio of anti-donor to anti-third party induced expression of CD154 in memory T cells has been reported as a predictive biomarker for early rejection (Ashokkumar et al., 2009). CD137 also has been reported to identify wide repertoire, including both naïve and memory subsets, of antigen-specific CD8⁺ T cells in short time activation (Wolfl et al., 2007). Despite specificity and quick detection, the frequency of detected alloreactive CD4⁺ and CD8⁺ T cells by CD154 and CD137 expression in allo-MLR was unsatisfactory, only 0.1 to 0.2%, probably because of the modest antigen presenting capacity of human peripheral blood mononuclear cells (PBMCs) even after freshly isolation (Litjens et al., 2009, 2013). PBMC, a readily accessible material to assess immune responses in humans. Ideally T cell activation assays should be performed immediately after sample isolation; however, this may be impractical, especially for multi-center clinical trials. It is more pragmatic to consolidate testing in a central laboratory and batch-test longitudinally collected cryopreserved PBMCs, minimizing the potential of inter-assay variability. Thus, there is a compelling need to develop an assay that is reliable, performed on cryopreserved PBMCs, and identifies and phenotypes donor-specific responding T cells.

Our initial studies found that cultures of freshly thawed human PBMCs expressed elevated levels of activation markers, creating an “activation noise” background present on most T cells. Here, we demonstrate that this background can be reduced by resting PBMCs prior to the assay. We also demonstrate that following culture with stimulated allogeneic PBMC, a substantial number of activated, allospecific T cells express a distinctive combination of CD69 and CD154/CD137 with a low background activation signal. Furthermore, we present a time course assay with polyclonal stimulation to optimize incubation time and marker selection for assays of specific interest. In summary, our protocol allows for the analysis of alloreactive T cells and has identified and excluded potentially confounding surface markers of non-specific activation. Understanding these assay conditions may allow for dissection of precise mechanisms of reactivity and unresponsiveness in transplant recipients.

2. Material and methods

2.1. Study subjects

This study was approved by the Partners Human Research Committee (2015P001580). Written form of consent was obtained from all the participants. Healthy donor buffy coat units were obtained from the Massachusetts General Hospital Blood Bank.

2.2. Isolation of peripheral blood mononuclear cells and cell culture

PBMCs were isolated by gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare), frozen in freezing medium (90% FBS and 10% DMSO), and stored in a liquid nitrogen freezer until use. PBMCs were cultured in 10% Human AB serum (Gemini Bio-Product) containing RPMI media (10%RPMI) or in serum-free AIM-V media supplemented with 5 mM HEPES (Gibco). PBMCs were rested after thawing at $2-3 \times 10^6$ cells/mL for the indicated time period.

2.3. Flow cytometry

The following Abs were used for phenotypic analysis: FITC-CD14 (M5E2), FITC-CD16 (3G8), FITC-CD19 (HIB19), FITC-HLA-A,B,C (W6/32), FITC-CD244 (C1.7), FITC-CD28 (28.2), PE-CD8a (SK1), PE-CD69 (FN50), PE-CTLA-4 (L3D10), PE-CD86 (IT2.2), PE-TCR vb9 (MKB1), PerCPCy5.5-CD3 (UCTH1), PerCPCy5.5-TCR Vb1 (BL37.2), PECy7-CD137 (4B4-1), PECy7-CD19 (HIB19), PECy7- KLRG1(SA231A2), APC-CD154 (Kutscher et al., 2013; Wilson and Blyth, 1968; Lindahl and Wilson, 1977; Ryser and MacDonald, 1979; Turka et al., 1989; DeWolf et al., 2018; Crespo et al., 2013; Sanchez-Fueyo and Markmann, 2016), APC-CD57 (HCD57), APC-LAG-3 (11C3C65), APCCy7-CD8 (HIT8a), APCCy7-CD45RA (HI100), BV421-CD4 (OKT4), BV421-PD-1 (EH12.2H7), BV421-CD127 (A019D5), and FOXP3 (206D), all purchased from Biolegend (San Diego, CA). FITC-CCR7 (150503), PerCPeFluor710-TIGIT (MBSA43), BV421 TCR Vb3 (JOVI-3), and BUV737 TCR Vb8 (JR2) were purchased from BD Biosciences (San Jose, CA). TIM-3 (344823) was purchased from R&D Systems (Minneapolis, MN). Data were collected on FACS Verse or FACS LSRII (BD Biosciences) and analyzed using Flowjo (Tree Star, Ashland, OR).

2.4. Alloreactive T cell detection assay

Alloreactive T cells were identified following MLR culture using allogeneic PBMCs as stimulators. Stimulator PBMCs were activated with a B cell-targeted cocktail consisting of multimeric human CD40L (100 ng/mL, Adipogen, San Diego, CA) and recombinant human IL-4 (10 ng/mL, Peprotech, Rocky Hill, NJ) for 2 days at 2×10^6 cells/mL in 24-well flat bottom plate. Prior to culture, stimulators were labeled with 5 uM CFSE for 5 min as described above and irradiated with 30 Gy. Responder PBMCs were rested for 1 day prior to culture. Responders and activated stimulators were co-cultured at 1:1 ratio (10^6 cells, each) in 96-well u-bottom plates with 200 uL media containing APC conjugated anti-CD154 labeling monoclonal Abs (clone 24-31, Biolegend) for the indicated time (Fig. 4A). Alloreactive CD4⁺ and CD8⁺ T cells were identified as CD154⁺ + CD69⁺ + CD4⁺

and CD137 + CD69 + CD8⁺ responder CD3⁺ cells, respectively. As a negative control, autologous PBMCs were simultaneously processed and co-cultured with responder cells.

2.5. HLA typing

Genomic DNA was extracted from PBMCs and HLA-A, HLA-B, and HLA-DR antigens were identified by reverse sequence-specific polymorphism-polymerase chain reaction – Luminex typing method using a LAB type RSSO1A, RSSO1B and RSSO2B1 kit (One Lambda, West Hills, CA), respectively. HLA mismatch status was determined according to comparison of HLA antigen on responder and stimulator samples.

2.6. Time course assay with polyclonal stimulation

Frozen PBMCs were thawed and rested for 2 days at $2-3 \times 10^6$ cells/mL in culture medium prior to the assay. PBMCs were cultured with anti-CD3 and anti-CD28 antibody coated beads (11141D, Invitrogen, Carls-bad, CA) at a 1:1 cell to bead ratio in 96-well u-bottom plates. Activated T cell phenotype was analyzed at serial time points (6, 12, 18, 24 h) by multi-parametric flow cytometry.

2.7. Statistical analysis

Statistical analyses were performed using Prism 7 (GraphPad Prism, San Diego, CA). Student's *t*-test or paired *t*-test was used to compare the parameters between the two groups. One-way ANOVA followed by the Tukey multiple comparison test was used for multi group analyses. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Reducing background T cell activation in media supplemented with human AB serum

Given the comparatively low frequency of alloreactive T cell precursors detected in PBMCs, it is essential to reduce non-specific activation as much as possible. However, we found that T cells in cryopreserved PBMCs express CD154, CD137, and CD69 without stimulation (Fig. 1). Upregulation of CD69 expression by human T cells following thawing and resting periods have been previously reported in cell cultures containing serum (Faint et al., 2011; Wang et al., 2016). Interestingly, the expression of CD69 and CD154 by CD4⁺ T cells was higher in serum free media (AIM-V) compared with human AB serum containing media (10% RPMI, Fig. 1A). CD69 expression by CD8⁺ T cells was also higher in serum free media (Fig. 1B).

After inclusion of serum in the culture media, it took 48 h for CD69 expression to diminish (0.5–1% CD4 + CD69⁺, 4–5% CD8 + CD69⁺ T cells, data not shown). Due to this high level of expression in the absence of stimulation and bystander activation following stimulation, CD69 alone is an unsuitable marker for detecting antigen specific responses. However, combining CD69 with CD154 or CD137 lowered the detection of non-specific activation compared to using CD154 or CD137 in isolation 12 h following stimulation with autologous cells (mean CD4⁺ 0.03% vs. 0.11%, or CD8⁺ 0.08% vs 0.16%, respectively, Supplementary Fig. S1). Resting responder cells for 1 day prior to inclusion in the MLR was instrumental in decreasing the background expression of CD154 and CD69 on CD4⁺

T cells. Without this rest, expression of these markers following autologous stimulation was significantly higher than that after 1 day rest and may confound the detection of alloreactive T cells (Fig. 2).

3.2. Stimulator B cell activation prior to MLR enhances alloreactive T cell stimulation

B cells are well recognized as effective antigen presenting cells (APCs) (Lapointe et al., 2003; Zand et al., 2005). To potentially enhance stimulation of responder alloreactive T cells, we activated stimulator B cells prior to their inclusion in the MLR. We observed that a 2 day incubation of bulk PBMCs with multimetric CD40L and human rIL-4 upregulated the expression of HLA Class I, II, and CD86 on both B cells and other potential APCs (Fig. 3).

3.3. CD69 and CD154 or CD137 expression identifies alloreactive CD4+ and CD8+ T cells

After 12 h of allo-MLR culture with random paired activating PBMCs from healthy donors (Fig. 4A, detail in material and methods), the proportion of CD154 + CD69+ cells in the CD4+ T cells and CD137 + CD69+ cells in CD8+ T cells were readily detected in multi-parametric flow cytometry analysis (all values mean $\pm$ s.d.; $0.38 \pm 0.20\%$ in CD4+ and $0.37 \pm 0.19\%$ in CD8+, respectively, Fig. 4B). The proportion of CD154 + CD69 + CD4+ and CD137 + CD69 + CD8+ T cells detected following MLR with activated autologous PBMCs was low ($0.04 \pm 0.02\%$ in CD4+ and $0.08 \pm 0.06\%$ in CD8+, respectively).

The percentage of T cells that were alloreactive peaked 18 h following stimulation for CD4+ T cells and 24 h following stimulation for CD8+ T cells (Fig. 4C). However, among the time points of 12, 18, 24, and 36 h, there are no statistically significant differences in the alloreactive T cell detection rate in multi-variate analysis. Following autologous stimulation, the rate of detection of CD154 + CD69 + CD4+ or CD137 + CD69 + CD8+ T cells remained uniformly low (Fig. 4C).

The detecting rate of both CD4+ and CD8+ alloreactive T cells was reproducible (Supplementary Fig. S2). Furthermore, we analyzed TCR Vb repertoires to test whether our assay can detect clonally consistent alloreactive cells. Representative, repeated experiments with a same allo-pair showed 3.1 (range 2.4–3.6) % and 2.1 (1.8–2.4)% of TCR Vb1+, 1.5 (1.3–1.7)% and 0.4 (0.3–0.4)% of TCR Vb3+, 5.2 (4.3–5.2)% and 1.1 (0.9–1.2)% of TCR Vb8+, and 4.6 (4.3–5.2)% and 0.4 (0.3–0.5) of TCR Vb9+ cells in CD4+ and CD8+ alloreactive cells, respectively (Fig. 5). The proportion of TCR Vbs positive cells were quite consistent in alloreactive cells despite small absolute number compared to non-alloreactive cells.

Consistent with the activated phenotype of stimulators after 2 days activation (Fig. 3), activated stimulators significantly enhanced detection of both CD4+ and CD8+ alloreactive T cells compared to non-activated PBMCs stimulators (% alloreactive cells in CD4+ and CD8+ T cells: $0.48 \pm 0.33\%$ vs $0.23 \pm 0.14\%$ ($P = 0.0031$) and $0.39 \pm 0.20\%$ vs $0.21 \pm 0.13\%$ ($P = 0.0056$), respectively, Fig. 6A, left panel). Background signal detected following responder culture with activated autologous stimulators was also significantly upregulated in CD4+ T cells compared with non-activated PBMC stimulators. (% CD154 + CD69+ cells $0.032 \pm 0.015\%$ vs $0.020 \pm 0.012\%$, $P = 0.0183$, Fig. 6B, left panel). Notably, the percentage of CD4+ T cells that became activated following autologous stimulation remained low

compared to the percentage of CD4⁺ T cells that became activated following allogeneic stimulation.

Activated vs. resting stimulators did not significantly affect the percentage of responder CD8⁺ T cells that were activated following autologous stimulation. Although a slightly higher percentage of responder CD8⁺ vs. CD4⁺ T cells become activated following stimulation with activated autologous PBMC (%CD4⁺ activated $0.032 \pm 0.015\%$ vs. %CD8⁺ activated $0.080 \pm 0.056\%$), this value is still below the mean percentage of responder CD8⁺ T cells that are activated following activated allogeneic stimulation (%CD8⁺ activated $0.39 \pm 0.20\%$, Fig. 6A and B, right panels).

We also examined the relationship between the number of HLA mismatches between stimulator and responder PBMCs and the frequency of the alloreactive response. A robust response across a spectrum of mismatches except one mismatch of HLA class I was observed (Fig. 7). Responder-stimulator pairs with zero HLA mismatches were intentionally avoided for this assay. Interestingly, although the number of HLA mismatches of HLA-DR loci increased the detection of CD4⁺ alloreactive T cells (Fig. 7A), we did not observe the same effect by those of HLA class I for detection of CD8⁺ alloreactive cells (Fig. 7B).

3.4. Defining optimal conditions to assess the immunologic status of responding T cells

We next sought to identify additional T cell surface markers that would be minimally affected by stimulation during 12 h of allo-stimulated culture. If a surface marker changed appreciably over the time course of the experiment, it may reflect a T cell population that is differentiating upon stimulation in vitro vs. a T cell population that has already been activated in vivo. To understand the kinetics of surface molecule expression after stimulation, we performed time course experiments over 24 h using anti-CD3 and anti-CD28 coated beads as pseudo stimulators. Similar kinetics of surface marker expression were observed following PHA stimulation (data not shown).

We observed 3 general patterns of surface marker expression following T cell stimulation: surface markers that increased over time following stimulation, surface markers that decreased over time following stimulation, and those that were largely unchanged over time following stimulation (Fig. 8).

Surface expression of activation induced inhibitory markers that are also suggestive of T cell exhaustion, such as PD-1, TIM-3, LAG-3, TIGIT and CTLA-4 increase over time following T cell stimulation. (Fig. 8A). Both CD4⁺ and CD8⁺ T cell expression of PD-1 (%CD4⁺ at 0 h vs. 18 h, $2.7 \pm 0.56\%$ vs. $14.6 \pm 4.3\%$; %CD8⁺ at 0 h vs. 18 h, $3.1 \pm 1.6\%$ vs. $12.1 \pm 6.3\%$), TIM-3 (%CD4⁺ at 0 h vs. 18 h, $0.18 \pm 0.17\%$ vs. $0.78 \pm 0.47\%$; %CD8⁺ at 0 h vs. 18 h, $0.093 \pm 0.056\%$ vs. $5.1 \pm 4.8\%$), and LAG-3 (%CD4⁺ at 0 h vs. 18 h, $0.053 \pm 0.052\%$ vs. $3.9 \pm 2.4\%$; %CD8⁺ at 0 h vs. 18 h, $0.032 \pm 0.032\%$ vs. $18.1 \pm 13.0\%$) remain low for the first 12 h of stimulation and begin to increase 18–24 h following stimulation. At 12 h following stimulation, CD4⁺ and CD8⁺ T cells begin to express slightly increased levels of TIGIT (%CD4⁺ at 0 h vs. 12 h, $6.2 \pm 2.3\%$ vs. $12.2 \pm 4.7\%$; %CD8⁺ at 0 h vs. 12 h, $22.6 \pm 13.0\%$ vs. $31.0 \pm 16.0\%$), and more noticeably, increased levels of CTLA-4 (%CD4⁺ at 0

h vs. 12 h, $14.3 \pm 5.5\%$ vs. $48.1 \pm 6.1\%$; %CD8⁺ at 0 h vs. 12 h, $2.0 \pm 0.86\%$ vs. $22.3 \pm 4.5\%$).

Surface expression of CD244 (%CD4⁺ at 0 h vs. 6 h, $0.78 \pm 0.60\%$ vs. $0.21 \pm 0.22\%$; %CD8⁺ at 0 h vs. 6 h, $24.9 \pm 15.9\%$ vs. $14.1 \pm 13.6\%$), CD28 (%CD4⁺ at 0 h vs. 6 h, $95.1 \pm 3.3\%$ vs. $55.6 \pm 9.2\%$; %CD8⁺ at 0 h vs. 6 h, $62.2 \pm 20.7\%$ vs. $44.4 \pm 12.6\%$), CD127 (%CD4⁺ at 0 h vs. 12 h, $88.5 \pm 8.8\%$ vs. $67.2 \pm 14.8\%$; %CD8⁺ at 0 h vs. 12 h, $78.7 \pm 14.3\%$ vs. $59.8 \pm 13.0\%$), and CD62L (%CD4⁺ at 0 h vs. 12 h, $76.4 \pm 5.1\%$ vs. $64.3 \pm 7.9\%$; %CD8⁺ at 0 h vs. %CD8⁺ at 12 h, $58.1 \pm 2.6\%$ vs. $49.7 \pm 13.5\%$) begin to decrease on both CD4⁺ and CD8⁺ T cells 6 or hours following stimulation, as indicated. As surface expression of these markers quickly changes in response to short-term stimulation, it is difficult to detect basal level expression vs. expression induced in response to stimulation (Fig. 8B).

Surface expression of markers of activation that overlap with markers suggestive of T cell memory, exhaustion, and senescence were also quantified over 24 h of stimulation. CCR7 (%CD4⁺ at 0 h vs. 24 h, $89.7 \pm 6.3\%$ vs. $90.0 \pm 3.1\%$; %CD8⁺ at 0 h vs. 24 h, $59.7 \pm 20.3\%$ vs. $78.2 \pm 15.4\%$), KLRG1 (%CD4⁺ at 0 h vs. 24 h, $27.1 \pm 16.2\%$ vs. $17.6 \pm 11.9\%$; %CD8⁺ at 0 h vs. 24 h, $51.4 \pm 17.9\%$ vs. $40.2 \pm 17.0\%$), and CD57 (%CD4⁺ at 0 h vs. % 24 h, $2.5 \pm 1.9\%$ vs. $2.2 \pm 2.0\%$; %CD8⁺ at 0 h vs. 24 h, $25.8 \pm 14.0\%$ vs. $20.3 \pm 12.7\%$), were relatively stable for up to 24 h following strong polyclonal stimulation (Fig. 8C). We also observed expression of CD45RA (%CD4⁺ at 0 h vs. 24 h, $17.3 \pm 12.2\%$ vs. $24.8 \pm 18.2\%$; %CD8⁺ at 0 h vs. 24 h, $36.1 \pm 13.9\%$ vs. $47.4 \pm 20.2\%$) and CD45RO (%CD4⁺ at 0 h vs. 24 h, $70.4 \pm 16.9\%$ vs. $62.3 \pm 18.1\%$; %CD8⁺ at 0 h vs. 24 h, $45.5 \pm 10.7\%$ vs. $45.2 \pm 19.1\%$), markers of naïve or memory T cells, to be relatively stable over 24 h of stimulation. Hence, we selected markers that changed minimally for the first 12 h following stimulation to phenotype the CD154⁺ + CD69⁺ + CD4⁺ and CD137⁺ + CD69⁺ + CD8⁺ alloreactive T cells detected following MLR.

Recently, CD4⁺ T cells that are CD137⁺ + CD154^{neg} following a short stimulation have been reported as antigen specific activated regulatory T cells (Tregs) (Schoenbrunn et al., 2012; Nowak et al., 2018). Although they represent a small population within total CD4⁺ T cells, we observed that the proportion of CD4⁺ T cells that were CD137⁺ + CD154^{neg} increased up to 36 h after incubation (Fig. 9A), and FOXP3⁺ T cells also accumulated in this population over time (Fig. 9B). The slight increase of FOXP3⁺ in CD137^{neg}CD154⁺ + CD4⁺ T cells may be due to activation of conventional T cells (Wang et al., 2007). The proportion of FOXP3⁺ + CD137⁺ + CD154^{neg} in CD4⁺ T cells after 36 vs. 12 h of activation was significantly increased ($0.26 \pm 0.10\%$ vs $0.080 \pm 0.061\%$, $P = 0.0034$, Fig. 9C). Collectively, our data support previous reports that this CD137⁺ + CD154^{neg}CD4⁺ T cell population represents alloreactive Tregs in the PBMCs. Thus, this assay may detect alloreactive Treg after 36 h of incubation.

4. Discussion

T cells play a central role in rejection and tolerance following solid organ transplantation. The T cell repertoire is selected in the thymus based on response to peptides bound to

self-MHC. Alloreactivity arises due to cross-reactivity of self-T cells to either intact, or peptides from, allogeneic MHC. Following transplantation alloantigens may be presented to recipient T cells directly in the form of whole, donor-derived MHC on donor-derived cells; indirectly in the form of processed donor peptide complexed with self-MHC on self-derived cells; or cross-presented in the form of donor membranes expressing donor MHC fused to self-cells expressing self-MHC (Marino et al., 2016). MHC-multimers allow accurate assessment of MHC-reactive T cells if the specific target antigen is known. Therefore, the processing (or lack thereof) of allogeneic MHC presentation to T cells in vivo, and the diversity of the T cell response, make detection of alloreactive T cells in vitro challenging.

Though difficult to detect in vitro, there is evidence of alloreactive T cell significance in vivo. Collins et al. has demonstrated that a single alloreactive CD4⁺ T cell clone detectable before stem cell transplantation expanded to compose almost the entirety of the circulating CD4⁺ T cell repertoire, and this expansion correlated with graft vs. host disease (Michalek et al., 2003). Conversely, T cell clonal deletion may contribute to operational renal transplant tolerance (Morris et al., 2015). Further confounding detection of alloreactive T cells, this repertoire may be impacted by thymic output and by exposure to viral and microbial pathogens (Wang et al., 2008). Identifying the alloreactive T cell population would allow for its phenotypic analysis both pre- and post-transplant and may provide invaluable information regarding the recipient's immune response to the allograft.

In this study, we have developed an assay that detects alloreactive T cells based on expression of the early activation markers CD69, CD154, and CD137. These alloreactive cells were detected following cryopreservation, allowing for batch-testing of participant cells collected over time. PBMCs are also relatively safe to collect from participants, and have been demonstrated to maintain antigen reactivity following cryopreservation elsewhere (Wang et al., 2016; Kreher et al., 2003). Previous studies have investigated several activating markers to identify alloreactive T cells. CD69 have been intensively investigated and found that it is induced very quickly within several hours, but their specificity is not satisfactory as we observed. CD25, CD71, and HLA-DR were induced later than CD69 (Caruso et al., 1997). Cell-cycle marker, Ki-67, also have been reported to detect recent activation (Ali et al., 2016), but we could not observe its upregulation within less than 24 h (data not shown). We chose the early activation markers based on rapid upregulation after stimulation, high sensitivity of detection, and low background. CD154 has been shown as rapid and sensitive marker of antigen-specific CD4⁺ T cell activation (Chattopadhyay et al., 2005; Frentsch et al., 2005). Co-culture of T cells with fluorescently labeled anti-CD154 antibody during their stimulation results in the capture and immediate labeling of any CD154 on the cell surface (Chattopadhyay et al., 2005). We used this co-culture method as it also maintains cell viability and function. While CD154 is expressed readily on CD4⁺ T cells following stimulation, the expression on CD8⁺ T cells is limited on a subset of CD8⁺ cells (Frentsch et al., 2013; Durlanik et al., 2016). CD137, however, has been reported as a rapid marker of both CD4⁺ and CD8⁺ T cell activation (Wolfl et al., 2007). For this assay to reliably and specifically detect alloreactive T cells, the selected markers must be expressed only after activation. However, we observed a non-specific upregulation of CD69, CD154 and CD137 expression on cryopreserved PBMCs T cells upon thawing in the absence of stimulation and. Resting cryopreserved PBMCs after thawing is commonly used to both eliminate dying cells

and recover viable cell responsiveness and, have been reported that they does not change the total number of antigen specific T cells as determined by MHC-multimer staining (Wang et al., 2016; Kutscher et al., 2013). Our data suggests that, during the resting period, thawed T cells are reprogrammed to upregulate activation pathways, primarily in response to intrinsic cues. Simultaneously, consistently observed cell death in resting precondition may lead bias that relate to test sensitivity, which live cell numbers below detection threshold, rather than selective death of alloreactive cells. We successfully reduce the background level of activation at resting phase with combination analyses of CD69 and CD154/CD137. Additionally, the expressions of CD137 on Treg and effector CD4+ T cells after stimulation have been reported to show differential kinetics (Schoenbrunn et al., 2012). Early expression of CD137, in the absence of CD154, has been reported as an antigen specific activation signature of CD4+ Treg (Nowak et al., 2018). Assessing expression of both CD154 and CD137 together allows for identification and analysis of alloreactive effector CD4+ T cells, alloreactive CD8+ T cells, and alloreactive CD4 + Tregs by CD4 + CD154 + CD69+, CD8 + CD137 + CD69+ and CD4 + CD137 + CD154-, respectively. This is, to our knowledge, the first report depicting alloreactivity in all three of these T cell subsets with a single assay.

In our assay, activation of stimulator cells by CD40L and IL-4 significantly enhanced detection of alloreactive T cells, perhaps explaining why we detected increased percentages of alloreactive T cells with CD154/ CD137, when compared to previous studies (Litjens et al., 2009, 2013). Classically, the proportion of alloreactive T cells has been reported as 1% –10% by varied assay conditions and read-outs in both murine and human T cell repertoires (Wilson and Blyth, 1968; Lindahl and Wilson, 1977; Ryser and MacDonald, 1979; Turka et al., 1989). More recently, high throughput sequencing has demonstrated the cumulative frequency of the alloreactive T cell repertoire detectable in total T cells to be approximately 0.8%, suggesting that previous estimates (up to 10%) may contain bystander responded clones or come from the dominators excluded the large part of clones that died during activating periods (DeWolf et al., 2018). These recent findings support that our short-term assay detecting around 0.5% alloreactive T cells does not miss significant part of alloreactive repertoire. Additionally, we analyzed the impact of clinically relevant HLA mismatch status in solid organ transplantation and observed that HLA-DR (class II) mismatch increased the detection of CD4+ alloreactive T cells, but HLA class I (A and B) mismatch did not increase that of CD8+ alloreactive T cells. The culture conditions, the preconditioning for both responder and stimulator PBMCs, and status of other HLA loci may be responsible of this differential reactivity. Since the detection of alloreactive T cells is highly dependent upon on the quality of both responder and stimulator cells, it would be advantageous to batch these assays which allows to use of the same stimulator cell preparation over multiple time points. Using cryopreserved PBMCs serves the opportunity to perform low variable assay between the institutions despite limitation of the potential difference from samples in vivo.

Additionally, this study describes the way to choose the phenotypic surface markers of interest, and their utility for identifying T cell subsets before their expression altered by activation. Polyclonal stimulation by anti-CD3 and anti-CD28 antibody coated beads allowed for kinetic modeling of changes in the expression levels of T cell surface markers, and identified the optimal time point following stimulation to evaluate specific surface

markers. This optimization is relevant as a current area of investigation is monitoring donor-specific hyporesponsiveness in recipient T cells following transplantation. Multiple inhibitory and activation markers have been reported as surface markers suggestive of T cell hyporesponsiveness (Crespo et al., 2013; Sanchez-Fueyo and Markmann, 2016). Predictably, we observed surface expression of some markers reported to be suggestive of T cell exhaustion, CTLA-4, CD244, CD28, and CD127, to rapidly change following stimulation. Thus, these markers were not well suited to phenotype alloreactive T cells in this assay. In contrast, we found that PD-1, TIM-3, LAG-3 and TIGIT, are not upregulated within 12 h, allowing us to assess pre-existing allospecific T cells with a surface phenotype suggestive of exhaustion with multi-color flow cytometry. Additional studies would be needed to confirm that these cells are indeed hyporesponsive.

5. Conclusion

In summary, this assay allows for highly specific identification of cryopreserved CD4+, CD8+, and regulatory alloreactive T cells based on the expression of cell surface markers. As it utilizes cryopreserved PBMCs, it can be performed in batches and performed centrally in multi-center clinical trials. It may also allow for exploring biomarkers of tolerance and provide mechanistic insights of the recipient anti-donor T cell response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

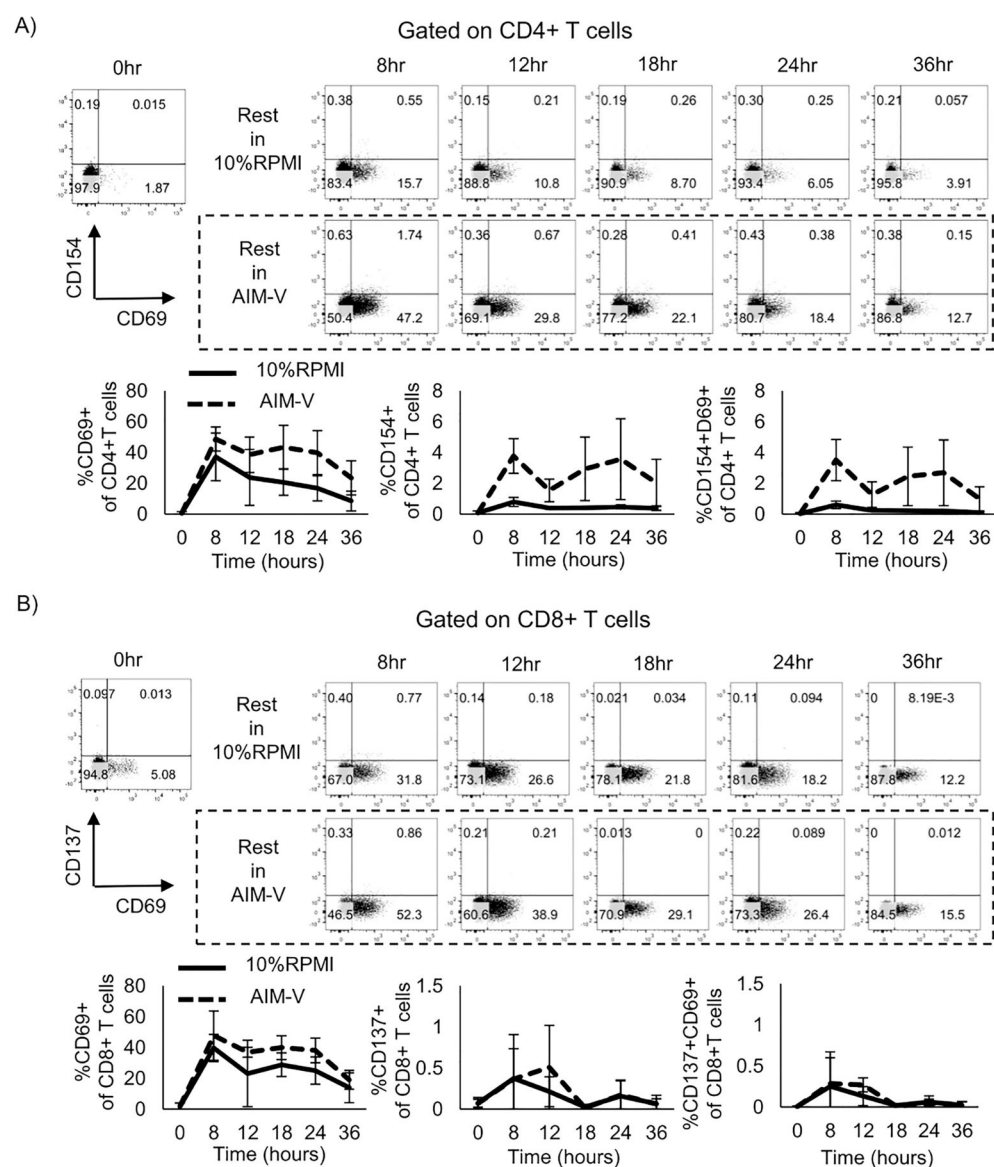
PBMC	peripheral blood mononuclear cell
MLR	mixed lymphocyte reaction
TNF	tumor necrosis factor
CFSE	carboxyfluorescein diacetate succinimidyl ester
APC	antigen presenting cell
MHC	major histocompatibility complexes

References

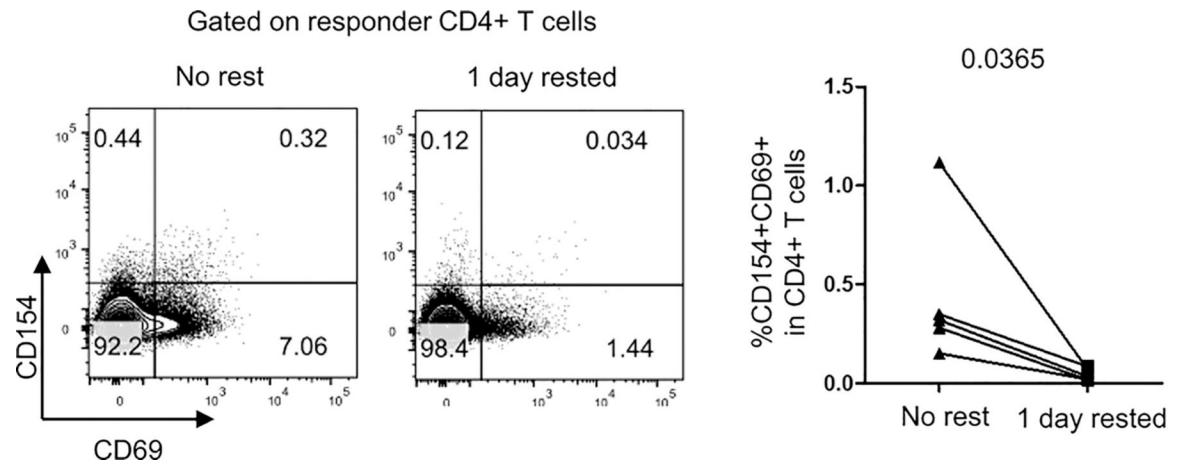
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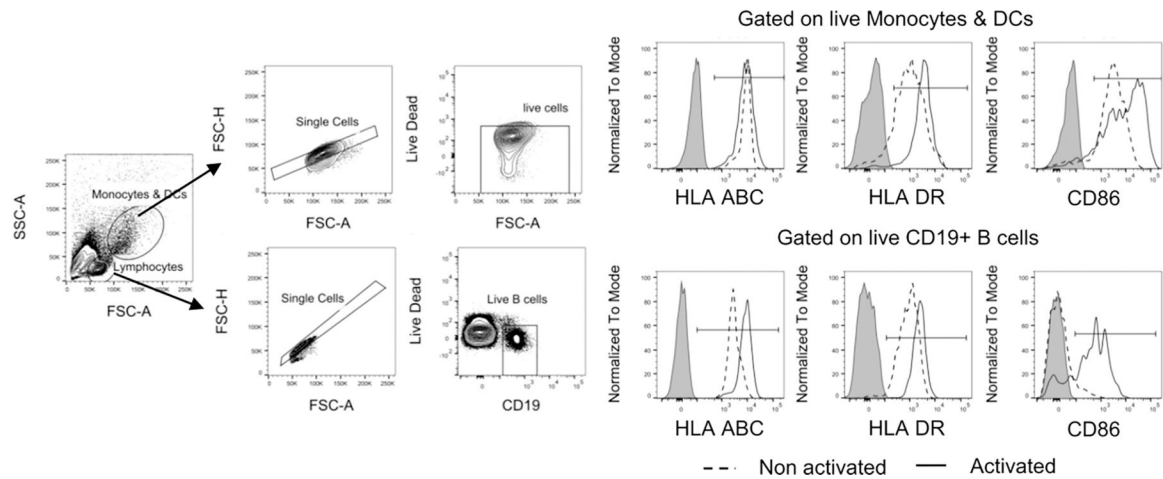
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**Fig. 1.**

T cells derived from cryopreserved PBMC have high basal levels of early activation markers. Cryopreserved PBMCs were thawed and rested in 10% human AB serum containing RPMI (10%RPMI) and serum-free medium (AIM-V) with anti-CD154 labeling antibody. The expression of CD154 and CD69 in CD4+ T cells (A) and CD137 and CD69 in CD8+ T cells (B) were quantified at indicated times before and during resting. Line plots show the percent of CD4+ (A) or CD8+ (B) T cells that are positive for markers, alone or in combination (mean $\pm$ s.d.). Data are representative of PBMCs from 6 different donor samples in 2 independent experiments.

**Fig. 2.**

Using the combination of CD69 and CD154 reduces the number of responder cells considered to be activated following autologous cell culture. Mixed lymphocyte reactions were performed with responders without resting (No rest) and after resting for 1 day (1 day rested) against autologous stimulators for 12 h. Representative dot plots indicate the expression of CD69 and CD154 on CD4⁺ responder T cell gated by exclusion of CFSE stained stimulators and dump (CD14⁺, CD16⁺, and CD19⁺) cells. The number indicates % of cells in each quadrant gate. Line plots show the proportion of CD154⁺CD69⁺ in CD4⁺ responder T cells from 5 different reactions in 2 independent experiments. Statistical analyses were performed by paired *t*-test. *P* values below 0.05 were considered significant.

**Fig. 3.**

Activation of stimulator cells prior to inclusion in MLR increases their surface expression of class I and class II HLA molecules. Representative dot plots show the gating strategy for analyzing B cells (CD19+) and potential antigen presenting cells, monocytes & dendritic cells (DCs, higher forward and side light scatter). Histograms depict the expression of HLA class I (ABC), II (DR), and CD86 on activated (solid) and non-activated (dash) stimulator cell subsets as compared to staining with isotype-matched control (grey filled). Data are representative of 3 independent experiments.

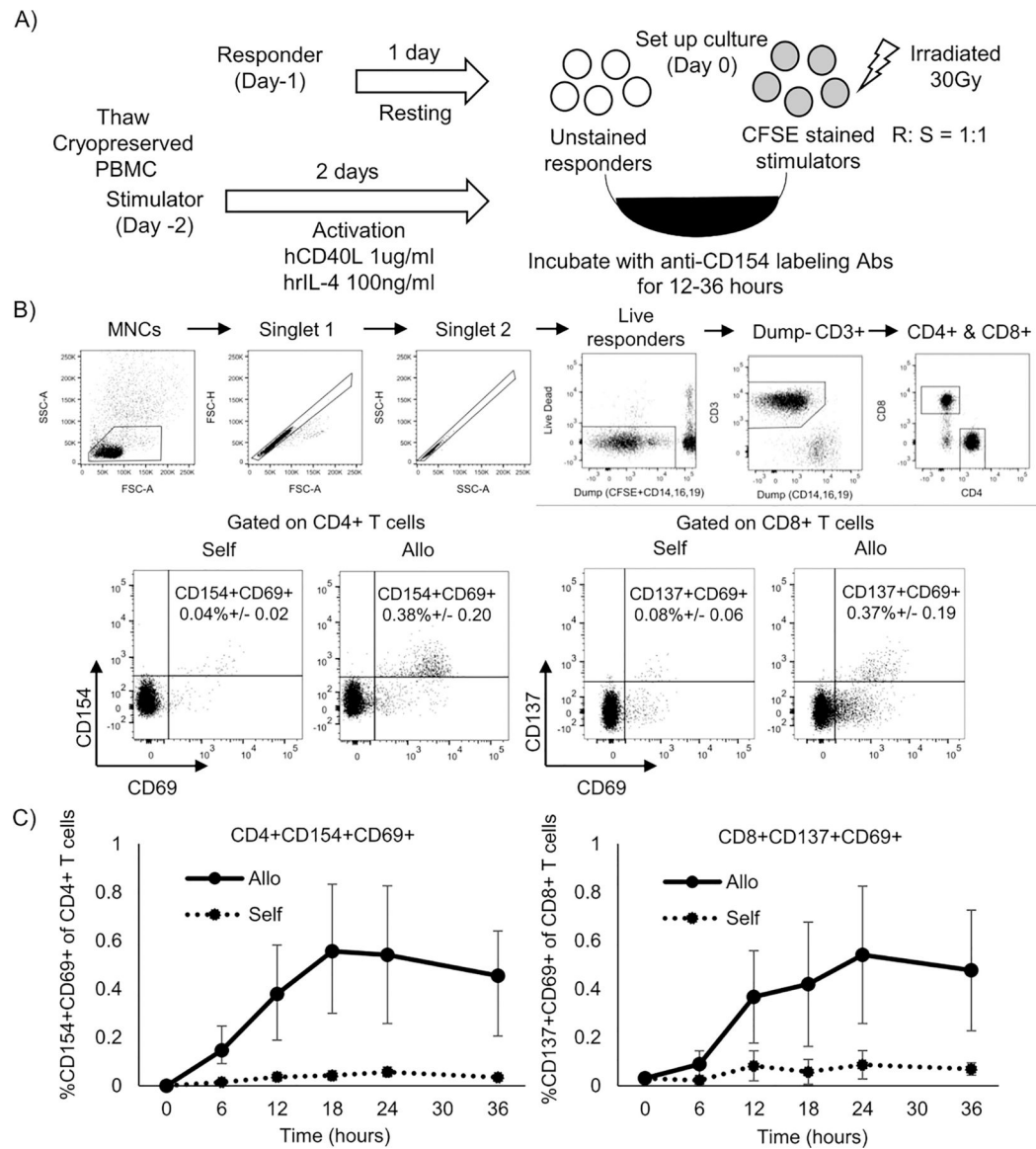


Fig. 4. Time dependent detection of CD4+ and CD8+ alloreactive T cells.

(A) Allogeneic or autologous stimulator PBMCs (S) are pre-activated with hCD40L + hIL-4 for 2 days, labeled with CFSE, and irradiated prior to MLR. Responder PBMCs (R) are rested 1 day prior to MLR. MLR are established by incubating R and S at a 1:1 ratio for 12–36 h as indicated in Results and Figure Legends. Fluorescently tagged anti-CD154 antibody was added to the culture media through the stimulation period. (B) Representative dot plots show the gating strategy to exclude CFSE stimulators and non-T cells from the analyzed CD4+ and CD8+ responder T cells (top row). Representative dot plots show the expression of CD69 and CD154/CD137 on CD4+ (bottom left 2 panels) and CD8+ (bottom right 2 panels) T cells, respectively, following culture with autologous stimulators (self) and allogeneic stimulator (allo). Numbers in panels indicate %double positive CD4+ or CD8+ T cells (CD69 + CD154+ or CD69 + CD137+, respectively, mean ± s.d.). (C) Line plots indicate the percent of CD4+ (left) and CD8+ (right) reactive T cells over time following

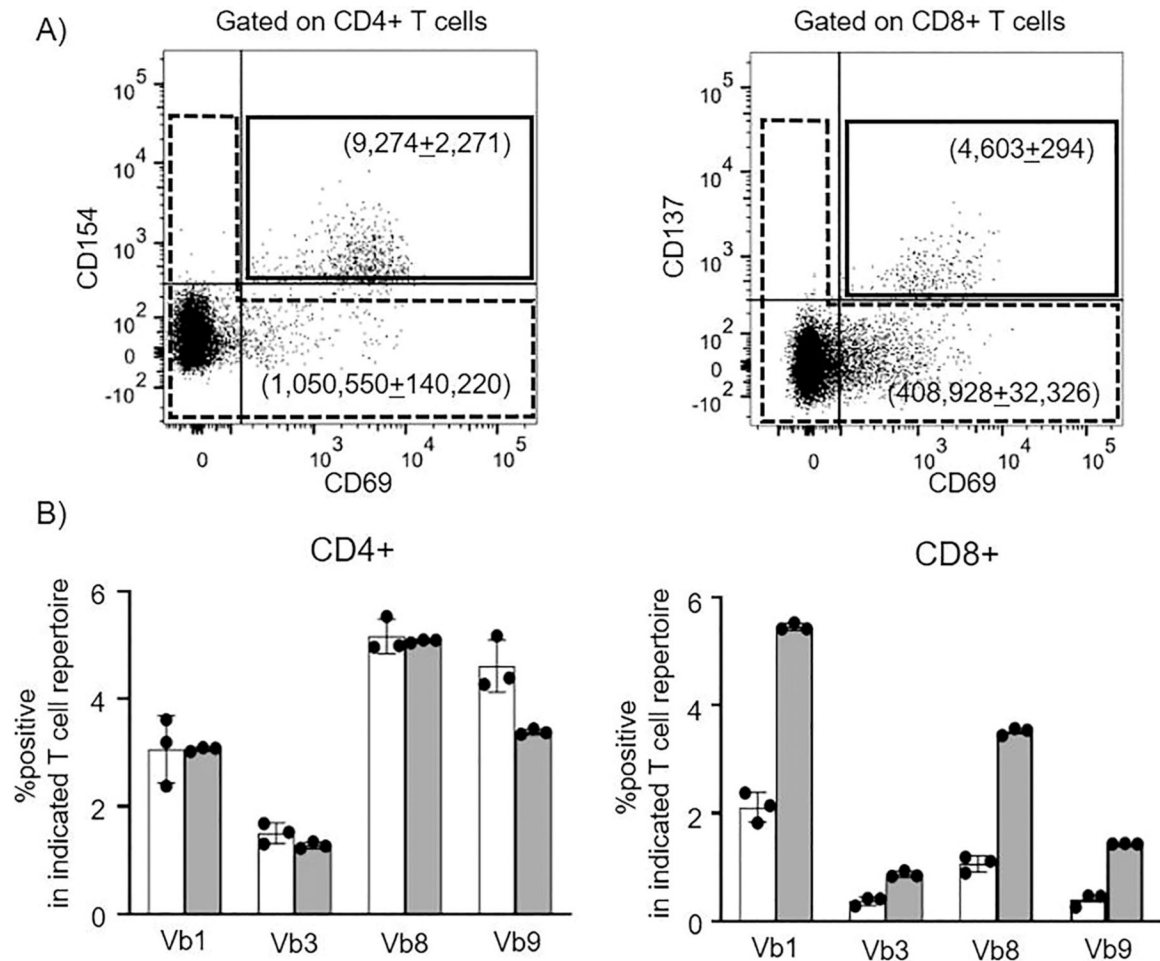
autologous stimulation (self, dotted line) and allogeneic stimulation (allo, solid line). Data from >6 reactions in 2 independent experiments (mean $\pm$ s.d.).

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**Fig. 5.**

TCR Vb usage in CD4⁺ and CD8⁺ alloreactive T cells. As detailed in Fig. 4, one-day rested PBMC responders were incubated for 14 h with CFSE-labeled allogeneic stimulators pre-stimulated with CD40L and IL-4. (A). Representative gating strategy carried out to identify allo-reactive (solid line) and non-alloreactive cells (dashed line). Dump^{neg} (CFSE, CD14, CD16, CD19) live CD3⁺CD4⁺ or CD3⁺CD8⁺ cells were gated on expression of CD69/CD154 or CD69/CD137 activation markers, respectively. Quadrants provided the absolute numbers of cells (mean ± S.D.). (B). Frequencies (% of Vb⁺ cells/ total cell number) of TCR Vbeta usage among alloreactive (open bars) and non-alloreactive CD4⁺ and CD8⁺ T cells (filled bars). Each dot represents the average of 3 independent experiments done on a single responder/stimulator pair.

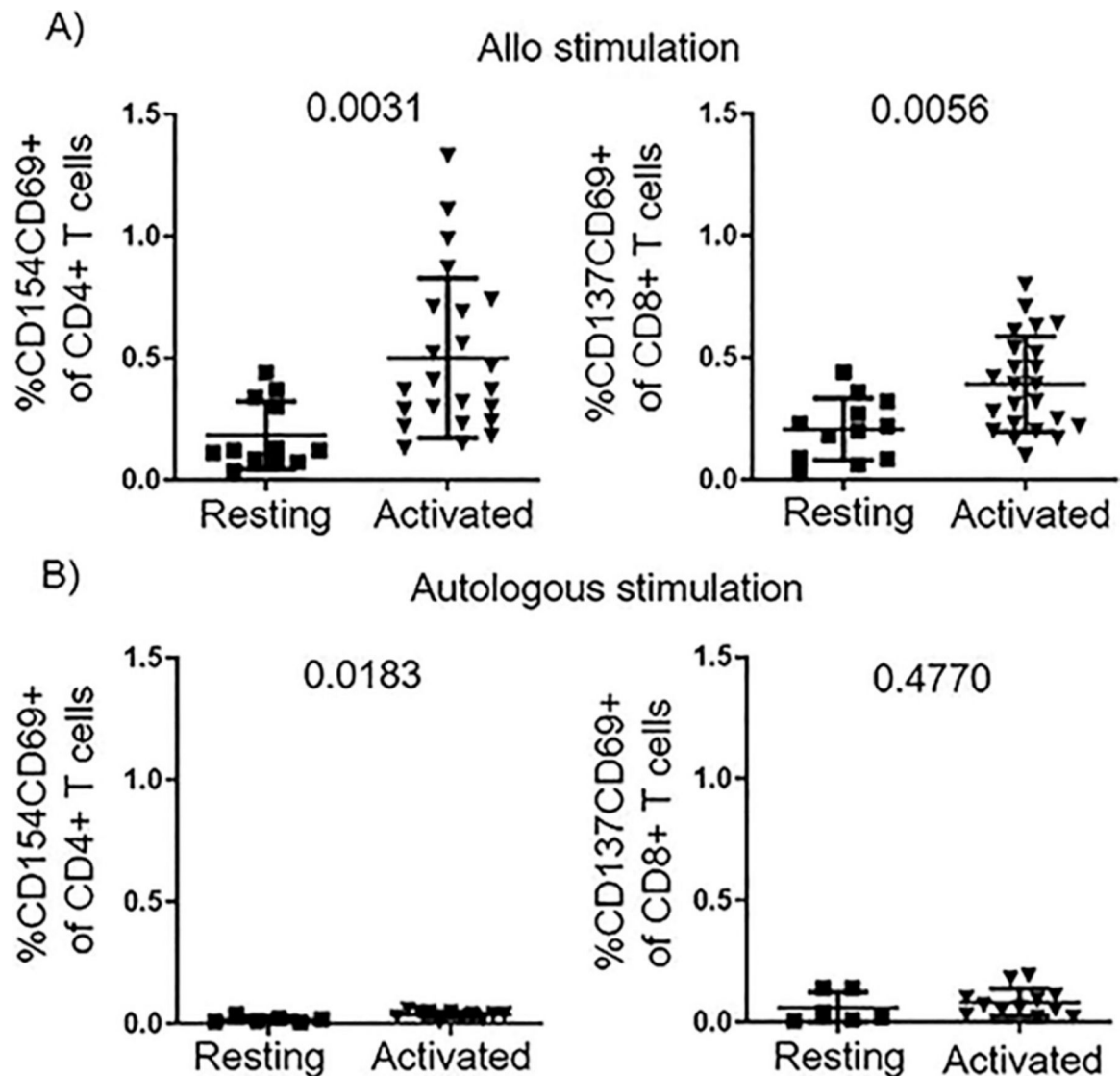


Fig. 6.

Activating stimulators prior to MLR enhances detection of alloreactive T cells in vitro. The proportion of CD4+ (CD154 + CD69+) and CD8+ (CD137 + CD69+) T cells that were reactive to allogeneic (A) or autologous (B) PBMCs after 12 h of co-culture. Stimulators were pre-activated for 2-days with CD40L and human rIL-4. (A) As compared to non-activated stimulators (Resting, $n = 12$), activated stimulators (Activated, $n = 28$) significantly increased detection of CD4+ and CD8+ alloreactive T cells. (B) Control experiments with autologous S/R pairs (self) show that the percentage of activated responder CD4+, but not CD8+ T cells, is increased following stimulation by activated autologous PBMCs (Activated, $n = 12$) in comparison with non-activated (Resting, $n = 6$). Vertical bars indicate the mean $\% \pm$ s.d. of indicated proportion.

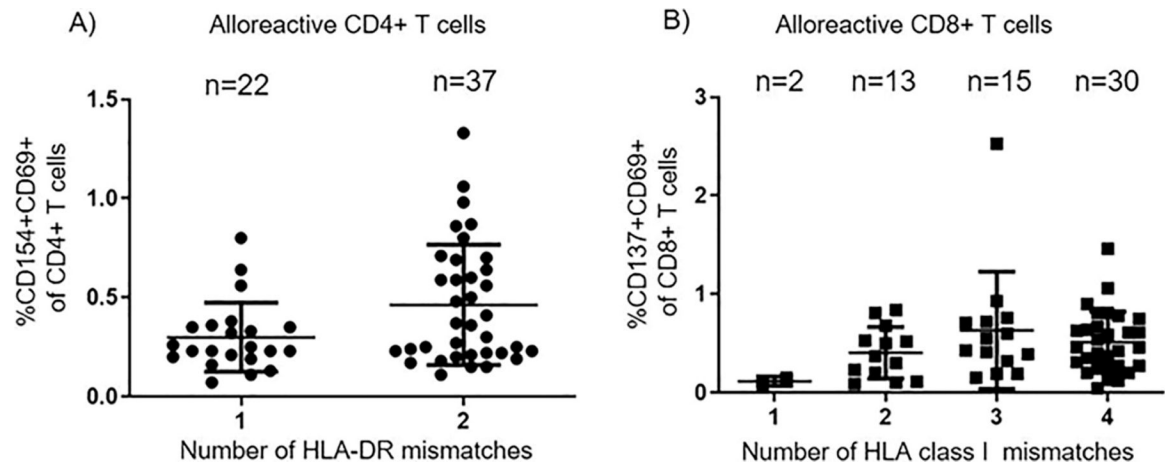


Fig. 7. The number of HLA mismatches between stimulator and responder cells increase detection of CD4+ alloreactive T cells, but not for CD8+ T cells.

(A) The proportion of alloreactive CD4+ (CD154 + CD69+) and (B) CD8+ (CD137 + CD69+) T cells was assessed following a 12 h MLR with randomly paired PBMCs (total $n = 60$) and categorized by the number of HLA mismatches present at HLA-DR and class I (A and B) loci. Data are presented from 4 independent experiments. Vertical bars indicate the mean $\pm$ s.d. of alloreactive T cells.

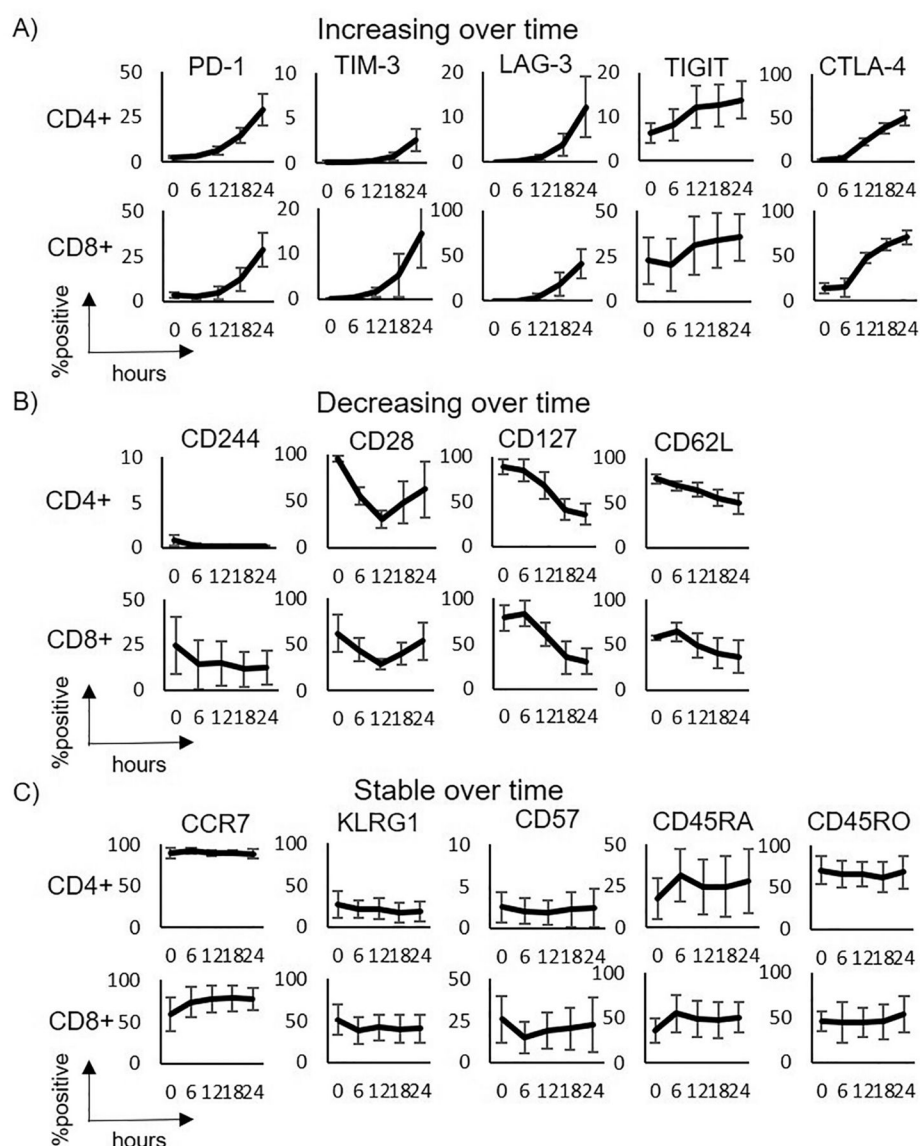


Fig. 8. Kinetic expression of T cell surface markers following stimulation. PBMCs were rested for 2 days, then cultured with CD3/CD28 beads at 1:1 cell to bead ratio for indicated periods of time. T cells were then assessed for expression of various surface markers by flow cytometry. Data were obtained on CD4+ (top row) and CD8+ (bottom row) followed by live CD3+ gate. Patterns of expression kinetics over time were categorized into (A) increasing over time (PD-1, TIM-3, LAG-3, TIGIT and CTLA-4) (B) decreasing over time (CD244, CD28, CD127, CD62L), and (C) stable over time (CCR7, KLRG1, CD57, CD45RA and CD45RO) according to their expression profile. Vertical bars indicate the mean % $\pm$ s.d. of positive cells based on isotype matched control.

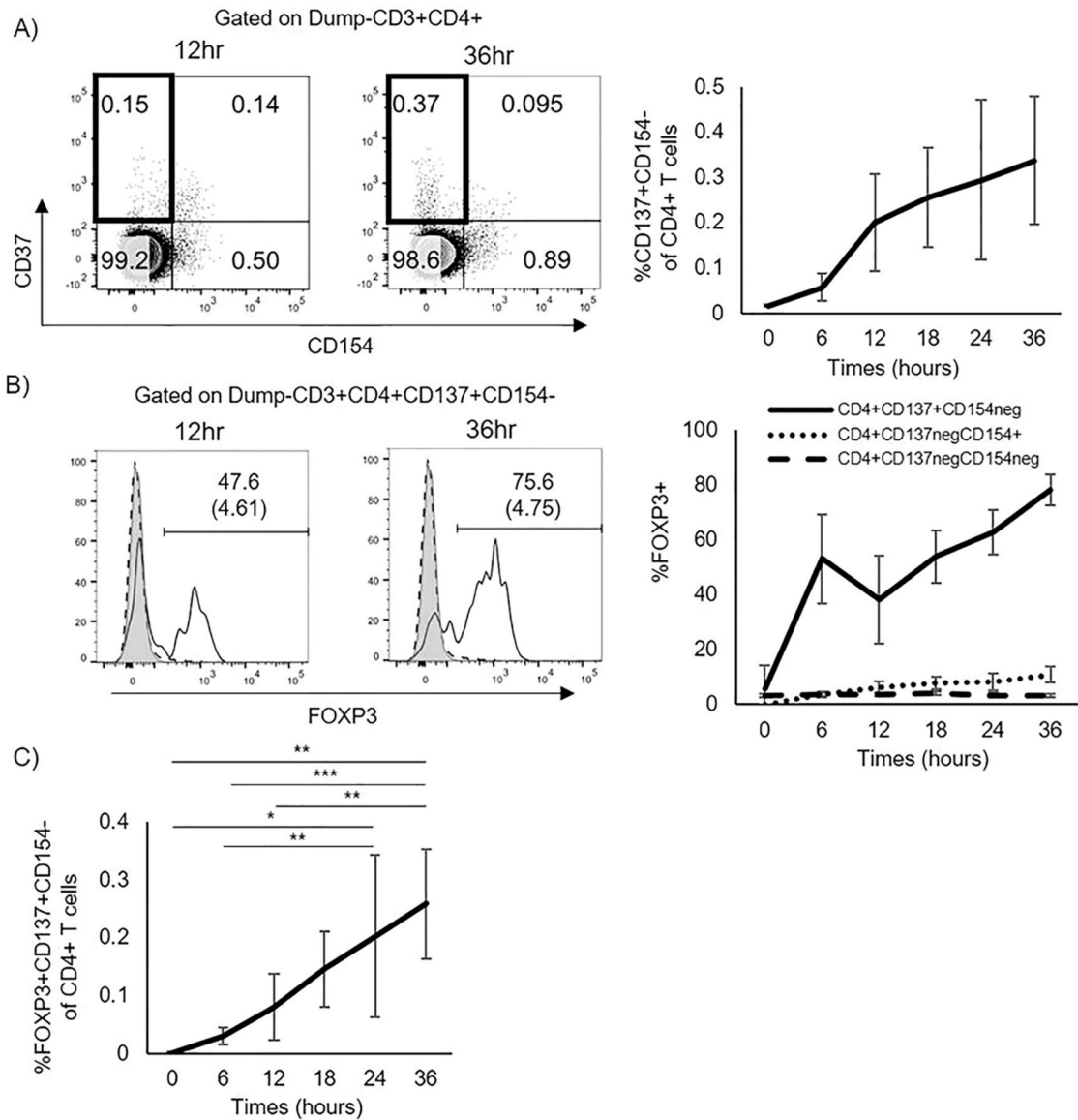


Fig. 9. FOXP3⁺ T cells accumulate within the CD137 + CD154⁻ CD4⁺ T cell subset.

(A) Representative dot plots show CD154 and CD134 expression on CD4⁺ T cells co-cultured with activated allogeneic stimulator cells for 12 h (left) and 36 h (middle). Line plot (right) depicts the mean % of CD137 + CD154^{neg}CD4⁺ T cells over time. Vertical bars represent s.d. (B) Representative histograms show the FOXP3 expression on CD137 + CD154^{neg}CD4⁺ T cells (solid line), CD137^{neg}CD154^{neg} CD4⁺ T cells (dashed line), and CD8⁺ T cell (grey filled, negative control) at 12 h (left) and 36 h (middle). Numbers indicate the %FOXP3⁺ in CD137 + CD154^{neg}CD4⁺ T cells and (in CD137^{neg}CD154^{neg} CD4⁺ T cells), respectively. Line plots show the mean %FOXP3⁺ in indicated populations (B, right) and %FOXP3 + CD137 + CD154^{neg} in total CD4⁺ T cells (C) following allogeneic stimulation. Vertical bars represent s.d. Statistical analysis shown only on significant data (* $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$, One way ANOVA followed by Tukey multiple comparison).