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BRIEF COMMUNICATION

# Use of polyvinyl alcohol for chimeric antigen receptor T-cell expansion

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**Serum albumin has long been an essential supplement for ex vivo hematopoietic and immune cell cultures. However, serum albumin medium supplements represent a major source of biological contamination in cell cultures and often cause loss of cellular function. As serum albumin exhibits significant batch-to-batch variability, it has also been blamed for causing major issues in experimental reproducibility. We recently discovered the synthetic polymer polyvinyl alcohol (PVA) as an inexpensive, Good Manufacturing Practice-compatible, and biologically inert serum albumin replacement for ex vivo hematopoietic stem cell cultures. Importantly, PVA is free of the biological contaminants that have plagued serum albumin-based media. Here, we describe that PVA can replace serum albumin in a range of blood and immune cell cultures including cell lines, primary leukemia samples, and human T lymphocytes. PVA can even replace human serum in the generation and expansion of functional chimeric antigen receptor (CAR) T cells, offering a potentially safer and more cost-efficient approach for this clinical cell therapy. In summary, PVA represents a chemically defined, biologically inert, and inexpensive alternative to serum albumin for a range of cell cultures in hematology and immunology.** **© 2019 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. This is an open access article under the CC BY license. (http://creativecommons.org/licenses/by/4.0/)**

Serum albumin has long been an essential supplement for ex vivo cultures of hematopoietic cells and lymphocytes [1]. However, serum albumin supplements represent a major source of biological contaminants and often display significant batch-to-batch variability, causing issues in experimental reproducibility and time-consuming batch testing [2]. Progressive attempts to limit these contaminants have involved moving from the use of serum, such as fetal bovine serum (FBS) or human serum, to the use of purified serum albumin (e.g., bovine serum albumin fraction V) and/or recombinantly derived human serum albumin (HSA) [2]. Additionally, serum albumin supplements represent a major cost in cell culture, particularly where Good Manufacturing Practice (GMP)-grade reagents are required.

We recently identified polyvinyl alcohol (PVA) in combination with insulin−selenium−transferrin−ethanolamine (ITSX) as a serum replacement for ex vivo hematopoietic stem cell (HSC) culture [3]. In comparison to serum albumin medium supplements, PVA represents an inexpensive, chemically defined, and GMP-compatible alternative. Here, we describe how PVA can also be used as a serum albumin replacement for in vitro culture of leukemia cells and T lymphocytes, including chimeric antigen receptor (CAR) T cells. Adaptive immunotherapies such as CAR T-cell therapies have become an exciting new therapeutic approach in the treatment and cure of various cancers [4−6].

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However, the large-scale ex vivo expansion of T lymphocytes [7] for these therapies currently relies on expensive pre-screened human serum. Our results suggest that PVA is a chemically defined alternative to serum albumin for CAR T-cell expansion and may offer advantages in terms of cost, safety, and reproducibility.

## Methods

*Cell line cultures*

Mouse 32D/MPL (previously generated by the laboratory [8]) and human K562 cells (purchased from the American Type Culture Collection) were cultured in RPMI-1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma), 1% ITSX (Gibco), 1 mg/mL recombinant human serum albumin (HSA; Albumin Biosciences), and/or 1 mg/mL PVA (Sigma P8136) at 37°C with 5% $CO_2$. Mouse 32D/MPL cells were supplemented with 10 ng/mL recombinant mouse thrombopoietin (TPO; R&D Systems or Peprotech). Both cell lines tested negative for mycoplasma using the MycoAlert Plus Mycoplasma Detection Kit (Lonza), following the manufacturer's instructions. Cell counting was performed using the Chemometec NC-3000 automated cell counter.

*Primary leukemia cell cultures*

Primary human acute myeloid leukemia (AML) samples were collected and stored by the Stanford Hematology Tissue Bank, after receiving informed patient consent, according to the Administrative Panel on Human Subjects Research Institutional Review Board (IRB)-approved protocols (Stanford IRB Nos. 18329, 6453, and 5637). Primary AML cells were cultured in MEMa media (containing 1% penicillin− streptomycin−glutamine; Gibco) supplemented with 10% FBS, 1% ITSX, or 1 mg/mL PVA at 37°C with 5% $CO_2$. The following cytokines were added to the culture (as described previously [9]): human TPO, human stem cell factor (SCF), human FLT3L, human interleukin (IL)-3, human IL-6, human granulocyte−macrophage colony stimulating factor (GM-CSF), and human granulocyte stimulating factor (all at 20 ng/mL).

*Primary T-cell cultures*

Peripheral blood mononuclear cells (PBMCs) from de-identified healthy donors were purchased from the Stanford Blood Center. CD3+ T cells were purified, stimulated with anti-CD3/CD28 Dynabeads (ThermoFisher Scientific), and then cultured in RPMI-1640 medium (Gibco) supplemented with 10 ng/mL human IL-15 (Peprotech), 10 ng/mL human IL-7 (Peprotech), 5% (v/v) human serum, 1% ITSX, and/or 1 mg/mL PVA at 37°C with 5% $CO_2$.

*CAR T-cell assays*

CD3+ T cells were purified and stimulated as above, before lentiviral transduction on days 2 and 3 with anti-CD19-CAR lentivirus (lentivirus plasmid was kindly provided by Dr. Crystal Mackall). Lentiviral infection was conducted with Retronectin coating (Clontech) with spinfection (1,000 *g* for 1 hour at 32°C) at a multiplicity of infection of 10. After an additional 7-day expansion, in vitro killing activity was determined by bioluminescence-mediated viability measurement [10] with firefly luciferase (FLuc)-transduced NALM6 cells.

*Enzyme-linked immunosorbent assays (ELISAs)*

Mouse TPO and human IL-15 ELISA Kits were purchased from R&D Systems (MTP00 and D1500) and used according to the manufacturer's instructions.

*Statistical analysis*

One-way and two-way analysis of variance (ANOVA) tests were performed as indicated in the figures using Prism 7 software.

## Results and discussion

As a direct replacement for HSA= in our HSC culture system, we hypothesized that PVA stabilizes proteins in the medium, such as the key HSC cytokine TPO [3]. Using an ELISA, we confirmed that PVA stabilized TPO concentrations similarly to HSA (Figure 1A), while significantly more TPO was lost from the RPMI-only and ITSX-only media (only ~5% remained after 48 hours at 37°C for ITSX-only media). Consistent with our previous results with HSC media [3] and the relative stability of TPO in these different media, the proliferation of the TPO-dependent 32D/MPL mouse cell line [8] was highest in PVA + ITSX or HSA + ITSX, comparable to expansion in FBS. By contrast, 32D/MPL cells more slowly proliferated under ITSX-only conditions, and essentially failed to grow in those media lacking ITSX (Figure 1B, C).

To expand the applications of PVA in hematology research, we next tested the use of PVA in primary human acute myeloid leukemia (AML) cultures. PVA + ITSX supported growth of primary human AML samples cultured in the presence of cytokines and was almost equivalent to FBS-containing media (Figure 1D). PVA + ITSX also supported more rapid proliferation of the human erythroleukemia K562 cell line [11], without addition of exogenous cytokines (Figure 1E). PVA may therefore have broad application in growing various hematological cell types beyond HSC cultures, although future work will need to determine how each cytokine and growth factor functions under albumin-free conditions. The PVA culture system also provides a useful platform for dissecting the biological components in serum that influence cell growth.

We also investigated the application of PVA to lymphocyte cell culture. We initially confirmed that the key T-cell cytokine IL-15 [12] was similarly stabilized in PVA + ITSX and serum + ITSX media (Figure 2A). In serum albumin-free media containing PVA + ITSX, primary human T cells expanded by ~8- to 22-fold over 11 days in culture (Figure 2B). This expansion was slightly lower than that in serum + ITSX cultures (~22- to 113-fold), suggesting human serum contains additional factors promoting T-cell growth, which remain to be identified. Consistent with the reduced stability of IL-15 in ITSX-only media, this condition supported only ~2-fold expansion of T cells over 11 days (Figure 2B).

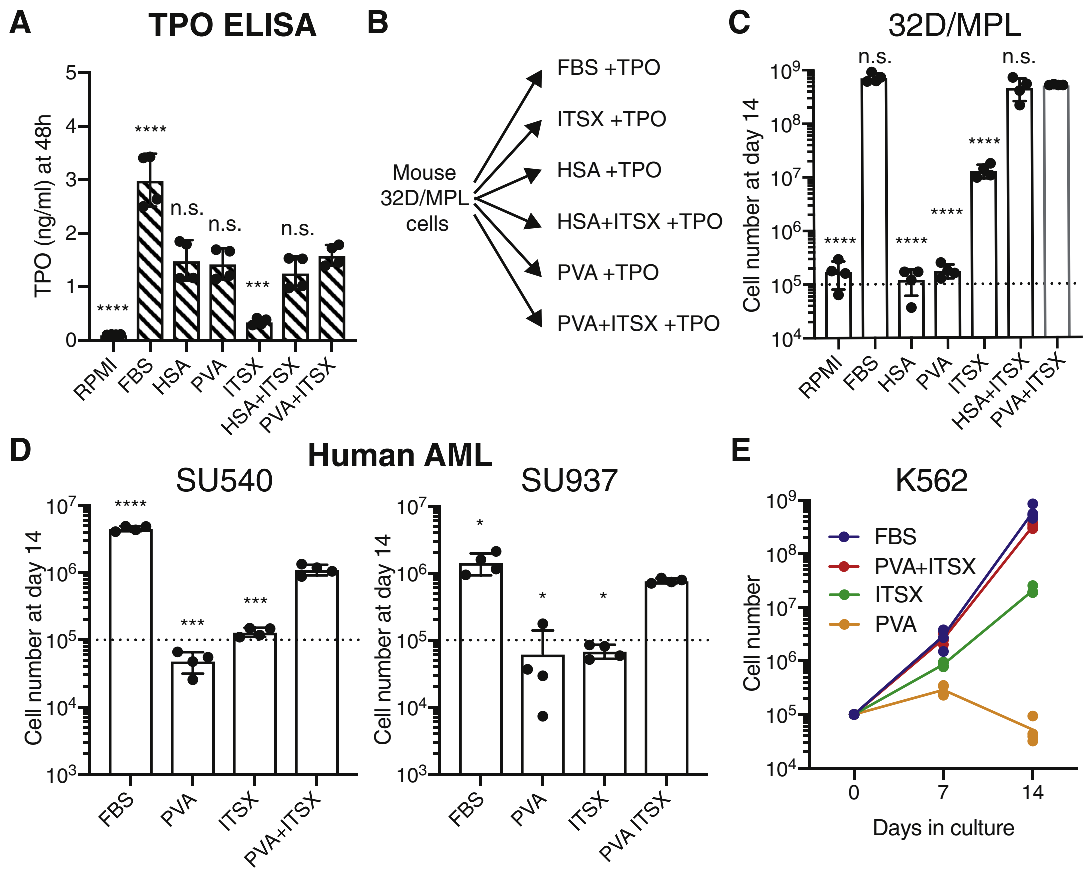


**Figure 1.** Polyvinyl alcohol can replace serum albumin in leukemic cell cultures. (**A**) ELISA assay for mouse TPO stability in RPMI-based culture medium after 48 hours (initiated at 10 ng/mL). Values are means ± SD of technical duplicates from biological replicates. (**B**) Schematic summary of TPO-dependent mouse 32D/MPL cells grown in various RPMI-based media supplemented 10 ng/mL TPO. (**C**) Total number of 32D/MPL cells after a 14-day culture in the RMPI-based media described in (**B**) with TPO at 10 ng/mL. Dotted line indicates starting cell concentration. Values are means ± SD of four independent cultures. (**D**) Total number of primary human AML cells (two AML samples from the Stanford Hematology Biobank) after a 14-day culture in MEMa-based medium with indicated supplements and cytokines (TPO, SCF, FLT3L, IL-6, IL-3, GM-CSF, and G-CSF, all at 20 ng/mL). Dotted line indicates starting cell concentration. Values are means ± SD of four independent cultures. (**E**) Total number of human K-562 cells over a 14-day culture in the RMPI-based medium with indicated supplements. Values are means of four independent cultures. In all panels, statistical analysis was performed by one-way ANOVA (analysis measured against the PVA + ITSX condition with significance indicated by *p < 0.05, **p < 0.01, ***p > 0.001, and ****p > 0.0001. *n.s.*=nonsignificant.

These results confirm that PVA can be used as a human serum replacement for expanding primary human T cells and identify an inexpensive and chemically defined carrier for human T-cell culture.

For CAR T-cell therapies, T cells must not only expand ex vivo, but must also function to kill target cancer cells. We therefore confirmed that PVA-expanded T cells retained full functionality by assessing the use of PVA for expanding anti-CD19 CAR T cells [13] (Figure 2C). PVA + ITSX exerted anti-CD19 killing activity comparable to that exerted in serum + ITSX (Figure 2D). The replacement of serum albumin with PVA may therefore provide significant improvements in terms of both cost and safety for ex vivo T-cell cultures.

In summary, PVA offers inexpensive and chemically defined culture conditions for a number of cell types in hematology and immunology. We expect that these serum albumin-free media will have important implications for how we culture cells for both basic research and clinical cell therapies.

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## Conflict of interest disclosure

RM is a co-founder, consultant, shareholder, and director of Forty Seven Inc. HN is a co-founder and shareholder of Century Therapeutics LLC.

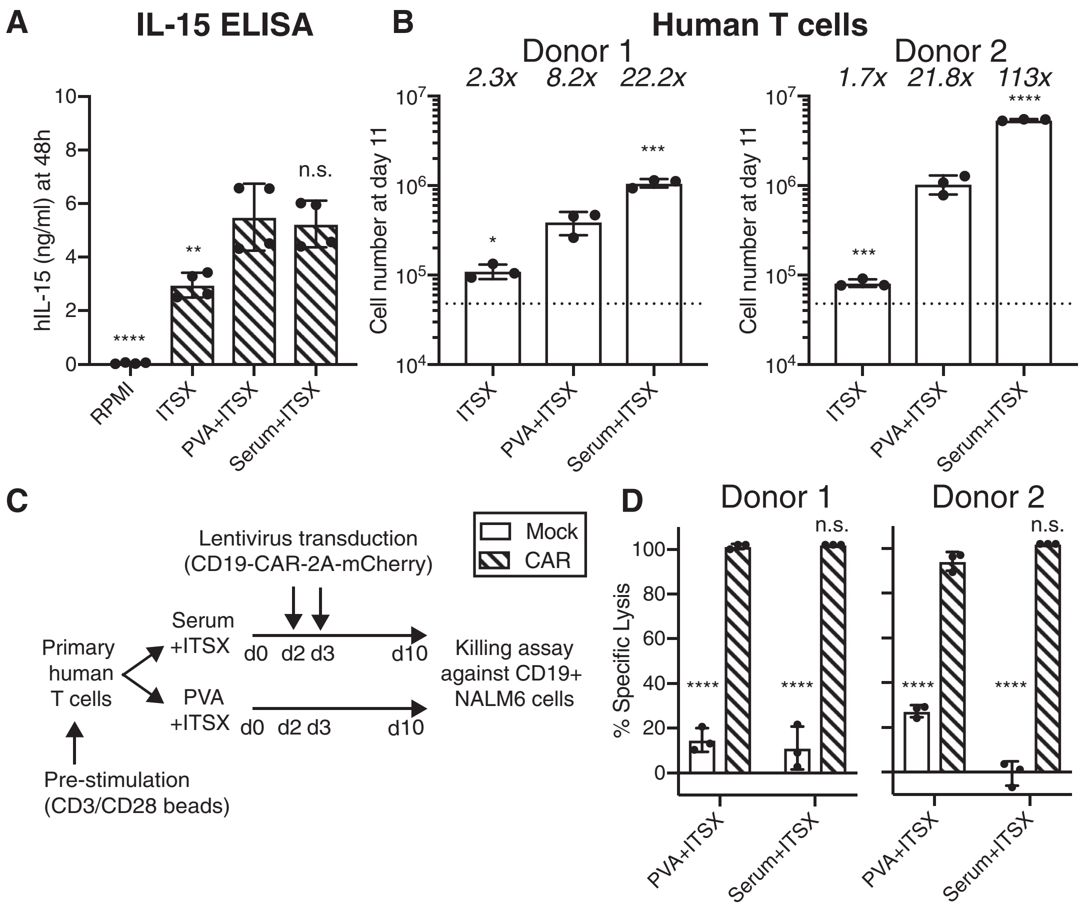


**Figure 2.** Polyvinyl alcohol can replace serum albumin in T-lymphocyte cell cultures. (**A**) ELISA assay for human IL-15 stability in culture medium after 48 hours (initiated at 10 ng/mL). Values are means ± SD of technical duplicates from biological replicates. (**B**) Total number of primary T cells after an 11-day culture in RPMI-based medium with the indicated supplements and cytokines (IL-15 and IL-7, 10 ng/mL each), following anti-CD3/CD28 Dynabead stimulation. Values are means ± SD of triplicated experiments for two healthy donors. (**C**) Schematic of CAR T-cell production by primary T-cell collection and stimulation with anti-CD3/CD28 Dynabeads at day 0, lentiviral transduction at days 2 and 3, and expansion until day 10, all in RMPI + PVA + ITSX and RPMI + serum + ITSX. At day 10, transduced mCherry+ anti-CD19 CAR T cells were used in a killing assay using CD19+ NALM6 target cells at a ratio of 5:1 (CAR T cells:target cells). (**D**) Killing activity of anti-CD19 CAR T cells targeting CD19-expressing NALM6 cells, described in (**C**). Values are means ± SD of technical triplicates for two separate donors. Representative of two biological replicates. Statistical analysis was performed by one-way (**A, B**) or two-way (**D**) ANOVA measured against the PVA + ITSX condition with significance indicated by *$p < 0.05$, **$p < 0.01$, ***$p > 0.001$, and ****$p > 0.0001$. *n.s.*=nonsignificant.

## Author contributions

TN conceptualized the research, performed experiments, analyzed data, and edited the article. IH, DCMK, and YN performed experiments, analyzed data, and edited the article. RM supervised the research and edited the article. SY conceptualized the research and edited the article. HN conceptualized the research, supervised the research, analyzed data, and wrote the article. ACW conceptualized the research, performed experiments, analyzed data, supervised the research, and wrote the article.

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