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A dual neutrophil-T cell purification procedure and methodological considerations in studying the effects of estrogen on human Th17 cell differentiation

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ABSTRACT

New procedures are required to optimize the use of blood samples to study different cell types. The purification of neutrophils and T cells from the same blood sample is not commonly described. We have previously used PolymorphPrep™ (P) or LymphoPrep™ (L) for purifying neutrophils or T cells, respectively. In this study, we describe a new method for purifying both of these cells using P and L from the same sample, and methodological considerations required to obtain consistent Th17 differentiation results. For T cell studies, we first isolated mononuclear cells from peripheral blood of healthy humans using either P alone, L alone or sequential isolation with P and then L (P + L). $CD3^+$ lymphocytes comprise up to 73% of peripheral blood mononuclear cells (PBMCs) obtained by sequential isolation, with 29% and 36% for P and L, respectively. T lymphocyte subsets, Th1, Th17 or double-positive (Th17/1), were then amplified. Four days of amplification culture after isolation by P alone led to over-expression of Th17/1 cells and of Th17 cells in comparison to cells isolated by L or by sequential P + L. Th17/1 cells comprised 11.0 ± 6.8% (P alone) *vs* 1.2 ± 0.28% (L alone) *vs* 0.45 ± 0.11% (P + L) and Th17 cells comprised 2.8 ± 0.4% (P alone) 0.88 ± 0.15% (L alone) *vs* 0.86 ± 0.14% (P + L). As the second step, we examined T cell purification and differentiation. A higher purity of 97.1 ± 0.44% naïve $CD4^+$ T cell was reached after P + L followed by immunomagnetic bead sorting in comparison to 70 ± 9.3% (L) *vs* 21.0 ± 8.5% (P). These cells grew well in the density range of 25, 000 to 100, 000 cells per well in 96-well plates during Th17 cell differentiation; higher or lower cell density did not support Th17 cell differentiation. Lastly, to investigate the effect of estrogen on Th17 cell differentiation, serum-free AIM V medium without phenol red was chosen to minimize the hormonal effects of the medium. We found that exogenous estrogen (1 nM) inhibited Th17 cell differentiation in this medium. Taken together, we devised a method to isolate both neutrophils and T cells from the same blood sample and show that high PBMC purity, selected culture medium and an optimal cell density of the initial cell culture produced the most robust and consistent results for Th17 differentiation.

1. Introduction

The T helper 17 (Th17) cells are a subset of $CD4^+$ T cells with unique functions. These cells have been extensively investigated since their discovery in 2005 (Langrish et al., 2005; Harrington et al., 2005). Early studies demonstrated an important role of Th17 cells in the protective inflammatory immune response to foreign pathogens such as extracellular bacteria and fungi (Guglani and Khader, 2010). More recent studies have demonstrated that Th17 cells are involved in infection, autoimmunity and immunodeficiency. Although activated Th17 cells provide a protective immune response, uncontrolled activation of Th17 cells is associated with inflammatory and autoimmune disorders through secretion of the cytokines interleukin (IL)-17A, IL-17F, tumor necrosis factor (TNF)-α and granulocyte macrophage-colony stimulating factor (GM-CSF; Marwaha et al., 2012; Noack and Miossec, 2014; Burkett et al., 2015; McGeachy, 2011). Th17 cell linage

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Table 1
Subject Demographics.

	PolymorphPrep (P)	LymphoPrep (L)	P + L
N	4	17	10
Age, mean (range)	19.5 (18–20)	24.9 (18–48)	25.0 (21–48)
Sex, male/female (%)	2/2 (50/50)	11/6 (70/30)	5/5 (50/50)
Number of Naïve CD4$^+$T/mL blood ($\times10^4$) (mean ± SEM, range)	1.07 ± 0.44 (0.29–2.3)	4.30 ± 1.12 (0–14.8)	6.40 ± 1.54 (0.7–15.5)
% Naïve T cells in Periphery (mean ± SEM, range)	10.7 ± 3.8 (5–18)	4.9 ± 1.0 (1.0–17.0)	5.48 ± 0.67 (1.1–10.8)
% CD3$^+$T cells in PBMC (mean ± SEM, range)	10.6 ± 6.1 (2.5–28.9)	18.2 ± 2.8 (1.8–35.7)	36.2 ± 2.93 (18.5–72.6)
% Purity of Naïve CD4$^+$T cells (mean ± SEM, range)	21 ± 8.5 (6.1–42.0)	69.6 ± 9.3 (18.7–96.6)	97.1 ± 0.44 (94.1–99.3)
% Peripheral Th1 (mean ± SEM, range)	41 ± 6.0 (31.3–58.4)	31.7 ± 3.2 (1.7–39.1)	17.2 ± 4.37 (1.9–39.4)
% Peripheral Th17 (mean ± SEM, range)	2.8 ± 0.4 (2.2–3.6)	1.3 ± 0.23 (0–2.6)	0.86 ± 0.14 (0–1.7)
Ratio of Th1/Th17 in periphery (mean ± SEM, range)	16.0 ± 5.0 (9.9–25.8)	35.5 ± 8.9 (5.5–88.5)	22.0 ± 5.02 (5.5–48.1)
% Peripheral Th17/1 (mean ± SEM, range)	11.0 ± 6.8 (0.78–30.9)	1.2 ± 0.28 (0–3.3)	0.45 ± 0.11 (0–1.9)
% Differentiated Th17 (mean ± SEM, range)	NA	0.88 ± 0.15 (0.1–1.8)	0.95 ± 0.22 (0.01–2.6)

has become the third major subset of effector Th cells, following the paradigmatic Th1 and Th2 cells.

Understanding the molecular mechanisms of Th17 cells' differentiation and the functional analysis of Th17 cells is very important for elucidating the nature of immune response in health and disease. Naïve T cells differentiate into Th17 cells when exposed to specific cytokine milieux that are created by activated antigen presenting cells upon recognizing pathogens. While the majority of effector Th17 cells die after antigen clearance, some of the remaining T cells differentiate into memory Th17 cells (Revu et al., 2018). Compared to naïve T cells, the long-lived and robust Th17 memory cells are more rapidly activated when exposed to certain pathogens (McGeachy, 2013). Therefore, there are two forms of functional analysis of human Th17 cells *ex vivo* in health and disease. One way is to expand memory Th17 cells in blood (McGeachy, 2013); the other way is to evaluate the intrinsic capacity of naïve CD4$^+$ T cells to proliferate and differentiate into effector Th17 cells *ex vivo*. The process of obtaining peripheral memory Th17 cells and differentiated Th17 cells typically includes multiple steps. Human peripheral blood mononuclear cells (PBMCs) are isolated simply by gradient separation. Those PBMCs can be directly cultured with the stimulation of anti-CD3 and CD28 antibodies for 4–5 days in order to expand the peripheral memory Th17 cells. For Th17 cell polarization, naïve CD4$^+$ T cells are purified further from PBMCs by an immunomagnetic beads-based cell sorting. Those cells are then expanded and/or differentiated into Th17 cells with a cocktail of specific antibodies and cytokines (Burgler et al., 2009; Revu et al., 2018; Kushwah et al., 2013; Kushwah et al., 2014; Djiadeu et al., 2017a).

Highly pure PBMCs and naïve T cells are essential for those Th17 studies. Standard protocols for isolating PBMCs and naïve CD4$^+$ T cells from peripheral blood yield a substantial proportion of undesired cells. One-step isolation using Ficoll-Paque or LymphoPrep is not sufficient to enrich PBMCs with lymphocytes free of other contaminating cells (*e.g.*, platelets, red blood cells, and polymorphonuclear granulocytes). Most of the time, CD45RA$^-$ CD4$^+$ lymphocytes (memory CD4$^+$ T cells) are the main contaminating cells in preparations of purified naïve CD4$^+$ T cells. To improve the purity, one approach is to purify naïve CD4$^+$ T cells with a flow sorter and the other is to use a more complicated purification process involving extra rounds of negative and positive selections (Blom and Poulsen, 2013). However, those strategies lead to substantial loss of cells, and make the isolation and purification costly and time consuming, and require access to a cell sorter.

PolymorphPrep™ (P) is a common one-step purification procedure used for isolating polymorphonuclear neutrophils and PBMCs. Neutrophils isolated using the P procedure have been used routinely for functional and cell death studies such as neutrophil extracellular trap (NET) formation (NETosis), apoptosis and ApoNETosis (Azzouz et al., 2018; Khan et al., 2018; Naffah de Souza et al., 2018; Khan et al., 2017; Djiadeu et al., 2017b). Removing neutrophils from the blood is expected to increases the purity of PBMCs; however, the PBMC layer is too close to the platelet cell layer in the gradient, requiring additional isolation steps before using the PBMCs isolated by P for T cell studies. Here, we describe a simple combined (P + L) protocol for isolation of PBMCs and purification of naïve CD4$^+$ T cells, and identify several factors that modulate Th17 cell differentiation. The P + L procedure is also useful for isolating both neutrophils and T cells from the same blood sample.

2. Materials, reagents and methods

2.1. Healthy blood donors

Venous blood (~40 mL) was obtained from 35 healthy adult donors (aged 20–48 years) of whom 10 (29%) were female. The demographics of the healthy subjects are shown in Table 1. All participants provided written informed consent. Ethical approval was obtained from the Hospital for Sick Children Research Ethics Board.

2.2. Antibodies, cytokines, reagents and culture media

Anti-human CD3-unconjugated (Clone: HIT3a; Cat: 16-0039-85), Anti human CD28-unconjugated (Clone: CD28.2; Cat: 16-0289-85), anti-human IL-4 (Clone: MP4-25D2; Cat: 16-7048-85), anti-human IFN-γ neutralizing antibodies (Clone: NIB42; Cat: 16-7318-85), anti-human CD3-APC (Clone: OKT3; 17-0037-41), anti-human CD4-PE (Clone: OKT4; 12-0048-41), anti-human IFN-γ-FITC (Cat: BMS107FI), anti-human CD45RA-FITC (Clone: JS-83; Cat: 11-9979-41), anti-human IL-17-APC (Clone: eBio64DEC17; Cat: 17-7179-42) and anti-human Foxp3- FITC (Clone: 236A/E7; Cat: 11-4777-41) are all from eBIOSCIENCE at ThermoFisher Scientific (USA). Human IL-6, Human IL-23, Human IL-1β, Human IL-2 and Human TGF-β1 are all from Peprotech (NJ, USA). LymphoPrep (L) and PolymorphPrep (P) are from Axis-Shield Diagnostics (Dundee, UK). Phorbol myristate acetate (PMA), ionomycin and Cytofix/Cytoperm with Golgistop are from BD Biosciences, USA. Dead/Live Fixable Violet was obtained from ThermoFisher Scientific (USA). Human Naïve CD4+ T Cell Isolation Kit was from STEMCELL Technologies Inc. (Cat: 19555). Estrogen was bought from Sigma and dissolved in DMSO. The stock solution (10 mM) was stored at −80 °C until thawed and diluted with culture media for use.

Fetal calf serum (FCS), charcoal-stripped FCS, serum-free AIM V medium (Cat: 12055091) and specially ordered serum-free, phenol red-free AIM V medium were purchased from Gibco at ThermoFisher Scientific (USA). Unless stated otherwise, all cells throughout this study were cultured with phenol red-free, serum-free AIM V media supplemented with 100 U/mL penicillin and 100 mg/mL streptomycin, 1 mM L-glutamine (Invitrogen) and 50 μM β-mercaptoethanol (β-ME; Sigma-Aldrich).

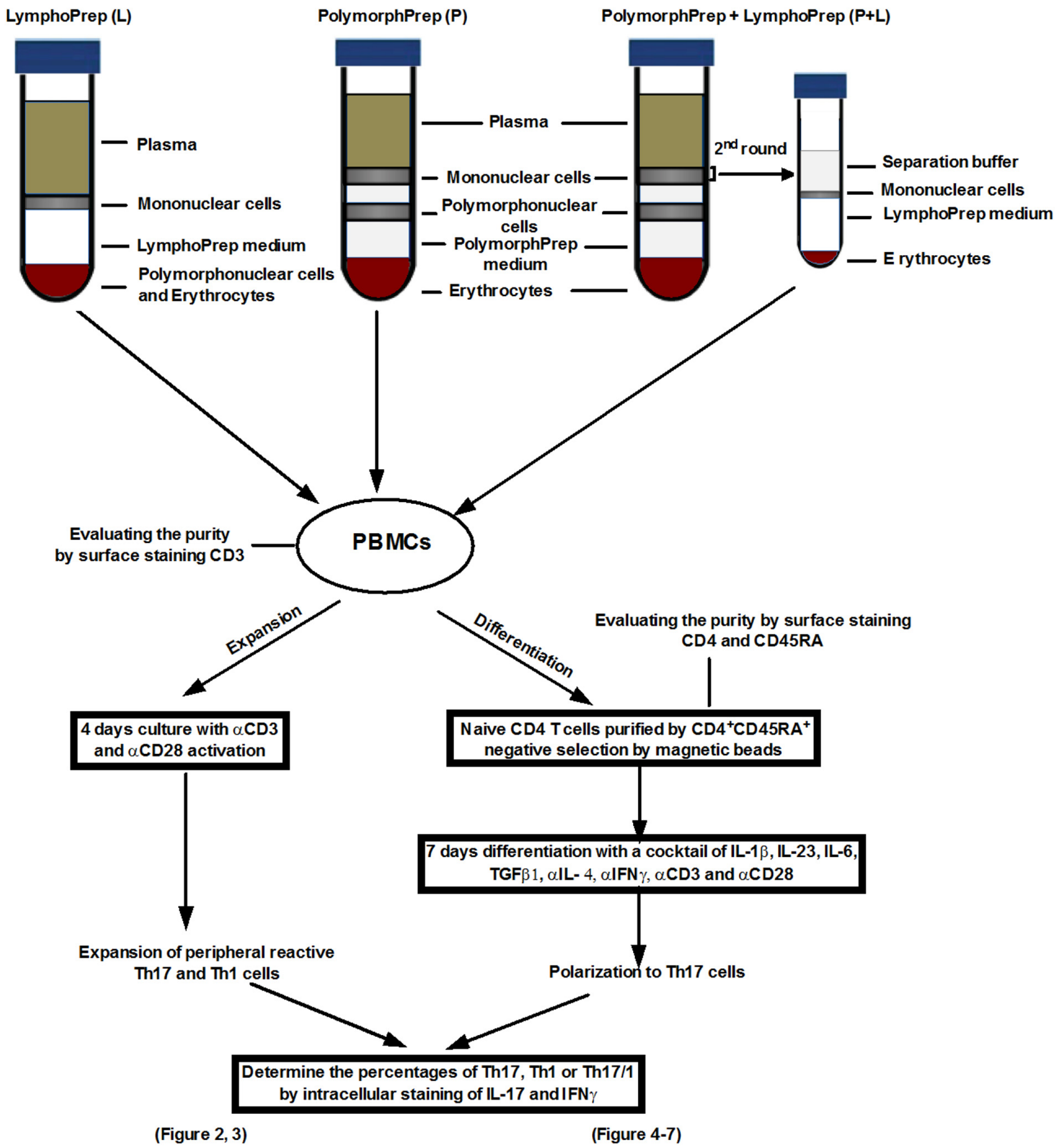


Fig. 1. Experimental schema for PBMC isolation, expansion of peripheral Th17 cells, naïve CD4$^+$ T cell purification, Th17 differentiation and their analysis by flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood using either PolymorphPrep (P) or LymphoPrep (L), or both methods sequentially (P + L). Some PBMCs were cultured with the stimulation of anti-CD3 and anti-CD28 antibodies for 4 days *in vitro* to expand peripheral memory Th17/Th1 cells. Most of the PBMCs were used for obtaining naïve CD4$^+$ T cells by a naïve CD4$^+$ T cell magnetic isolation kit followed by *ex vivo* culture with a cytokine cocktail of IL-1β, IL-6, TGF-β1, IL-23, neutralizing antibodies of IL-4 and IFN-γ, plus anti-CD3 and anti-CD28 antibodies for 7 days for Th17 cell polarization. The efficiency of the isolation and purification processes was determined by surface staining of their respective markers. The percentages of IL-17$^+$ CD4$^+$ Th17 or IFN-γ$^+$ CD4$^+$Th1 cells were determined by intracellular cytokine staining.

2.3. PBMC isolation and naïve CD4$^+$ T cells purification

PBMCs were isolated freshly from blood samples using P or L or P followed by L. In the latter, the PBMC layer collected from P separation were resuspended with separation buffer (PBS with 2% FCS and 1 mM EDTA) followed by L separation. After a few low speed (230 ×*g*, 10 min) centrifugations to remove the remaining contaminated cells, those PBMCs were used for the following culture or further purification to obtain naïve T cells.

Human naïve CD4$^+$ T cells were isolated using immunomagnetic beads either from Miltenyi Biotech or from Stemcell Technologies according to the manufacturer's instructions. The purity of T cells in PBMCs and naïve CD4$^+$ T-cells in purified end products are determined by staining with anti-human CD3-APC for the former, with anti-human CD45RA and anti-human CD4-PE for the latter (for Flow cytometry analysis refer to section 2.5).

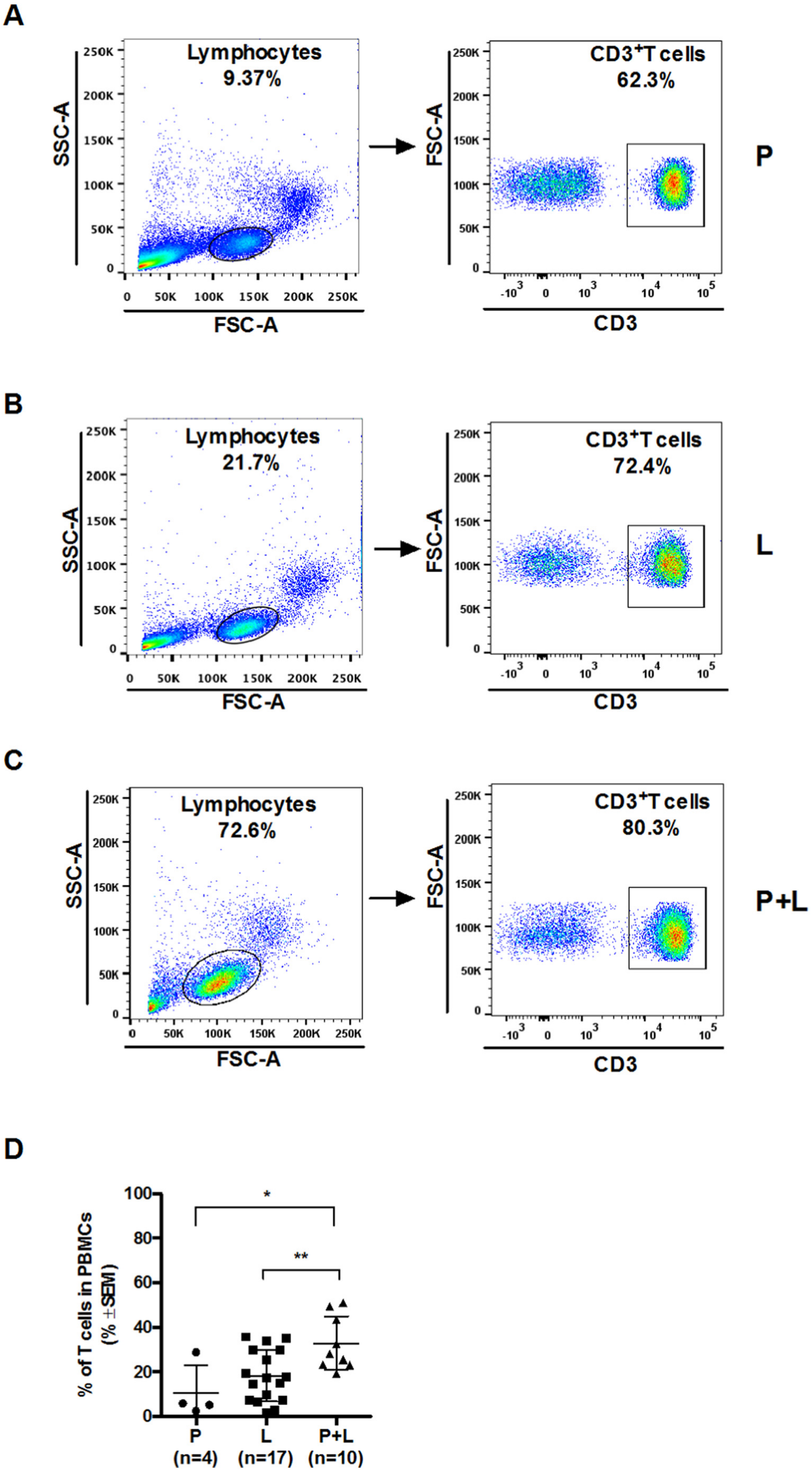


Fig. 2. Isolation by either (A) P alone or (B) L alone yielded lower purity PBMCs and CD3$^+$ T cells than (C) sequential purification by P then L. (A–C) Representative flow cytometry dot plots. (D) Summary graphs comparing the % yield by each purification approach. *$p < .05$; **$p < .01$. Comparison of P alone *vs* L alone: p ns.

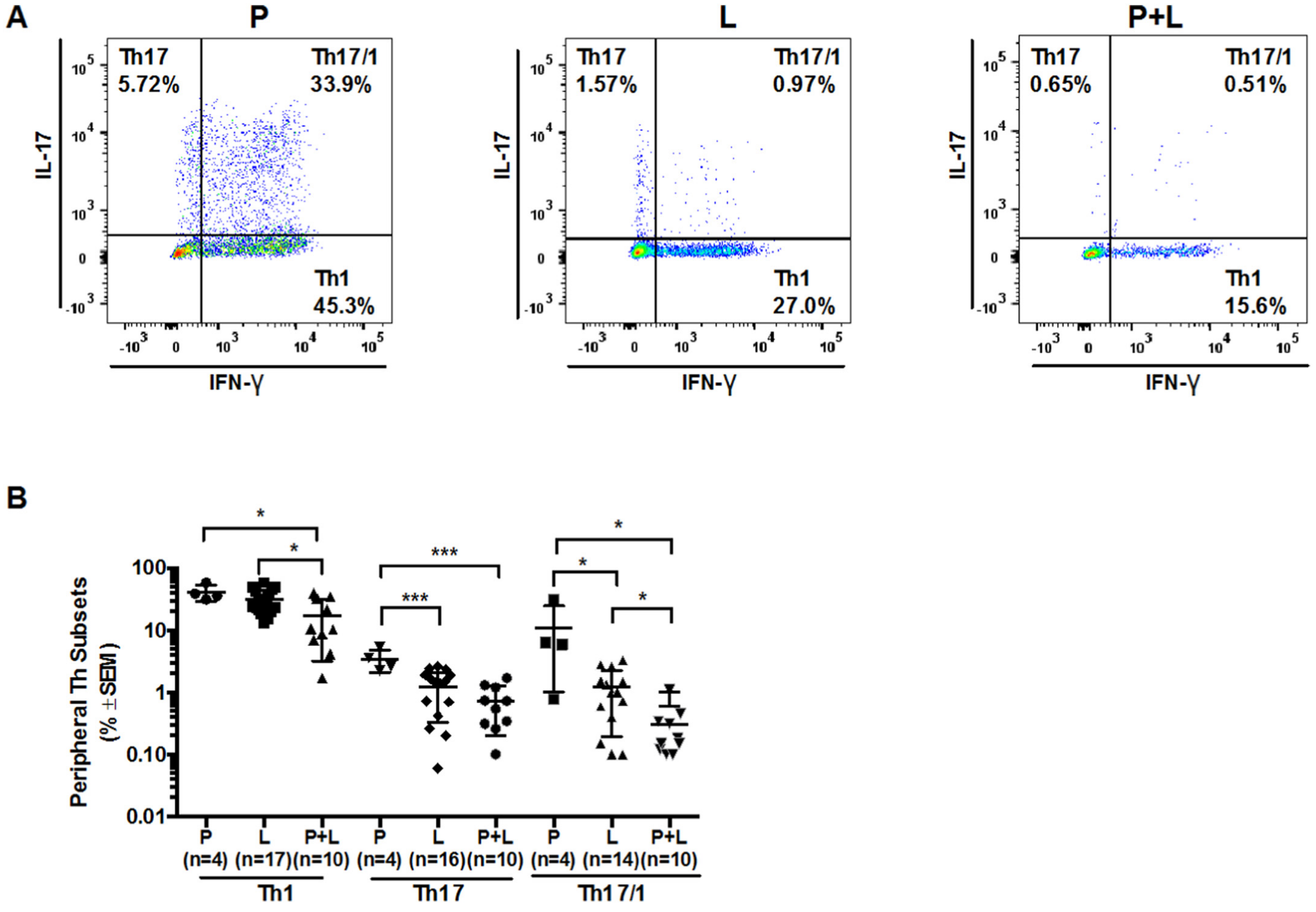


Fig. 3. Lower purity of PBMCs after isolation by P or L alone is associated with over-expansion of Th17, Th1 and Th17/1 cells after 4 days of culturing *ex vivo*. (A) Representative flow cytometry dot plots. (B) Summary graphs comparing the % yield of differentiated peripheral T helper subsets by each purification approach. $^*p < .05$; $^{***}p < .001$.

2.4. Expansion of peripheral memory Th17/Th1 cells in vitro and Th17 differentiation

For the expansion of peripheral Th17/Th1 cells *in vitro*, PBMCs were seeded into a 12 well plate pre-coated with anti-human CD3 antibody (10 μg/mL) at a density of 5×10^5/mL (unless otherwise stated), with freshly added anti-human CD28 antibody (final concentration 10 μg/mL) in 1 mL culture media and the plate was incubated at 37 °C and 5% (v/v) of CO_2 for 4 days.

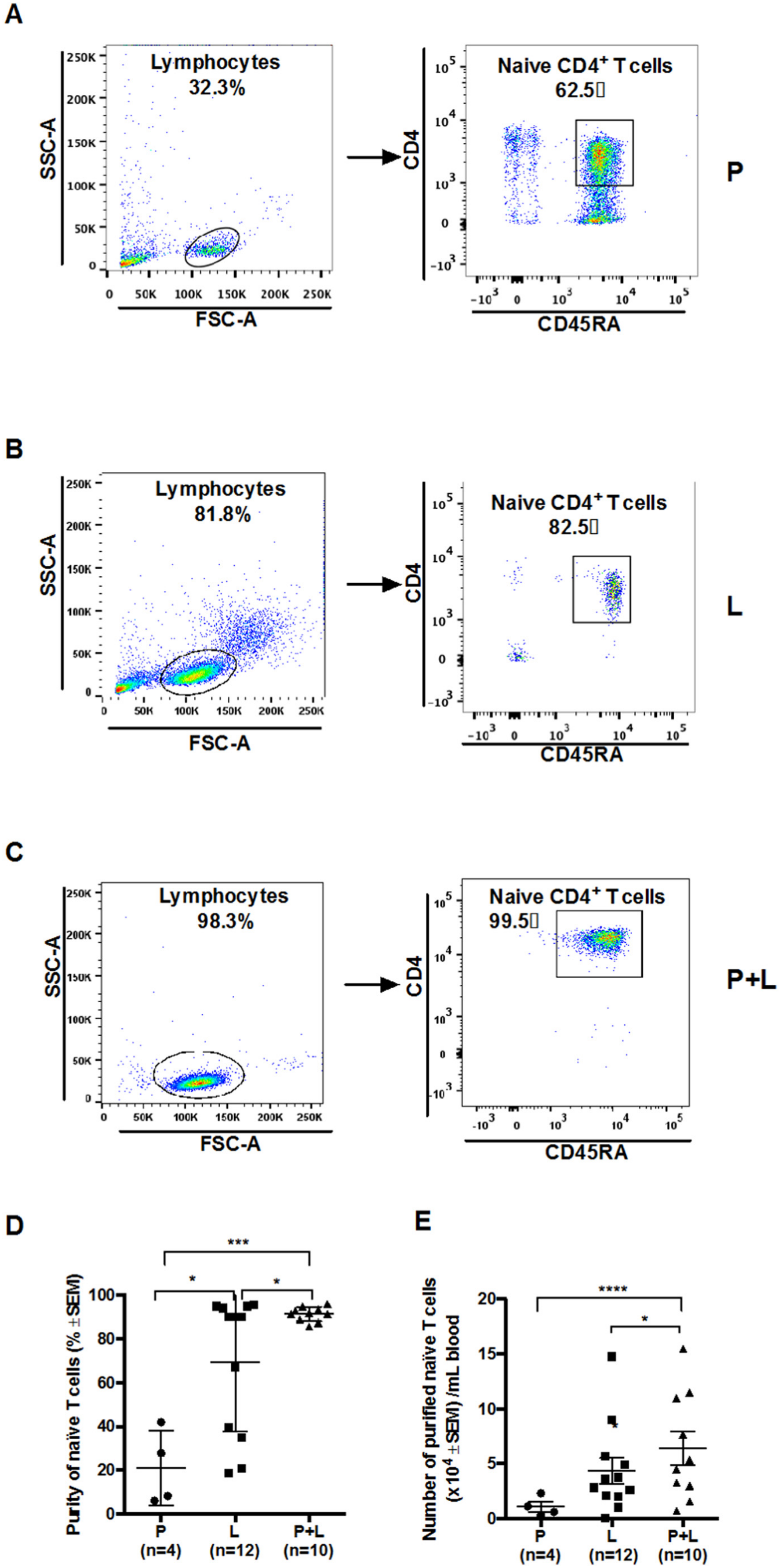
To differentiate Th17 cells, purified naïve CD4 T cells were seeded into a round bottom 96 well plate pre-coated with anti-CD3 antibody (10 μg/mL) together with soluble anti-CD28 antibody (1 μg/mL), IL-6 (10 ng/mL), IL-1β (10 ng/mL), TGF-β1 (5–10 ng/mL), IL-23 (10 ng/mL), and the neutralizing antibodies anti-IL-4 (10 μg/mL) and anti-IFN-γ (10 μg/mL) in serum-free AIM-V media without phenol red for 7 days (Manel, 2008; Blom and Poulsen, 2013). The cell density was 5×10^4/well of a 96-well plate and the medium was AIM V without phenol red unless otherwise specified. Half of the medium was replaced at day 4 when necessary. Before harvesting for intracellular cytokine staining, cells were treated with PMA (50 ng/mL) and ionomycin (1 μg/mL) in the presence of transport inhibitor Golgistop (final concentration 1 μg/mL) for 4 h. To determine estrogen's effect on Th17 differentiation, naïve T cells were cultured with the polarization cytokine cocktail (mentioned above) and treated with 1 nM E2 at the same time. The medium was replaced when necessary.

2.5. Flow cytometry analysis

For surface staining of PBMCs with anti-CD3-APC and naïve T cells with anti-CD45RA-FITC and anti-CD4-PE, cells were stained on ice in the dark for 30 min and then washed once with FACS buffer (PBS containing 2 mM EDTA and 1% (v/v) FBS). Intracellular staining for IL-17 or IFN-γ was performed following the manufacturer's protocol (eBioscience). Briefly, cells were first stained with Dead/Live fixable violet for 30 min and were then permeabilized with Cytofix/Cytoperm for 20 min. After thoroughly washing twice, cells were stained with anti-IL-17, anti-IFN-γ and anti-CD4 on ice for 30 min. Appropriate gating was based on FMO (Fluorescence Minus One) information. Samples were acquired on an LSR II flow cytometer using FACSDIVA software (BD) at the SickKids-UHN Flow and Mass Cytometry Facility in Toronto. Analysis was performed using FlowJo V_10 (Tree Star). Th17, Th1 and Th17/1 cells were defined as IL-17^+ CD4^+ cells, IFN-γ^+ CD4^+T cells, and IL-17^+ IFN-γ^+ CD4^+, respectively.

2.6. Statistical analysis

All statistical analyses were performed using Prism 6 (Graph Pad) software. Results are representative of at least three independent experiments. Student's *t*-test or paired *t*-test were used as appropriate, with $p < .05$ as the criterion for detecting significant differences between two groups.



(caption on next page)

Fig. 4. Purification by P alone (A) yielded a lower purity of naïve CD4$^+$ T cells than L alone B), which in turn yielded a lower purity of naïve CD4$^+$ T cells than sequential isolation by P + L (C). (A–C) The final purification step in each of used an immunomagnetic beads kit. Representative flow cytometry dot plots. (D) Summary graphs comparing the % of Th cell subsets differentiated from naïve CD4$^+$ T cells isolated by P, L or P + L. *p < .05; ***p < .001. (E) Compared to isolation by L alone, isolation of naïve CD4$^+$ T cells from whole blood by P + L increased the yield of naïve CD4 T cells.

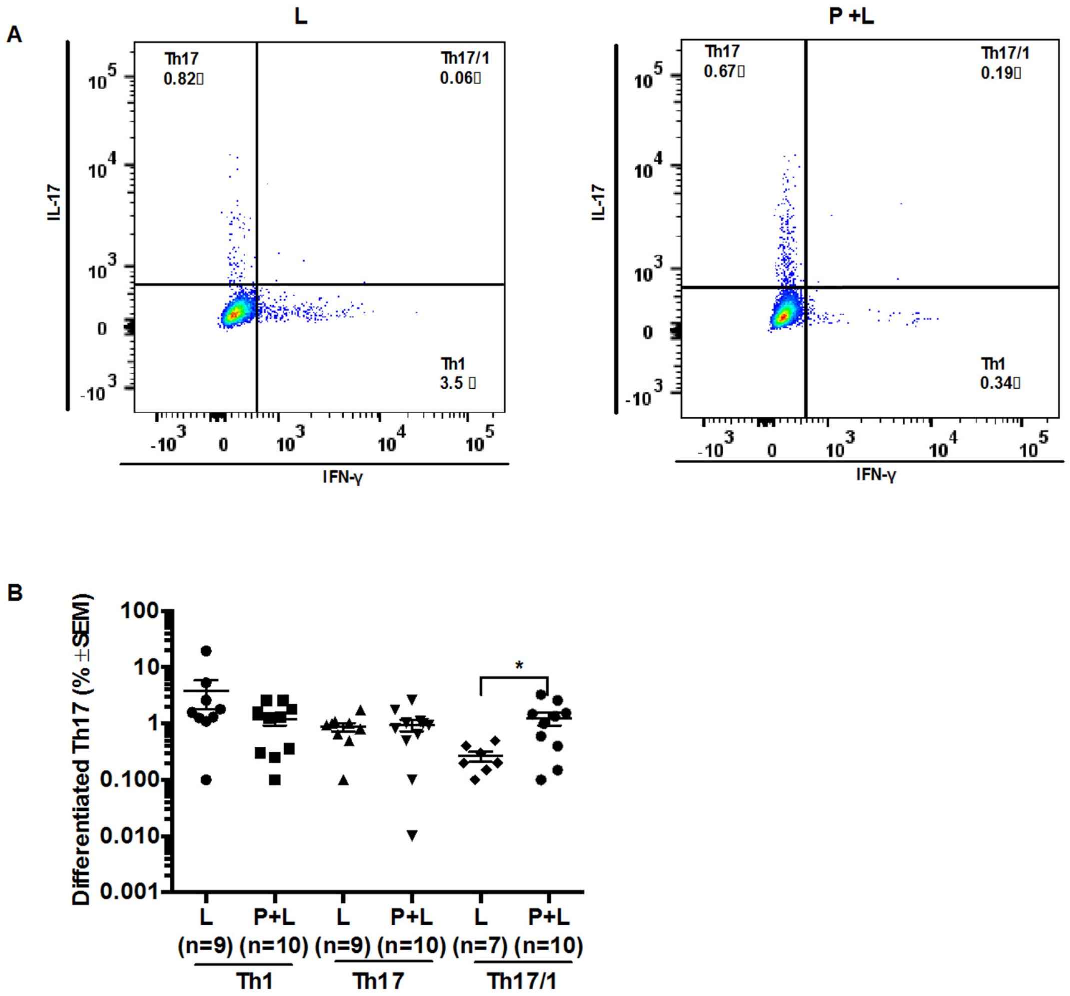


Fig. 5. Th1, Th17 and Th17/1 cells differentiated from naïve CD4$^+$ T cells isolated by L or P + L. (A) Representative flow cytometry dot plots of the expression of Th17, Th1 and Th17/1 after differentiation culture conditions for 7 days. (B) The percentages of differentiated Th17, Th1 and Th17/1 cells after 7 days differentiation culture.

3. Results and discussion

3.1. Sequential use of PolymorphPrep and LymphoPrep (P + L) leads to significant enrichment of T cells in PBMC preparations

Firstly, we tested the isolation of PBMCs from human peripheral blood using either PolymorphPrep (P) or LymphoPrep (L), or both methods sequentially (P + L) (Fig. 1). The combination of P + L was superior to either P or L alone in terms of the percentage of CD3$^+$ T cells in PBMCs (Fig. 2, A–D). The major contaminating cells isolated using the P protocol alone are platelets (Fig. 2, A; small size); using L protocols, contaminating cells are erythrocytes, platelets, granulocytes and memory lymphocytes (Fig. 2, B). Most of those “polluting cells” were removed after sequential rounds of separation by P followed by L (Fig. 2, C). Therefore, the P + L procedure enriched the peripheral T lymphocytes in the PBMC preparations. Neutrophils isolated from PolymorphPrep have been used routinely by our lab and several other labs (> 95% purity; Azzouz et al., 2018; Khan et al., 2018; Naffah de Souza et al., 2018; Khan et al., 2017; Djiadeu et al., 2017b; Caudrillier et al., 2012; Oh et al., 2008; Branitzki-Heinemann et al., 2016). The sequential use of P + L permits studies of both neutrophils and lymphocytes from the same sample of blood.

3.2. Lower purity of PBMCs over-expanded Th17 cells and Th17/1 cells

We next selectively expanded the T cells present in the PBMC preparations by culturing in anti-CD3-coated plates for 4 days in the presence of anti-CD28. Analysis of cells by gating for T cells (CD3 marker) showed that the presence of an increased proportion of non-T cells was associated with increased amplification of peripheral Th1, Th17 and Th17/Th1 cells (Fig. 3, A). In comparison to L and P + L, P alone produced a higher percentage of Th subsets: 2.8 ± 0.4% (P) *vs*, 1.3 ± 0.23% (L) *vs* 0.86 ± 0.14% (P + L) for Th17 cells; 41.0 ± 6.8% (P) *vs* 31.7 ± 3.2% *vs* (L) *vs* 17.2 ± 4.37% (P + L) for Th1 cells, and 1.05 ± 0.22% (P) *vs* 0.45 ± 0.11% (L) *vs* 0.95 ± 0.22% (P + L) for Th17/1 cells, respectively.

Overall, the PBMC preparations with the lowest purity, isolated by P alone, led to the highest over-expansion of all three Th cell subsets in comparison to P + L (Fig. 3, B). Although we did not determine the cytokine levels in the culture supernatant, cytokines secreted from the

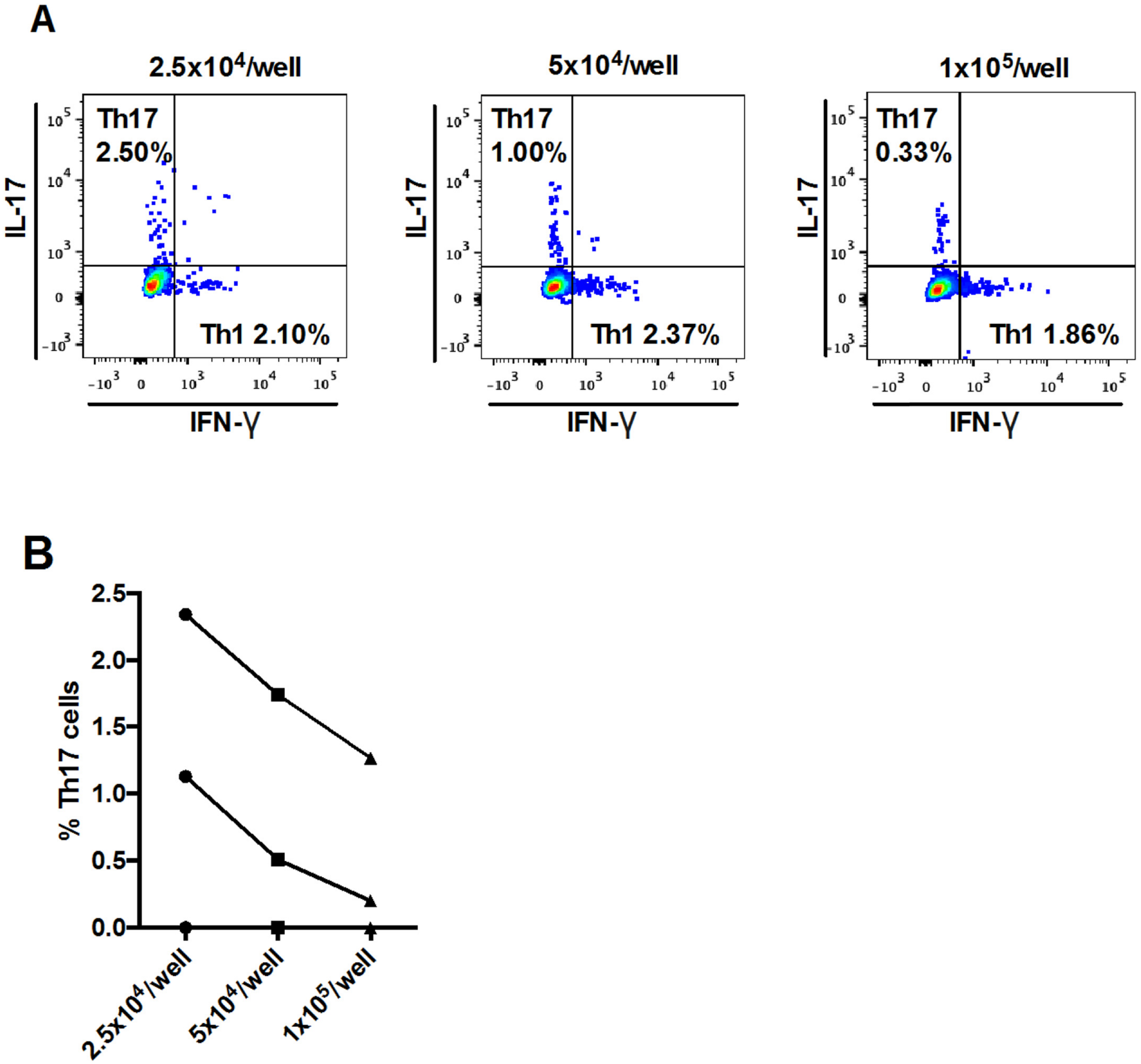


Fig. 6. The density of initial culture influences the differentiation of Th17 cells from naïve CD4$^+$ T cells. Assessment of the effect of cell densities (2.5 × 10^4/well, 5 × 10^4/well and 1 × 10^5/well in a 96-well plate) on the percentages of Th17 cells after 7 days of differentiation from naïve CD4$^+$ T cells. (A) Dot plot of data of a representative experiment. (B) Summary data of 3 independent experiments.

contaminating cells might play a role. Furthermore, platelets might mediate their effect by their derived microparticles (Chakraverty, 2014; Dinkla et al., 2016). Granulocytes present in the PBMC preparations could release granules to alter the degree of expansion of various Th cell subsets. The percentage of Th17 cells by L alone is similar to the (1.60 ± 0.56)% reported after isolation from the blood of healthy subjects using Ficoll-Hypaque (Shen et al., 2009).

3.3. Sequential use of PolymorphPrep and LymphoPrep (P + L) leads to the consistently of isolating high purity naïve CD4$^+$ T cells from the PBMC preparations

We also used the PBMCs isolated from these three methods for purifying naïve CD4$^+$ T cells using magnetic bead-based negative selection. The P + L procedure gave the highest proportion of lymphocytes (Fig. 4, A–C), and consistently very high purity naïve CD4$^+$ T cells (Fig. 4, D). The purity of our naïve CD4$^+$ T cells isolated from P + L high purity PBMCs followed by immunomagnetic bead separation (97.1 ± 0.44%, Table 1) was significantly higher than the corresponding values after isolation using L (69.6 ± 9.3%) and P (21.0 ± 8.5%). The purity of naïve CD4$^+$ T cells that are based on sequential use of P and L compares favorably with the published purities (75%–96%) of naïve CD4$^+$ T cells isolated using immunomagnetic beads alone (Revu et al., 2018; Schmidt et al., 2016). The presence of a higher proportion of other cells in the PBMC preparations markedly reduced the final purity of naïve CD4$^+$ T cells. This is likely due to the consumption of antibodies binding to the large number of target cells. Therefore, the negative selection was not sufficient to completely eliminate all the other cells to yield high purity naïve CD4$^+$ T cells. Compared to isolation by L alone, isolation of naïve

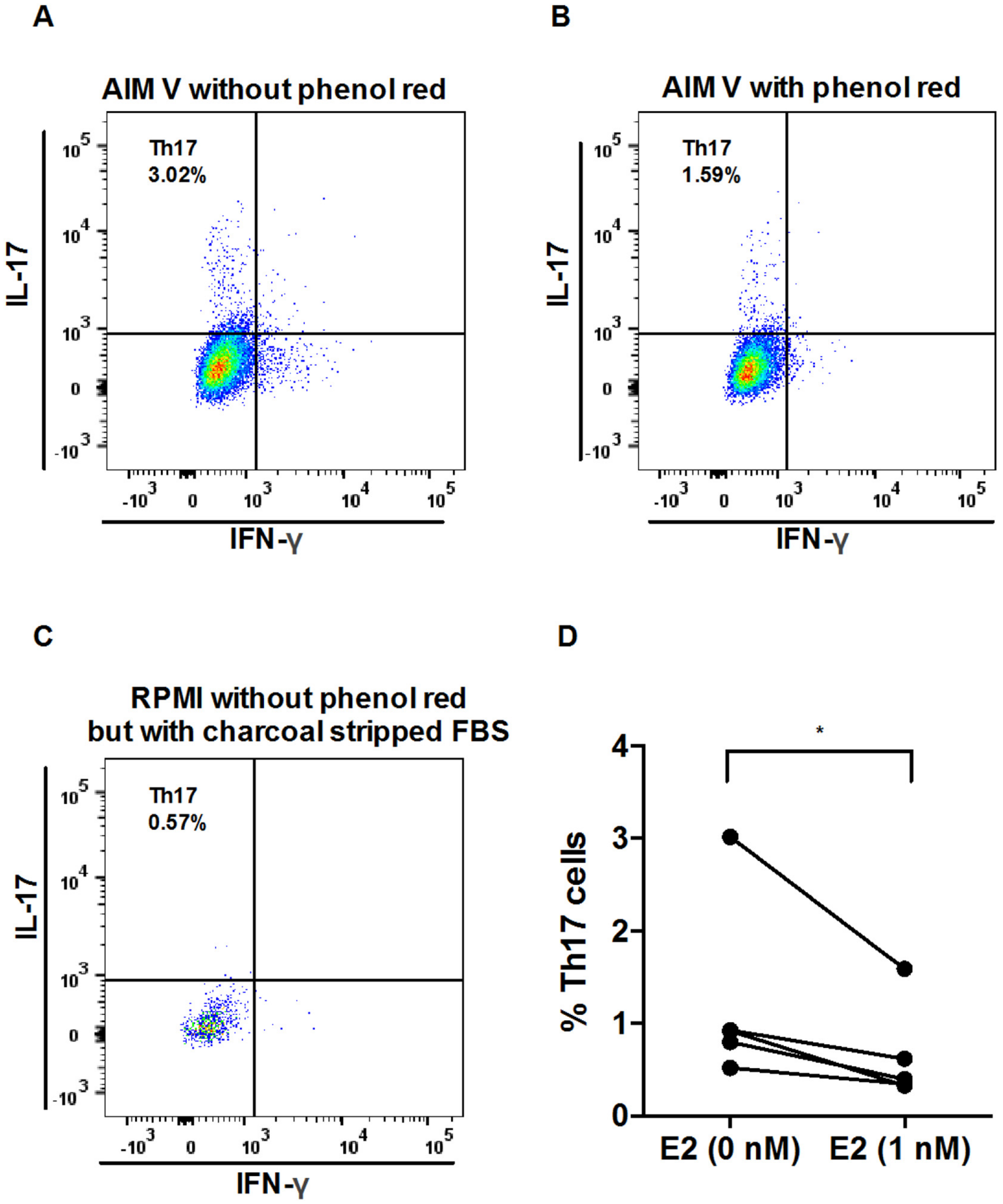


Fig. 7. The influence of medium composition on Th17 differentiation. Naïve CD4$^+$ T cells were differentiated for 7 days at a density of 5 × 10^4/well in (A) serum-free AIM V medium without phenol red, (B) serum-free AIM V with phenol red, or (C) RPMI 1640 supplemented with charcoal stripped FBS and free of phenol red. (A-C) show the proportions of Th17 cells after isolation in each medium ($n = 3$ independent experiments). (D) Estrogen (E2; 1 nM) inhibited Th17 differentiation in serum-free AIM V medium without phenol red; $n = 5$; *$p < .05$, paired *t*-test.

CD4$^+$ T cells from whole blood by P + L increased the yield of naïve CD4$^+$ T cells (Fig. 4, E).

3.4. *Effect of purification protocols on Th 17 differentiation from naïve CD4$^+$ T cells*

The yield of Th17 cells differentiated from pure naïve CD4$^+$ T cells isolated by P + L was (0.95 ± 0.22)%. This was very similar to the corresponding yield after differentiation from naïve CD4$^+$ T cells isolated by L alone: (0.88 ± 0.15%, p ns; Fig. 5). The percentage of Th1 cells in Th17 differentiation medium were respectively 3.9 ± 2.0% (L) and 1.2 ± 0.3% (P + L) (p ns; Fig. 5, A). However, the differentiated Th17/1 cell population in P + L is significantly higher than the cells isolated by L alone (Fig. 5, B). Therefore, the purity of the naïve CD4$^+$ T cells used in the cultures can affect the differentiation of Th cell subtypes.

3.5. *Cell culture density influences Th17 differentiation from naïve CD4$^+$ T cells*

Next, we examined the association between cell density and Th17 phenotype. To this end, we tested the effects of cell density effect on Th17 differentiation. Naïve CD4$^+$ T cells were seeded at 2.5 × 10^4, 5 × 10^4 and 1 × 10^5 cells/well in a 96-well plate pre-coated with anti-human CD3 antibody and cultured for 7 days. There was a cell density-dependent effect: lower density (2.5 × 10^4/well) of the culture leading to more Th17 cells (2.5%), with only 0.33% Th17 cells at higher density (1 × 10^5/well) (Fig. 6). No cells grew with < 1 × 10^4/well in a 96-well plate (not shown). Therefore, unless specified otherwise, for our standard throughout this study we used a cell density of 5×10^4/well in a 96-well plate for Th17 differentiation.

It has been reported that the survival of normal T cells and a number of leukemic cell lines depend on cell density (Ma et al., 2010; Pilling et al., 2000). Resting T cells are totally dependent on serum and cell-cell contact since they die rapidly if deprived of either of them. Pilling et al., (2000) showed that cell survival is proportional to serum concentration in the range of 0.5–2×10^6/mL, but cells die at low density even with high concentrations of serum. This also applies to activated T cells, which grow rapidly *ex vivo* at high cell density; however, lower density leads to more activated T cell death mediated by oxidative stress (Ma et al., 2010). We report here that a low density of naïve CD4$^+$ T cells favours Th17 polarization, which is inhibited by a higher density of naïve T cells. These results highlight the importance of the initial cell culture density on the differentiation of naïve T cells. Th17 differentiation requires an optimal cell density (2 to 5×10^4/well in 96 well plate) to ensure an adequate cell-cell interaction.

3.6. Estrogen inhibited human Th17 differentiation from naïve CD4$^+$ T cells

To better evaluate the effects of sex hormones on Th17 cell differentiation, we tested the suitability of various cell culture media for study of the effects of sex hormones. Phenol red-free RPMI 1640 medium supplemented with 10% charcoal-stripped FCS is one option. The pH-responsive dye phenol red is commonly included in culture media, but may be removed for studies of the effects of sex hormone due to its partial estrogenic activity (Welshons et al., 1988). In order to reduce or eliminate confounding effects from serum hormones, serum may be exposed to activated charcoal (“stripped”) prior to adding to the culture medium, or the serum may simply not be added (serum-free). Serum-free media such as AIM V that contains specific T cell growth factors can be used for *ex vivo* T cell expansion and proliferation (Medvec et al., 2017).

Fig. 7 shows representative data of three independent experiments using the following media: AIM V without phenol red (medium M1, Fig. 7, A), AIM-V with phenol red (medium M2, Fig. 7, B), and RPMI 1640 without phenol red with 10% charcoal stripped FCS (medium M3, Fig. 7, C). After differentiation from naïve CD4$^+$ T cells, there were 3.02%, 1.59% and 0.57% Th17 cells in media M1, M2 and M3, respectively. Charcoal-stripped FBS has been deprived of lipid-like components including but not limited to virus, certain growth factors, hormones and cytokines. Therefore, it is not surprising that naïve CD4$^+$ T cells did not proliferate and differentiate well with medium M3 at a density of 5×10^4/well in a 96-well plate (Fig. 7, C). Increasing the density up to 1×10^5/well permitted the cells to proliferate (data not shown), but the limited number of naïve CD4$^+$ T cells obtained from a healthy subject makes M3 unsuitable for our purposes. M1 was chosen over M2 for the following experiments due to its better yield of Th17 cells.

Previous studies showed that estrogen inhibits Th17 differentiation from naïve CD4$^+$ T cells in mouse models (Tyagi et al., 2012; Chen et al., 2015), but the effect on human Th17 differentiation has not been fully investigated. We found that 1 nM estradiol significantly inhibited Th17 differentiation (Fig. 7, D). The only difference between media M1 and M2 was the phenol red content of M2. The smaller yield of Th17 cells in M2 medium is consistent with competitive inhibition of estrogen activity due to the weak estrogenic agonist phenol red. Overall, serum free media without phenol red is the best option to explore estrogen's effect on Th17 differentiation.

4. Conclusion

In this report, we present a modified protocol to isolate peripheral blood neutrophils, mononuclear cells and naïve CD4$^+$ T cells with high purity. The protocol is an efficient and cost-effective alternative to the use of cell sorters. The consistency of initial cell purity and culture density would be suitable for multiple center patient-based studies. We found that cell density modulates Th17 differentiation from naïve CD4$^+$ T cells. For studies of the effects of sex hormones on Th17 differentiation from naïve CD4 T cells, the most suitable culture medium was free of serum and free of phenol red. A physiological dose of estrogen inhibited Th17 differentiation. Overall, the new sequential dual purification protocol proved useful for isolating highly pure neutrophils and naïve CD4$^+$ T cells for various experiments from the same blood sample.

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Author contributions

FX conducted experiments, interpreted the data, drafted figures and the manuscript. ZSA isolated neutrophils and edited the manuscript. NP conceived the idea and planned experiments. NS is the principal investigator. NS and NP supervised the study, interpreted the data and edited the manuscript.

References

Azzouz, D., Khan, M.A., Sweezey, N., Palaniyar, N., 2018 Apr 27. Two-in-one: UV radiation simultaneously induces apoptosis and NETosis. Cell Death Discov. 4 (51). https://doi.org/10.1038/s41420-018-0048-3. eCollection 2018. PMID: 29736268.

Blom, L., Poulsen, L.K., 2013. In vitro Th1 and Th2 cell polarization is severely influenced by the initial ratio of naïve and memory CD4+ T cells. J. Immunol. Methods 397 (1–2), 55–60. https://doi.org/10.1016/j.jim.2013.08.008.

Branitzki-Heinemann, K., Möllerherm, H., Völlger, L., Husein, D.M., de Buhr, N., Blodkamp, S., Reuner, F., Brogden, G., Naim, H.Y., von Köckritz-Blickwede, M., 2016 Nov 25. Formation of neutrophil extracellular traps under low oxygen level. Front. Immunol. 7, 518 (eCollection 2016).

Burgler, S., Ouaked, N., Bassin, C., Basinski, T.M., Mantel, P.Y., Siegmund, K., Meyer, N., Akdis, C.A., Schmidt-Weber, C.B., 2009. Differentiation and functional analysis of human T(H)17 cells. J. Allergy Clin. Immunol. 123 (3), 588–595. 595.e1-7. https://doi.org/10.1016/j.jaci.2008.12.017.

Burkett, P.R., Meyer zu Horste, G., Kuchroo, V.K., 2015. Pouring fuel on the fire: Th17 cells, the environment, and autoimmunity. J. Clin. Invest. 125 (6), 2211–2219. https://doi.org/10.1172/JCI78085.

Caudrillier, A1., Kessenbrock, K., Gilliss, B.M., Nguyen, J.X., Marques, M.B., Monestier, M., Toy, P., Werb, Z., Looney, M.R., 2012. Platelets induce neutrophil extracellular traps in transfusion-relatedacute lung injury. J. Clin. Invest. 122 (7), 2661–2671. https://doi.org/10.1172/JCI61303.

Chakraverty, R., 2014. An unexpected role for platelets in blocking Th17 differentiation. J. Clin. Invest. 124 (2), 480–482. https://doi.org/10.1172/JCI74231.

Chen, R.Y., Fan, Y.M., Zhang, Q., Liu, S., Li, Q., Ke, G.L., Li, C., You, Z., 2015. Estradiol inhibits Th17 cell differentiation through inhibition of RORγT transcription by recruiting the ERα/REA complex to estrogen response elements of the RORγT promoter. J. Immunol. 194 (8), 4019–4028. https://doi.org/10.4049/jimmunol.1400806. (Epub 2015 Mar 13).

Dinkla, Sip, van Cranenbroek, Bram, van der Heijden, Wouter A., He, Xuehui, Wallbrecher, Rike, Dumitriu, Ingrid E., van der Ven, Andr ´e J., Bosman, Giel J.C.G.M., Koenen, Hans J.P.M., Joosten, Irma, 2016. Platelet microparticles inhibit IL-17 production by regulatory T cells through P-selectin. Blood 127 (16), 1976–1986. https://doi.org/10.1182/blood-2015-04-640300.

Djiadeu, P., Kotra, L.P., Sweezey, N., Palaniyar, N., 2017 Mya. Surfactant protein D delays Fas- and TRAIL-mediated extrinsic pathway of apoptosis in T cells. Apoptosis 22 (5), 730–740. https://doi.org/10.1007/s10495-017-1348-4. PMID: 28168327.

Djiadeu, P., Azzouz, D., Khan, M.A., Kotra, L.P., Sweezey, N., Palaniyar, N., 2017 Mar 10b. Ultraviolet irradiation increases green fluorescence of dihydrorhodamine (DHR) 123: false-positive results for reactive oxygen species generation. Pharmacol. Res. Perspect. 5 (2), e00303. https://doi.org/10.1002/prp2.303. eCollection 2017 Apr. PMID: 28357129.

Guglani, L., Khader, S.A., 2010. Th17 cytokines in mucosal immunity and inflammation. Curr. Opin. HIV AIDS 5 (2), 120–127. https://doi.org/10.1097/COH.0b013e328335c2f6.

Harrington, L.E., Hatton, R.D., Mangan, P.R., Turner, H., Murphy, T.L., Murphy, K.M., Weaver, C.T., 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol. 6 (11), 1123–1132.

Khan, M.A., Farahvash, A., Douda, D.N., Licht, J.C., Grasemann, H., Sweezey, N., Palaniyar, N., 2017 Jun 13. JNK activation turns on LPS- and gram-negative bacteria-induced NADPH oxidase-dependent suicidal NETosis. Sci. Rep. 7 (1), 3409. https://doi.org/10.1038/s41598-017-03257-z.PMID: 28611461.

Khan, M.A., Philip, L.M., Cheung, G., Vadakepeedika, S., Grasemann, H., Sweezey, N., Palaniyar, N., 2018 Feb 13. Regulating NETosis: increasing pH promotes NADPH oxidase-dependent NETosis. Front Med (Lausanne). 5 (19). https://doi.org/10.3389/fmed.2018.00019. eCollection 2018.PMID: 29487850.

Kushwah, R., Gagnon, S., Sweezey, N.B., 2013 Dec 17. Intrinsic predisposition of naïve cystic fibrosis T cells to differentiate towards a Th17 phenotype. Respir. Res. 14, 138. https://doi.org/10.1186/1465-9921-14-138. PMID: 24344776.

Kushwah, R., Gagnon, S., Sweezey, N.B., 2014 Jan 17. T cell unresponsiveness in a pediatric cystic fibrosis patient: a case report. Allergy Asthma Clin Immunol. 10 (1), 2. https://doi.org/10.1186/1710-1492-10-2. PMID: 24438707.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., Cua, D.J., 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J. Exp. Med. 201 (2), 233–240.

Ma, Q., Wang, Y., Lo, A.S., Gomes, E.M., Junghans, R.P., 2010. Cell density plays a critical role in ex vivo expansion of T cells for adoptive immunotherapy. J. Biomed. Biotechnol., 386545. https://doi.org/10.1155/2010/386545.

Manel, Nicolas, 2008. *In vitro* differentiation of human Th-17 CD4+ T cells. Protocol Exchange. https://doi.org/10.1038/nprot.2008.122.

Marwaha, Ashish K., Leung, Nicole J., McMurchy, Alicia N., Leving, Megan K., 2012. TH17 cells in autoimmunity and immunodeficiency: protective or pathogenic? Front. Immunol. https://doi.org/10.3389/fimmu.2012.00129.

McGeachy, M.J., 2013 Nov. Th17 memory cells: live long and proliferate. J. Leukoc. Biol. 94 (5), 921–926. https://doi.org/10.1189/jlb.0313113. Epub 2013 Sep 4 Review.

McGeachy, Mandy J., 2011. GM-CSF: the secret weapon in the TH17 arsenal. Nat. Immunol. 12, 521–522. https://doi.org/10.1038/ni.2044.

Medvec, A.R., Ecker, C., Kong, H., Winters, E.A., Glover, J., Varela-Rohena, A., Improved Expansion, Riley J.L., 2017. *In vivo* function of patient T cells by a serum-free medium. Mol Ther Methods Clin Dev. 7 (8), 65–74. https://doi.org/10.1016/j.omtm.2017.11.001. eCollection 2018 Mar 16.

Naffah de Souza, C., Breda, L.C.D., Khan, M.A., de Almeida, S.R., Câmara, N.O.S., Sweezey, N., Palaniyar, N., 2018 Jan 9. Alkaline pH promotes NADPH oxidase-independent neutrophil extracellular trap formation: a matter of mitochondrial reactive oxygen species generation and citrullination and cleavage of histone. Front. Immunol. 8 (1849). https://doi.org/10.3389/fimmu.2017.01849. eCollection 2017. PMID: 29375550.

Noack, M., Miossec, P., 2014. Th17 and regulatory T cell balance in autoimmune and inflammatory diseases. Autoimmun. Rev. 13 (6), 668–677. https://doi.org/10.1016/j.autrev.2013.12.004.

Oh, H., Siano, B., Diamond, S., 2008. Neutrophil isolation protocol. J. Vis. Exp.(17). https://doi.org/10.3791/745. pii: 745.

Pilling, D., Akbar, A.N., Shamsadeen, N., Scheel-Toellner, D., Buckley, C., Salmon, M., 2000. High cell density provides potent survival signals for resting T-cells. Cell Mol Biol (Noisy-le-grand) 46 (1), 163–174.

Revu, S., Wu, J., Henkel, M., Rittenhouse, N., Menk, A., Delgoffe, G.M., Poholek, A.C., McGeachy, M.J., 2018. IL-23 and IL-1β drive human Th17 cell differentiation and metabolic reprogramming in absence of CD28 costimulation. Cell Rep. 22 (10), 2642–2653. https://doi.org/10.1016/j.celrep.2018.02.044.

Schmidt, A., Éliás, S., Joshi, R.N., Tegnér, J., 2016. In vitro differentiation of human CD4+FOXP3+ induced regulatory T cells (iTregs) from naïve CD4+ T cells using a TGF-β-containing protocol. J. Vis. Exp. 118. https://doi.org/10.3791/55015.

Shen, H., Goodall, J.C., Hill Gaston, J.S., 2009. Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis. Arthritis Rheum. 60 (6), 1647–1656. https://doi.org/10.1002/art.24568.

Tyagi, A.M., Srivastava, K., Mansoori, M.N., Trivedi, R., Chattopadhyay, N., Singh, D., 2012. Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis. PLoS One 7 (9), e44552. https://doi.org/10.1371/journal.pone.0044552.

Welshons, W.V., Wolf, M.F., Murphy, C.S., Jordan, V.C., 1988. Estrogenic activity of phenol red. Mol. Cell. Endocrinol. 57 (3), 169–178.