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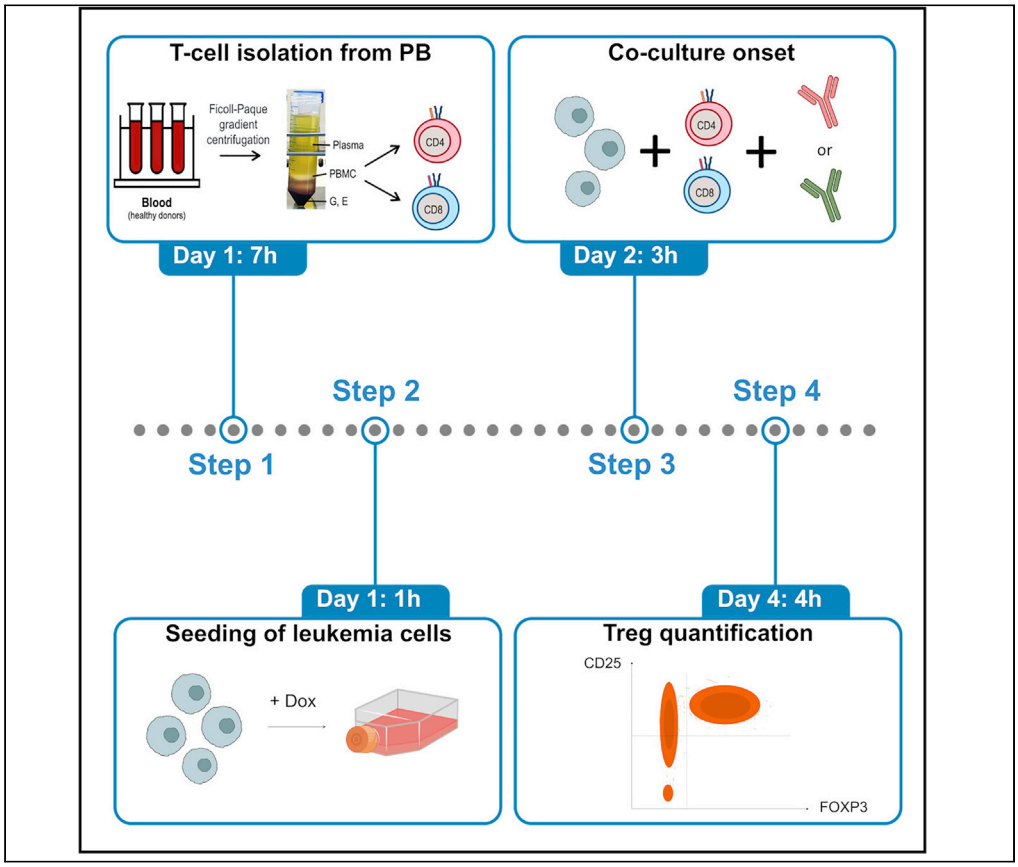
Co-culture of primary human T cells with leukemia cells to measure regulatory T cell expansion



The expansion of regulatory T cells (Tregs) is known to be mediated by cytokines including IL-10 and TGFβ but has additionally been shown to depend on the interaction of the immune receptors ICOSLG and ICOS. Here, we describe a co-culture system which enables quantification of the ability of leukemia cells to induce Treg expansion through secreted cytokines and direct receptor interactions. The protocol is applicable for MHC-matched and -unmatched experiments and allows assessment of Treg expansion without using a mouse model.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

MHC-unmatched co-culture of primary T cells with leukemia cells

Quantification of the ability of leukemia cells to induce regulatory T-cell expansion

Investigation of ICOSLG-mediated regulatory T-cell induction

Regulatory T-cell characterization using flow cytometry and ELISA

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Co-culture of primary human T cells with leukemia cells to measure regulatory T cell expansion

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SUMMARY

The expansion of regulatory T cells (Tregs) is known to be mediated by cytokines including IL-10 and TGFβ but has additionally been shown to depend on the interaction of the immune receptors ICOSLG and ICOS. Here, we describe a co-culture system which enables quantification of the ability of leukemia cells to induce Treg expansion through secreted cytokines and direct receptor interactions. The protocol is applicable for MHC-matched and -unmatched experiments and allows assessment of Treg expansion without using a mouse model.

For complete details on the use and execution of this protocol, please refer to Külp et al. (2022).

BEFORE YOU BEGIN

This protocol describes an *in vitro* approach to assess the ability of leukemia cell lines to induce the development of Tregs.

The t(4;11) acute lymphoblastic leukemia (ALL) cell line SEM was stably transfected with the open reading frame (ORF) of the *EGR3* gene (SEM::EGR3) which has been identified as a transactivator of ICOSLG (Külp et al., 2022). The empty vector transfected cell line SEM::mock served as a control.

In the literature, ICOSLG expression on healthy and tumor cells has been shown to foster the development of adjacent Tregs (Martin-Orozco et al., 2010; Faget et al., 2012; Lee et al., 2017; Han et al., 2018; Iwata et al., 2019). In this protocol, we exemplify how we proved that the EGR3-mediated ICOSLG transactivation led to Treg expansion in the t(4;11) proB-ALL cellular context.

Of note, we describe the set-up of an MHC-unmatched co-culture system. In contrast to MHC-matched (isogenic) co-cultures, MHC-unmatched co-cultures require artificial T cell activation allowing T cell receptor (TCR)-independent experiments.

In brief, $CD4^+$ and $CD8^+$ T cells will be extracted from peripheral blood mononuclear cells (PBMC) of healthy donors and subsequently activated using antibody-coated beads enabling a non-isogenic co-culture of T cells with the previously generated ALL model cell lines SEM::EGR3 and SEM::mock. Tregs will be quantified using flow cytometry and the cultured supernatants will be measured on an ELISA cytokine quantification assay (Figure 1). The co-culture includes not only $CD4^+$ but also $CD8^+$ T cells because leukemia cells have been characterized to interact with both T -cell subtypes in the

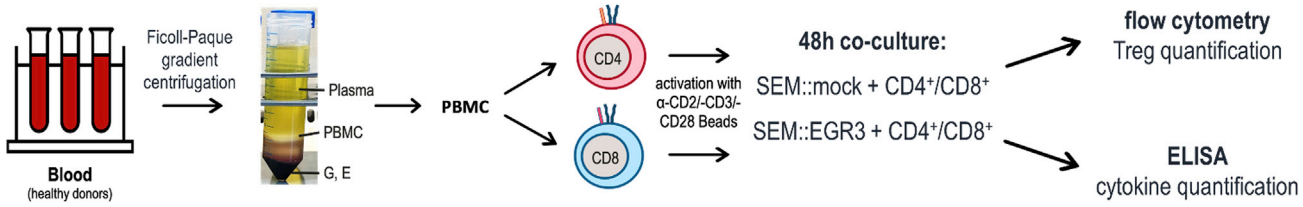

Figure 1. Operational workflow of T cell co-culture
PBMC = peripheral blood mononuclear cells. G = granulocytes. E = erythrocytes.

bone marrow (Feuerer et al., 2003; Pastorczak et al., 2021). In principle, the experiment could also be performed without the $CD8^+$ fraction but would then reflect the bone marrow's cellular composition to a lesser extent.

Institutional permissions

Use of pseudonymized donor material, including the donor informed consent documentation was assessed and formally approved by the Ethics Committee of Goethe University Medical Center (approval #329/10).

Please acquire all permissions needed for the use of human T cells from relevant authorities of your institution.

Test cultivation in T cell media

Timing: 1 week

This protocol is applicable to any leukemia cell culture model, cell line or even primary cells, as long as these cells are cultivatable in T cell media which consists of DMEM high glucose supplemented with 10% human plasma, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

We checked the SEM::EGR3 and SEM::mock cell culture models for viability and changes in the expression of different genes of interest including *EGR3* and *ICOSLG* upon cultivation in T cell media as the SEM cell lines were usually cultivated in RPMI supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin.

If the leukemia cells intended for T cell co-culture are usually cultivated in another media as in our case, we recommend performing a test cultivation of leukemia cells in the described T cell media prior to co-culture to enable assessment of viability and detection of potential expression differences. Through this, you can avoid a potential bias e.g., due to the usage of human plasma instead of FBS. It is appropriate to compare the cultivation of leukemia cells in T cell media with the cultivation in conventional media.

1. Cultivate and expand the leukemia cells intended for subsequent T cell co-culture in usual media.
2. For half of the leukemia cells, exchange usual media with T cell media to enable a comparison of both cultivations.
   a. Aliquot half of the cells.
   b. Centrifuge with cell-dependent conditions, e.g., SEM cells at 200 × *g* for 5 min.
   c. Remove supernatant and resuspend in T cell media.
3. Cultivate leukemia cells for three times of their doubling time, so they pass at least three cell cycles.
4. Check leukemia cells for normal behavior using viability or proliferation assays, cell cycle analysis, transcriptomic analyses, western blotting, or other appropriate methods.

⚠ CRITICAL: In case of adaption of the protocol to an isogenic co-culture without T cell activation, the media should additionally be supplemented with 50 U/mL human IL-2.

Bead loading for T cell activation

Timing: 3 h

5. In case of usage of the T Cell Activation/Expansion Kit human (130-091-441, Miltenyi Biotec) for activation of T cells, loading of the Anti-Biotin MACSiBead Particles with CD2-Biotin, CD3-Biotin and CD28-Biotin is needed. To facilitate the experimental procedure, we recommend to perform bead loading in advance according to the manufacturer's protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Recombinant Anti-ICOS Ligand/ICOSL, monoclonal antibody, unconjugated (WB: 1/1,000)	Abcam	ab209262 RRID not found
Mouse Anti-Human CD3, monoclonal antibody, APC-H7 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 560176, RRID: AB_1645475
Mouse Anti-Human CD4, monoclonal antibody, PE-Cy7 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 557852, RRID: AB_396897
Mouse Anti-Human CD8, monoclonal antibody, BV510 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 563919, RRID: AB_2722546
Mouse Anti-Human CD25 (IL-2 Receptor α), monoclonal antibody, BV421 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 564033, RRID: AB_2738555
Mouse Anti-Human FoxP3, monoclonal antibody, AF647 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 561184, RRID: AB_10584328
Mouse Anti-Human CD278 (ICOS), monoclonal antibody, BB515 conjugated (flow cytometry: 5 µL / 1 × 10^6 cells)	BD	BD Biosciences Cat# 564549, RRID: AB_2738840
Mouse Anti-Human B7-h2, monoclonal antibody, neutralizing, unconjugated (neutralization assays: 20 µg/mL)	R&D Systems	R and D Systems Cat# MAB165, RRID: AB_2122734
Mouse IgG1 kappa isotype control, functional grade (neutralization assays: 20 µg/mL)	Thermo Fisher Scientific	Thermo Fisher Scientific Cat# 16-4714-85, RRID: AB_470162
Chemicals, peptides, and recombinant proteins		
Annexin V Pacific Blue	Invitrogen	Invitrogen A35122
7-AAD	Miltenyi Biotec	Miltenyi Biotec 130-111-568
Critical commercial assays		
IL-10 (human) ELISA Kit	Enzo	Enzo ADI-900-036
IL-2 ELISA Kit (human)	Aviva	Aviva OKBB00179
Doxycycline hydrochloride	Sigma-Aldrich	Sigma Aldrich D3447
Lymphosep	Biowest	Biowest L0560-500
CD4 MicroBeads human	Miltenyi	Miltenyi 130-045-101
CD8 MicroBeads human	Miltenyi	Miltenyi 130-045-201
T Cell Activation/Expansion Kit human	Miltenyi	Miltenyi 130-091-441
Bovine serum albumine (BSA)	Roth	Roth 8076.5
Dulbecco's PBS	Capricorn	Capricorn PBS-1A
RBC lysis buffer	Alfa Aesar	Alfa Aesar J62990.AP
Transcription Factor Buffer Set	BD	BD 562574
Experimental models: Cell lines		
SEM::EGR3; human (female) t(4;11) proB cell line, stably transfected with an Doxycycline-inducible *EGR3* open reading frame expression cassette	Külp et al. (2022)	N/A
SEM::mock; human (female) t(4;11) proB cell line, stably transfected with an Doxycycline-inducible empty expression cassette	Külp et al. (2022)	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
GraphPad Prism 7.01	GraphPad Software Inc.	GraphPad Prism 7.01
BD FACSuite™ Application	BD	BD Biosciences

MATERIALS AND EQUIPMENT

T cell media for co-culture		
Reagent	Final concentration	Amount
DMEM high glucose (DMEM-HPA, Capricorn)	88%	44 mL
Human plasma	10%	5 mL
L-glutamine (STA-B, Capricorn)	2 mM	0.5 mL
Penicillin/Streptomycin (PS-B, Capricorn)	100 U/mL penicillin, 100 µg/mL streptomycin	0.5 mL
Total	**N/A**	**50 mL**
The media can be stored up to 6 months at 2°C–8°C.		

SEM cell media for passaging		
Reagent	Final concentration	Amount
RPMI 1640 (RPMI-HA, Capricorn)	88%	44 mL
FBS (FBS-11A, Capricorn)	10%	5 mL
L-glutamine (STA-B, Capricorn)	2 mM	0.5 mL
Penicillin/Streptomycin (PS-B, Capricorn)	100 U/mL penicillin, 100 µg/mL streptomycin	0.5 mL
Total	**N/A**	**50 mL**
The media can be stored up to 6 months at 2°C–8°C.		

Doxycycline stock solution		
Reagent	Final concentration	Amount
Doxycycline hydrochloride	1 mg/mL	100 mg
Milli-Q water	N/A	100 mL
Total	**N/A**	**100 mL**
The Doxycycline stock solution can be stored frozen at −20°C up to 6 months and should be protected from light.		

Doxycycline working solution		
Reagent	Final concentration	Amount
Doxycycline stock solution	1 µg/mL	10 µL
Milli-Q water	N/A	9.990 mL
Total	**N/A**	**10 mL**
The Doxycycline working solution can be stored frozen at −20°C up to 6 months and should be protected from light.		

⚠ CRITICAL: Incubate SEM cells at 37°C, 5% CO_2 and saturated humidity in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL Penicillin and 100 µg/mL Streptomycin, preheated to 37°C prior to use. Passage SEM cells twice per week with complete media exchange and maintain a density of 1–3 × 10^6 cells/mL.

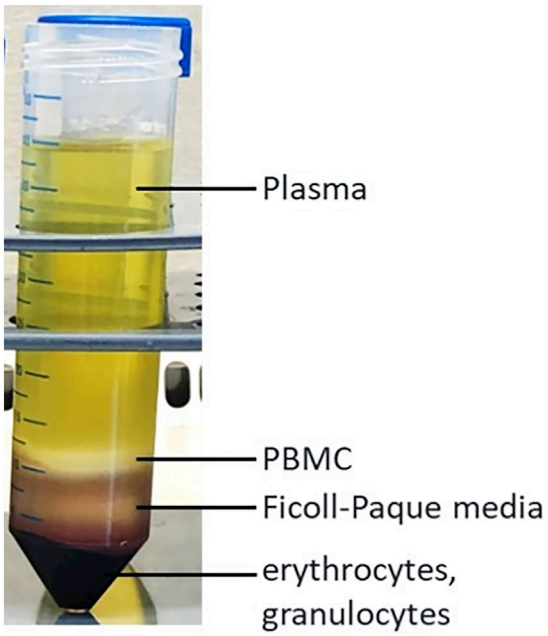

Figure 2. Phase separation of PB after Ficoll-Paque density gradient centrifugation
PBMC = Peripheral blood mononuclear cells.

Alternatives: Instead of using leukemia cell lines, a co-culture with primary leukemia cells can be performed similarly.

STEP-BY-STEP METHOD DETAILS

T cell isolation from peripheral blood

Timing: 7 h

$CD4^+$ and $CD8^+$ T cells are magnetically isolated from peripheral blood mononuclear cells (PBMC) after Ficoll-Paque density gradient centrifugation of peripheral blood (PB) derived from healthy adult donors.

Alternatives: Other methods of T cell isolation are also suitable, e.g., FACS-sorting.

1. Ficoll-Paque density gradient centrifugation.
   a. Aliquot 8–15 mL of PB into 50 mL tubes.
   b. Dilute PB with PBS/0.5% BSA to a volume of 35 mL.
   c. Prepare one 50 mL tube with 10 mL Lymphocyte Separation Media (MS00ZZ1002, Biowest) for each PB tube.
   d. Carefully overlay the separation media with the diluted PB from step b sustaining clear phase separation. See also troubleshooting 1.
   e. Centrifuge at 20°C for 30 min at 400 × *g* **without brake** to enable separation of PBMC.

   Note: PB is now separated in four phases. The supernatant is the plasma diluted with PBS/0.5% BSA, followed by the white layer of PBMC, the Lymphocyte Separation Media and a pellet consisting of erythrocytes and granulocytes (Figure 2).

2. Carefully pipette the plasma into another 50 mL tube without disturbing the white PBMC layer.

3. Use a 1,000 µL pipette to transfer the PBMC into a separate 50 mL tube. The remaining layers can be discarded.

⚠ CRITICAL: Avoid co-transfer of erythrocytes.

4. Add PBS/0.5% BSA until reaching a volume of 50 mL to wash the PBMC.
5. Centrifuge at 400 × *g* for 5 min with brake.
6. During centrifugation, the plasma from step 2 can be sterile filtered using a 50 mL syringe capped with a 0.20 µm sterile filter.

⚠ CRITICAL: Store filtered plasma below −20°C. The plasma will be used as cell culture media supplement. See also troubleshooting 2.

Optional: Plasma can be heat inactivated at 56°C for 30 min in a preheated water bath prior to storage as beneficial effects of heat inactivation on T cell proliferation were reported (Fante et al., 2021). In our study, we did not perform heat inactivation and the Treg expansion was still efficient.

Optional: If the PBMC pellet generated in step 5 appears to be red in color, erythrocytes were co-transferred during step 3 and a red blood cell (RBC) lysis step can be performed.

a. Discard supernatant and resuspend pellet in RBC lysis buffer and incubate at 20°C for 10 min.
b. Add 30 mL PBS/BSA 0.5%.
c. Centrifuge at 400 × *g* for 5 min with brake.

7. Discard supernatant and resuspend pellet in 7 mL PBS/BSA 0.5%.
8. Count the total number of PBMC.
9. Use CD4 and CD8 MicroBeads human (130-045-101, 130-045-201, Miltenyi Biotec) together with MS columns (130-042-201, Miltenyi Biotec) and OctoMACS™ separator (130-042-109, Miltenyi Biotec) to magnetically separate $CD4^+$ and $CD8^+$ T cells according to the manufacturer's protocol.

⚠ CRITICAL: Use at least 66% of total PBMC suspension for $CD8^+$ enrichment to achieve approximately equal numbers of isolated $CD4^+$ and $CD8^+$ T cells as $CD4^+$ T cells are more abundant than $CD8^+$ cells in PB.

Alternatives: If available, also the autoMACS® system can be used for automatic T cell isolation.

10. Perform T cell activation using the T Cell Activation/Expansion Kit human (130-091-441, Miltenyi Biotec) according to the manufacturer's protocol.

⚠ CRITICAL: Use at least 1×10^6 $CD4^+$ and 1×10^6 $CD8^+$ T cells.

a. Additionally, keep at least 1×10^6 $CD4^+$ and 1×10^6 $CD8^+$ T cells for purity assessment in step 12.
b. Use a bead-to-cell ratio of 1:2 and follow the T cell activation protocol, following the T cell expansion protocol is not necessary.
c. Cultivate activated T cells in T cell media 16 h at 37°C and 5% CO_2 and saturated humidity.

11. Perform flow cytometry with 1×10^6 $CD4^+$ and 1×10^6 $CD8^+$ non-activated T cells (step 11) to check purity of MACS enrichment.

Note: Staining of CD3, CD4 and CD8 is sufficient to discriminate between T cell populations and other cells. Use unstained T cells in comparison to the isolates to set the gates as shown in Figure 3.

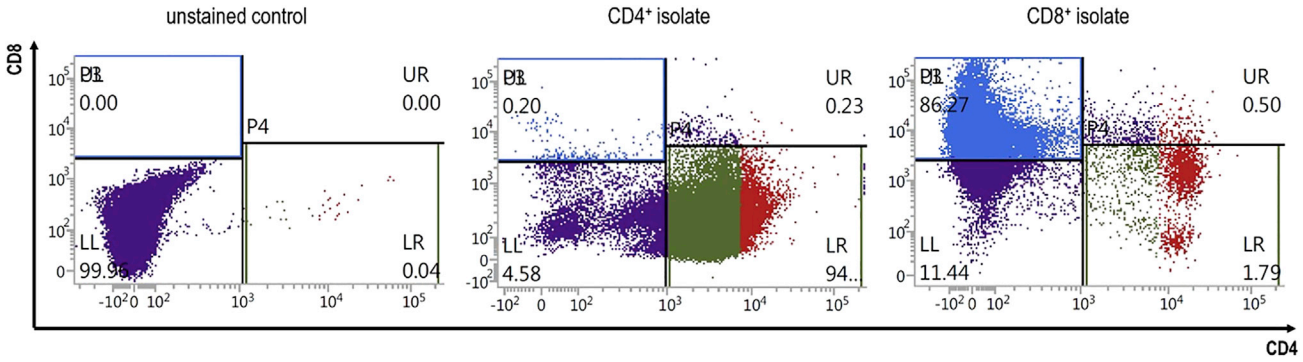

Figure 3. Exemplary dot plots visualize purity assessment of isolated T cells

⚠ CRITICAL: A minimal purity of 85% is tolerable. See also troubleshooting 3.

Seeding of leukemia cells in T cell media

Timing: 1 h

On the day of T cell isolation and activation, seeding of leukemia cells in T cell media should be done in parallel. This allows for equilibration of the cells. Culture leukemia cells and T cells in the same media with plasma supplement from the same donor to avoid any kind of interference between soluble factors from two different donors. We used our established cell culture models SEM::mock and SEM::EGR3 which were generated with the sleeping beauty transposon system. In this system, the transgene is under control of a tetracycline responsive promotor which requires induced transcription the day before co-culture through addition of Doxycycline (Kowarz et al., 2015).

12. Seed 2 × 10^6 SEM::mock and 2 × 10^6 SEM::EGR3 cells in 2 mL T cell media in a 6-well plate.
13. Add 2 µL of a 1 µg/µL Doxycycline working solution to reach a final concentration of 1 µg/mL in the media.
14. Cultivate cells 16 h at 37°C and 5% CO_2 and saturated humidity.

Co-culture onset

Timing: 3 h

The next day, two hours prior to co-culture onset, SEM::mock and SEM::EGR3 cells are treated either with a neutralizing α-ICOSLG antibody or an IgG1 isotype control. EGR3 overexpression fosters *ICOSLG* transactivation and antibody receptor blockade enables evaluation of the effect of ICOSLG expression on Treg differentiation.

15. Count SEM::mock and SEM::EGR3 cells.
16. Seed two wells of a 24-well plate with 1 × 10^6 cells of each cell line in 880 µL T cell media supplemented with 1 µg Doxycycline to sustain transgene expression.
17. Treat each cell line with 40 µL of a 0.5 µg/µL working solution of neutralizing α-ICOSLG antibody or IgG1 isotype control to reach a final concentration of 20 µg/mL and cultivate for 2 h at 37°C and 5% CO_2 and saturated humidity.
18. Count the number of activated $CD4^+$ and $CD8^+$ T cells and adjust to 1,25 × 10^7 T cells/mL.
19. Add 40 µL $CD4^+$ (=5 × 10^5 cells) and 40 µL $CD8^+$ (=5 × 10^5 cells) T cells to each well with SEM cells and carefully mix by pipetting up and down.
20. Cultivate the co-culture for 48 h at 37°C and 5% CO_2 and saturated humidity.

Table 1. Flow cytometry antibody master mix

Viability staining (1×)	Treg staining (1×)
CD3 5 μL	CD3 5 μL
CD4 5 μL	CD4 5 μL
CD8 5 μL	CD8 5 μL
Brilliant Stain Buffer 50 μL	CD25 5 μL
	Brilliant Stain Buffer 50 μL

Treg quantification using flow cytometry

Timing: 4 h

The flow cytometry analysis consists of two different staining batches. Half of the co-culture undergoes a viability staining, the other half a Treg quantification staining. The viability assessment is performed as a surface staining of CD3, CD4 and CD8 followed by Annexin V and 7-AAD. Treg quantification is conducted as a surface staining of CD3, CD4, CD8 and CD25 followed by an intracellular staining of the Treg-specific transcription factor FOXP3.

Note: In our case, flow cytometry was performed using the BD FACSVerse™ flow cytometer with three lasers and four blue, two red and two violet fluorescence channels. The selected antibodies are conjugated with those fluorophores displaying the lowest level of interference and are listed in the key resources table. Please select your own FACS panel that fits your instrument.

21. After 48 h of co-culture, the whole cell suspension is transferred to a tube and centrifuged at 300 × *g* for 10 min.
22. Transfer each supernatant into another tube and store at −80°C.

Note: Use the supernatants for subsequent cytokine analysis e.g., using ELISA quantifying IL-2 and IL-10.

23. Resuspend cell pellet in 100 μL PBS/0.5% BSA.
24. Add 5 μL of Human BD Fc Block (564220, BD Biosciences) and incubate for at least 10 min at 20°C.
25. Prepare two antibody master mixes with 5 μL of each antibody in 50 μL Brilliant Stain Buffer (563794, BD Biosciences) according to Table 1, mix and dispense among FACS tubes.

Note: FACS gating of CD25 and FOXP3 requires fluorescence minus one (FMO) controls. Accordingly, prepare two additional Treg FMO staining master mix, one without CD25 and one without FOXP3. These FMO controls will be used to set the gates as shown in Figure 4. CD3, CD4 and CD8 gates were set at step 11 (Figure 3).

26. Add 50 μL of blocked cell suspension to each FACS tube with antibody mix and incubate for 30 min at 20°C protected from light.
27. Wash by adding 2 mL PBS/0.5% BSA, centrifuge at 400 × *g* at 20°C for 5 min and discard the supernatant.

Note: Beginning with the next step, the Viability staining is performed differently to the Treg staining. Both staining can be carried out simultaneously due to incubation times, or successively with cells stored on ice in PBS/0.5% BSA protected from light.

28. Viability staining.

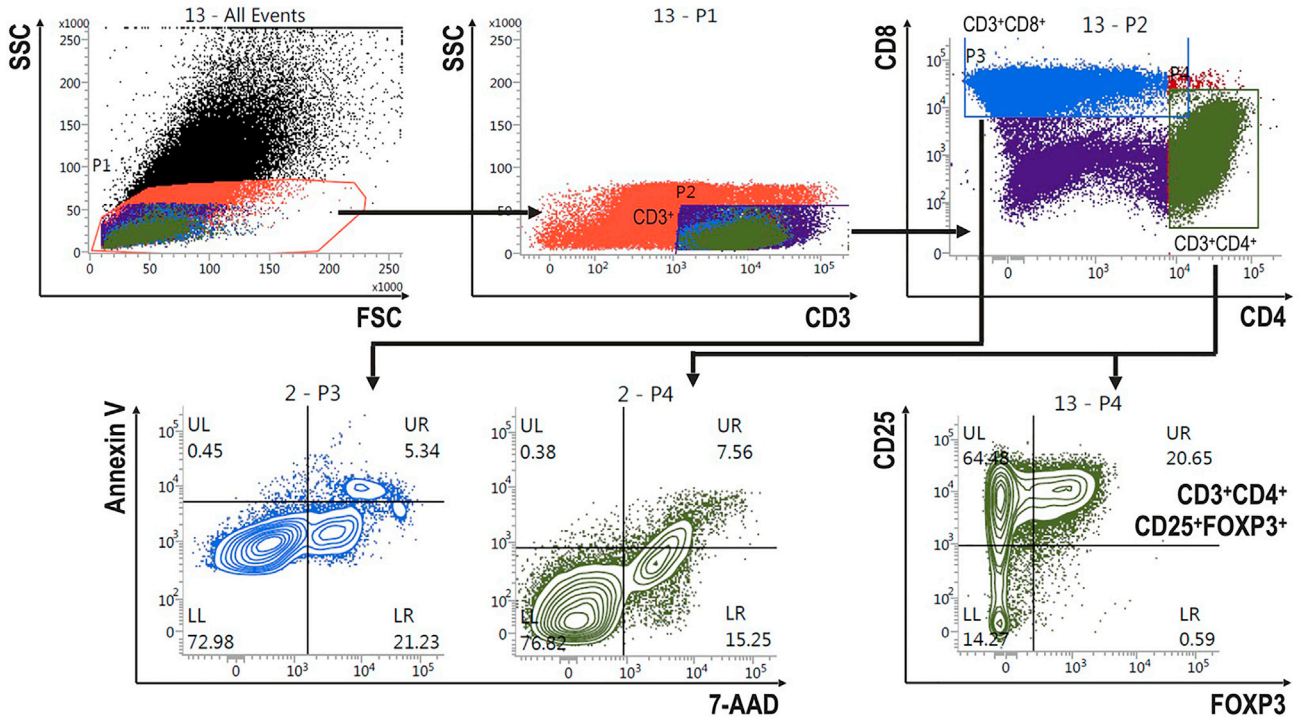

Figure 4. Flow cytometry gating strategy

First, low granularity cells were gated and $CD3^+$ T cells selected to separate $CD4^+$ and $CD8^+$ cells. Following this, viability of these populations was assessed using Annexin V and 7-AAD staining to ensure survival of T cells throughout co-culture. $CD25^+FOXP3^+$ Tregs were gated and their percentage among the $CD4^+$ population was quantified. Figure taken from Külp et al. (2022).

a. Wash by adding 2 mL PBS/0.5% BSA, centrifuge at 400 × *g* at 20°C for 5 min and discard the supernatant.
b. Resuspend pellet in 100 µL 1× Annexin Binding Buffer (V13246, Invitrogen).
c. Add 5 µL Annexin V Pacific Blue (A35122, Invitrogen).
d. Incubate 15 min at 20°C protected from light.
e. Wash by adding 2 mL PBS/0.5% BSA, centrifuge at 600 × *g* at 20°C for 6 min and discard the supernatant.
f. Resuspend in 300 µL 1× Annexin Binding Buffer.
g. Add 3 µL 7-AAD (130-111-568, Miltenyi Biotec).
h. Incubate 5–15 min on ice. Avoid an incubation longer than 15 min until flow cytometry.
i. Perform flow cytometry with gating as explained in Figure 4. A viability above 70% can be regarded as tolerable. See also troubleshooting 4.

29. Treg staining.
a. For fixation and permeabilization of cells, prepare a 1× Fix/Perm and Perm/Wash working solution of the Transcription Factor Buffer Set (562574, BD Biosciences) according to the manufacturer's protocol.
b. Vortex pellet from step 27, resuspend in 1 mL Fix/Perm and vortex again.
c. Incubate 40–50 min at 2°C–8°C protected from light.
d. Wash through addition of 1 mL Perm/Wash.
e. Centrifuge at 600 × *g* for 6 min at 4°C and discard supernatant.
f. Wash through addition of 2 mL Perm/Wash.
g. Centrifuge at 600 × *g* for 6 min at 4°C and discard supernatant.
h. Resuspend in 80 µL Perm/Wash.

i. Add 5 μL of FOXP3 antibody except to the FOXP3 negative control batch prepared at step 25.
j. Incubate at 2°C–8°C for 40–50 min protected from light.
k. Vortex and wash through addition of 2 mL Perm/Wash.
l. Centrifuge at 600 × *g* for 6 min at 4°C and discard supernatant.
m. Wash through addition of 2 mL Perm/Wash.
n. Centrifuge at 600 × *g* for 6 min at 4°C and discard supernatant.
o. Resuspend pellet in 350 μL PBS/0.5% BSA.
p. Perform flow cytometry with gating as explained in Figure 4.

Note: Use the CD25 and FOXP3 negative controls to set the respective gates. CD3, CD4 and CD8 gates were set during T cell isolation (Figure 3).

q. Calculate the percentage of the $CD4^+CD25^+FOXP3^+$ population among the $CD4^+$ population.

Alternatives: Instead of the BD Biosciences Transcription Factor Buffer Set, the eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher, 00-5523-00) could also be used.

Assessment of cytokine levels

Timing: 7 h

To further analyze the function of Tregs and non-Tregs, a cytokine quantification can be performed using ELISAs of the frozen co-culture supernatants from step 23. IL-10 is secreted by Tregs and therefore indicates their function (Maynard et al., 2007), whereas IL-2 represents general T cell activation. Particularly $ICOS^+$ Tregs were reported to mainly sustain their suppressive function through IL-10 secretion (Löhning et al., 2003; Ito et al., 2008) which renders an IL-10 ELISA an important means to investigate the ICOSLG/ICOS-induced Treg expansion.

You can use either timesaving precoated ELISA plates or the cheaper ELISA development kits where plate-coating is performed by the experimenter. Perform ELISAs according to the manufacturer's protocol of your kit of choice. We performed ELISAs using the IL-2 ELISA Kit (human) (OKBB00179, Aviva) and IL-10 (human) ELISA Kit (ADI-900-036, Enzo) according to the manufacturer's protocol.

Alternatives: Instead of using ELISA, ELISpot assays or cytokine bead arrays might also be suitable.

EXPECTED OUTCOMES

The rapid expansion of Tregs after co-culture of primary T cells with SEM::EGR3 in comparison to SEM::mock is shown in Figure 5A. Six healthy donors (HD1 – HD6) displayed a relative outgrowth of Tregs between 7.96 and 23.94% with a geometric mean of SEM::EGR3 Treg percentage to SEM::-mock Treg percentage of 1.14 ± 0.009991 ($p=0.0023$). Thereby, we showed that EGR3 overexpression leads to Treg induction in the t(4;11) proB-ALL cellular context.

Replication of the experiment with addition of 20 μg/mL neutralizing monoclonal α-ICOSLG antibody or an IgG1 isotype control antibody revealed that EGR3-mediated Treg induction became impaired upon ICOSLG antibody blockade (Figure 5B). This led to the conclusion that Treg expansion was due to the EGR3-mediated upregulation of ICOSLG.

Assessment of the IL-2 (Figure 5C) and IL-10 (Figure 5D) cytokine levels uncovered that ICOSLG antibody blockade did not affect general T cell activation represented by approximate IL-2 levels,

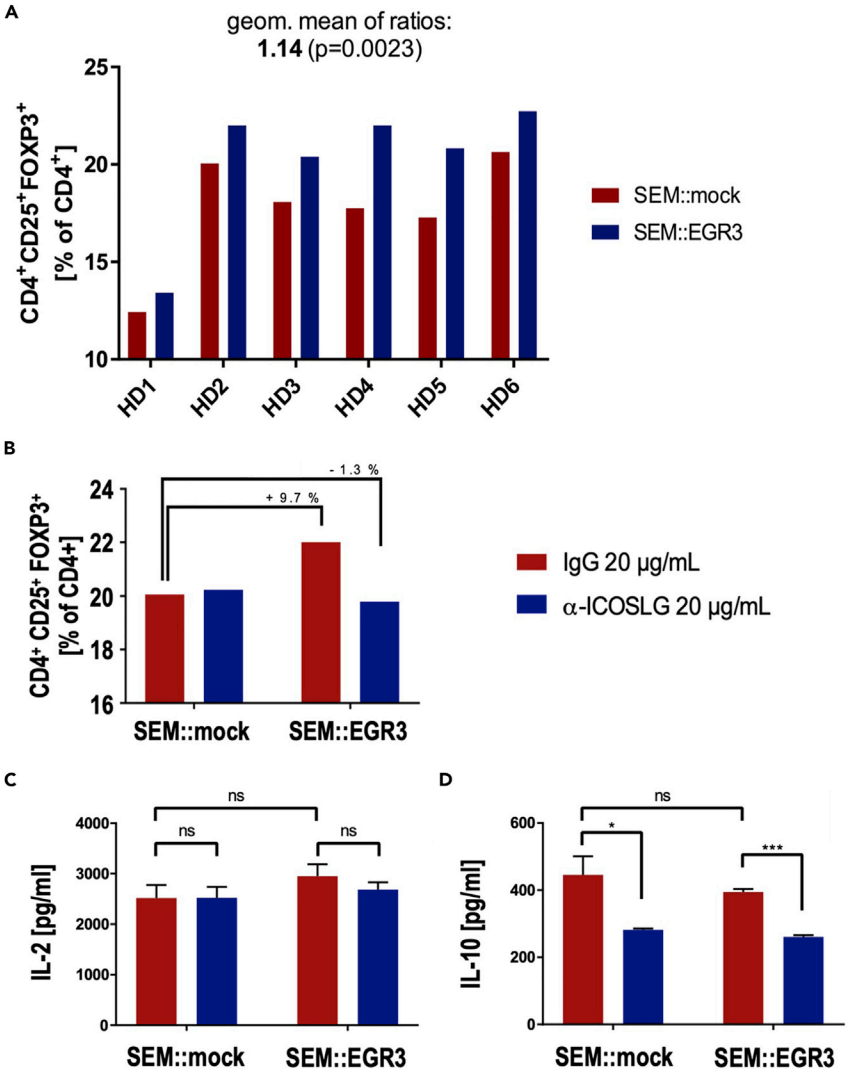

Figure 5. Experimental outcomes

(A) Percentages of $CD25^+FOXP3^+$ Tregs among $CD4^+$ cells for healthy donors 1–6 (HD1 – HD6) following co-culture with SEM::mock (red) or SEM::EGR3 (blue), respectively.

(B) Replication of co-culture experiment under addition of IgG control antibody (red) or neutralizing α-ICOSLG antibody exemplary for HD2.

(C and D) Cytokine quantification of IL-2 and IL-10 using ELISA for HD1, HD2, and HD3 revealed that ICOSLG antibody blockade strongly decreased IL-10 levels of SEM::mock (p=0.0424) and SEM::EGR3 (p=0.0002) co-cultures (multiple t-tests). Error bars represent the standard error of mean of HD1, HD2, and HD3. Figures taken from Külp et al. (2022).

whereas Treg function became strongly impaired indicated by decreased IL-10 levels of the α-ICOSLG treated co-cultures.

Taken together, flow cytometric and ELISA analysis of T cell co-cultures with leukemia cell lines demonstrated ICOSLG checkpoint inhibition as a promising strategy to prevent Treg expansion.

LIMITATIONS

Figure 5A shows that relative Treg outgrowth as well as absolute percentages of Tregs differed among the evaluated six healthy donors. Therefore, results might fluctuate depending on the donor's T cell constitution. Consequently, valid results can be generated through inclusion of a higher number of donors included in your study.

The protocol does not evaluate T cell activation by leukemia cell lines as this is performed using antibody-coated beads prior to co-culture. Therefore, it should be considered for interpretation of results that a detected Treg expansion not only requires co-stimulation e.g., via ICOS/ICOSLG binding but also prior activation of T cells e.g., through specific antigen exposure.

TROUBLESHOOTING

Problem 1

Overlay of PB with lymphocyte separation media does not result in a clear phase separation.

During step 1d, quick or harsh overlay of PB with Ficoll-Paque media frequently leads to a mixture of both phases at their interface. This results in a weaker PBMC separation after centrifugation and therefore provokes a decreased yield of separated PBMC.

Potential solution

Hold the falcon with PB inclined in order to increase the phase's surface. Pipette lymphocyte separation media very gently at the inner wall of the tube with lowest adjustable pipetting velocity. Slowly erect and move the tube into the centrifuge.

Problem 2

Filter clogging during sterile filtration of plasma.

In some cases, during step 6 the 0.20 μm syringe filter gets clogged and only part of the plasma can be filtered.

Potential solution

Prefilter the plasma with a 0.45 μm pore size sterile filter and subsequently re-filtrate using a 0.20 μm filter. In case of greater volumes of plasma, use a threaded bottle top filter and vacuum filtration.

Problem 3

T cell purity is below 85% following magnetic separation.

The measurement of T cell purity after magnetic separation during step 11 is important to verify the selectivity of enrichment. According to our experiences, a purity of 85% or higher does not negatively affect the co-culture, especially considering that following bead-based activation, T cells massively proliferate and overgrow unintendedly co-isolated cells.

Potential solution

If you do not reach approximately 85% T cell purity after magnetic separation, you should re-separate the cells with another column thereby increasing the purity of intended $CD4^+$ or $CD8^+$ T cells, respectively. Alternatively, FACS sorting can be performed to increase the T cell purity.

Problem 4

Viability of T cells after co-culture is below 70%.

If the viability of T cells after co-culture measured with 7-AAD and Annexin V staining during step 28i does not reach approximately 70%, the measured Treg percentage could be over- or underestimated and reliability of results should be questioned.

Potential solution

A common reason for T cell apoptosis, exhaustion and stress is overstimulation of the cells. If your viability is below 70%, you should decrease the bead-to-cell ratio to stimulate T cells more gently. The here recommended bead-to-cell ratio of 1:2 is very high and could be adjusted to 1:4 or 1:6.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rolf Marschalek (rolf.marschalek@em.uni-frankfurt.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets/code.

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AUTHOR CONTRIBUTIONS

M.K., H.B., and R.M. conceptualized the protocol; M.K. wrote the manuscript and performed experiments; L.D. and H.B. provided blood samples and contributed to T-cell isolation; R.M. provided funding; all authors reviewed and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare that they have no competing interests.

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