How Pure is Pure Enough: Effects of Red Blood Cell and Platelet Contamination on T-cell Culture

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ABSTRACT

Red blood cells (RBCs) and platelets (PLTs) have historically been considered immunologically inert. Recently, enough evidence has accumulated to suggest an important immunoregulatory role for these cells, both *in vivo* and *in vitro*. The latter is particularly important in the context of manufacturing novel cellular immunotherapies, including chimeric antigen receptor (CAR) T-cell therapies. T-cells are a sub-set of lymphocytes, a type of white blood cells that play a major role in the adaptive immune response. By reprogramming these cells to seek out and destroy target malignant cells, CAR T-cell therapies for cancer achieve remission rates as high as 90%. To manufacture such a highly effective treatment, T-cells must first be separated from whole blood and ultimately expanded in culture. RBCs serve as dynamic reservoirs of immunological signaling proteins, able to absorb and release at least 46 different cytokines. The presence of RBCs during culture has a significant impact on T-cell proliferation and survival. Contamination of T-cell culture with residual PLTs results in significant suppression of CD4+ cells and upregulation of regulatory T-cells, a type of T-cell that suppresses the function of other T-cells to prevent autoimmune responses. Additionally, residual PLTs modulate a variety of cytokines including IL-2, IL-5, IL-10, IL-17, IFN-γ and TNF-α. There is a wide range of cell separation methods currently employed in both research and clinical settings. And the number of residual RBCs and PLTs present during T-cell culture is almost entirely determined by the method used to isolate T-cells from whole blood. Given their significant immunoregulatory effects, the level of RBC and PLT contamination necessitates careful consideration when choosing a T-cell isolation method, in addition to the typical performance metrics such as T-cell

recovery and purity, separation throughput, and operating costs. This thesis examines the known effects of RBCs and PLTs on expansion of T-cells in culture to help evaluate the allowable number of these contaminating cells, and facilitate a well-informed decision when selecting a separation method for manufacturing CAR T-cell therapies.

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CHAPTER I. INTRODUCTION

Lymphocyte or T-cell culture has applications ranging from *in vitro* immunological research to *in vivo* cellular immunotherapy. The ability to expand T-cells *in vitro* is a cornerstone of immunological research and has facilitated major discoveries. T-cell culture has also enabled numerous clinical therapies including virus-specific T-cell therapy and CAR T-cell therapy. Before lymphocytes can be cultured, they must first be isolated from whole blood. The most common and cost-effective method to isolate lymphocytes is the use of density gradient separation with a reagent such as Ficoll. Density gradient methods produce a sample of peripheral blood mononuclear cells (PBMC). While this produces a purity around 70% lymphocytes, it is often pure enough to begin a T-cell culture for a wide range of purposes. A basic T-cell culture from PBMC will often be activated with anti-CD3 monoclonal antibodies (mAbs), often in conjunction with anti-CD28 MAbs, either attached to magnetic beads or coated on the surface of the expansion environment. For pure T-cell cultures, where monocytes are not present, an additional TCR stimulus such as anti-CD28 is required [1].

There is a wide selection of lymphocyte isolation methods, each with trade-offs in the key areas of recovery, purity, cost, and time. Naturally, differing resources and goals will often dictate what approach is used when conducting a T-cell culture. For example, Ficoll is very cheap but produces moderate recovery and low purity in around one hour. On the other hand, a positive-selection immunomagnetic method can

produce extremely pure samples with a comparable recovery rate but at a much higher cost. As such, it is clear that there are many variables that need to be accounted for when making a decision on how to isolate lymphocytes. One variable that has been severely neglected is purity, particularly the number of Red blood cells (RBCs) or platelets that are allowable in a given culture.

Traditionally RBCs and platelets have not been viewed as immunoregulatory cells. However, enough evidence has recently accumulated to suggest immunoregulatory roles and functions of these two cells [2-10]. RBCs are able to modulate T-cell proliferation and survival while platelets alter T-cell function and phenotype [3, 11]. RBCs and Platelets can have major impacts on T-cells both *in vitro* and *in vivo* and in order to make a fully informed choice on blood separation and culture methods it is crucial to understand their impact on T-cells. This review will cover the effects of RBCs and Platelets in T-cell cultures and evaluate what number of these contaminating cells are acceptable for a given application in order to guide culture-based decision making. A particular example that will be given a large focus is the manufacturing of CAR-T cells.

CHAPTER II. RED BLOOD CELL CONTAMINATION

Red blood cells are the major constituent of whole blood, accounting for about 45% by volume. The primary function of RBCs is the transport of oxygen and CO2. Though historically thought to be immunologically inert, red blood cells have demonstrated powerful immunoregulatory capabilities, particularly in relation to T-cells [2-8]. Recently, it has been shown that RBCs are reservoirs for at least 46 different cytokines and may play an important role in cytokine signaling [9, 10]. Red blood cells are capable of both releasing cytokines and absorbing them [4, 9]. Furthermore, autologous and heterologous RBCs have been repeatedly shown to enhance human T-cell proliferation and inhibit activation induced cell death [5, 6]. Red blood cells have also demonstrated the ability to increase IL-1 Beta, TNF-α, and IFN-γ production in a dose-dependent manner *in vitro* [8]. These and other immunoregulatory capabilities have major implications *in vivo* and *in vitro* that are only recently being realized [9, 10, 12].

Red blood cells are able to increase T cell proliferation by more than 2-fold in ideal conditions. At least 10 RBCs per T-cell are needed to see maximum proliferation increase, and this increase remains similar up to 100 RBC per T-cell [6, 13]. This proliferation enhancing effect is independent of TCR/CD3 stimuli as it can also be seen when T-cells are activated with TCR/CD3 independent stimuli such as IL-15 and IL-2 [2]. Meanwhile, RBC mediated survival enhancement is not seen when T-cells are activated with IL-2 or IL-15, likely due to the fact that these cytokines have their own survival enhancing properties [2].

Red blood cell bioactivities are not fully dependent on cell-to-cell contact and are a result of Erythrocyte-derived growth and survival factors (EDGSF). Red blood cells spontaneously release protein factors without relation to phosphatidylserine externalization or hemolysis. Phosphatidylserine externalization refers to the relocation of the phosphatidylserine phospholipid to the outer leaflet of the cell membrane, an indication of cell apoptosis Indeed, RBCs do not even need to be present to exert their culture enhancing effects. While optimal responses required intact RBCs with cell-to-cell contact, simply culturing T-cells in media that has previously contained RBCs for at least 90 minutes is enough to see some proliferation and survival benefits. As with RBCs, RBC-conditioned media exerts its effects regardless of activation method, including non-TCR mediated methods. Furthermore, proliferation and survival benefits are exclusive to CD3+ cells and preferential to CD8+ cells [4].

While RBCs provide a significant benefit to T-cells *in vitro*, these benefits are at least somewhat dependent on their proper form and function [14, 15]. The modulation of RBC bioactivities is often correlated disease activity [10, 12, 13]**.** Red blood cell's proliferation enhancement is significantly reduced just one day after total hip arthroplasty [12]. Additionally, RBCs from atherosclerosis patients lose their ability to rescue T-cells from apoptosis [13]. The side effects of both surgery and atherosclerosis cause the release of oxygen free radicals which bind to RBCs causing oxidative stress [12, 13]. This oxidative stress is thought to be responsible for the subsequent reduction or removal the RBC's beneficial bioactivities [12, 13]. However, oxidative stress isn't the only way for RBC's to lose their

immunoregulatory function. The blood-banking processes has some element that reverses the benefits of RBC's as packed red blood cells (PRBC) have been shown to have a strong suppressive effect on T cell proliferation**,** although this effect requires direct cell-to-cell contact [15]. Red blood cell bioactivities can also be negated by treating T cells with the immunosuppressive drug cyclosporine A [2].

Effect on T-cell proliferation and survival

Red blood cells are most effective at enhancing T-cell proliferation when phytohemagglutinin (PHA) is used as the source of T cell activation. Phytohemagglutinin over-activates T-cells and causes significant activation induced cell death [5]. The ability of RBCs to both enhance proliferation and rescue T cells from activation induced death makes it an ideal pairing with PHA [6]. This effect seems to correlate with the concentration of PHA, where lower concentrations of PHA require more RBCs to see the same benefit. When activated with .0625% PHA, peripheral blood mononuclear cells (PBMC) required an RBC to PBMC ratio of 5:1 to achieve a 2.6-fold 3H-Thymadine uptake increase while a .125% PHA activated culture achieved a 1.8x increase with only half the number of RBCs [3]. The optimal dose of PHA is 1% or 5ug/ml [16]. At this optimal dose, purer samples of lymphocytes, such as a >85% CD3+ peripheral blood lymphocyte (PBL) composition, experience an increase in the percent of dividing cells from 40% to over 85% after 7 days in culture with 10 RBCs per PBL [5]. Normally T-cells require accessory cells to achieve high levels of proliferation when activated by PHA [5]. However, after a 7-day culture at a ratio of 10:1 RBC to pure T-cell, RBC bioactivities were able to restore T-cell proliferation to levels above a PBL culture that was free of RBCs [5].

This shows that RBC bioactivities act directly on T-cells without the need for accessory cells, likely through the preservation of the antioxidant state of activated T-cells [5]. In another experiment, an RBC to PBL ratio of 100:1 saw a 3H-Thymadine uptake increase of almost 6-fold over 5 days [6]. Thus, RBCs are beneficial in T-cell culture, up to and possibly beyond 100 RBCs per T-cell.

Effect on CD8+ cells

CD8+ cells are majorly affected by the presences of RBCs in culture. Red blood cells increase CD8+ cell proliferation, hasten their entrance into the cell cycle, and rescue them from apoptosis induced cell death and oxidative stress [6]**.** In a 5-day culture activated by PHA with an RBC to pure T-cell ratio of 100:1, the number of dividing CD8+ positive quadrupled from 8% to 63% [7]. In a PBL culture with the same conditions, the % of CD4+ saw an increase from 34% to 53% [7]. Thus CD8+ cells are preferentially expanded in PBL cultures, while in pure T-cell cultures both CD4+ and CD8+ cells achieve about the same percent of dividing cells, CD8+ gains a much larger increase in division through the effect of RBCs. CD8+ cells also see a significant (3.8x reduction) decrease in the number annexin-V positive cells when cultured in PHA at a ratio of 100:1 RBC to PBL [7]. Meanwhile, when activated by OKT-3, secondary PBMC cultures saw no significant change in the percent of CD8+ cells for ratios of 0:1, 2:1, 10:1, and 50:1 RBC to PBMC [8].

Effect on CD4+ cells

CD4+ cells seem to be less affected by the positive effects of RBCs than CD8+ cells. In a 5-day culture activated by PHA with an RBC to pure T-cell ratio of 100:1, the number of dividing CD4+ positive doubled from 36% to 63% [7]. Meanwhile, in

a 5-day culture activated by PHA with an RBC to PBL ratio of 100:1, the number of CD4+ positive cells saw no statistically significant increase over 19 experiments [7]. Additionally, 5-day OKT-3 activated secondary PBMC cultures showed no significant difference in the percent of CD4+ positive cells for ratios of 0:1, 2:1, 10:1, and 50:1 RBC to PBMC [8]. It is unknown if the loss of proliferation enhancement is due to the presence of accessory cells or if it has something to do with the OKT-3 activation methods. CD4+ cells do seem to gain some benefit from the survival enhancement of RBCs as the percent of annexin-V positive cells was lower on days 3 and 5 of a 100:1 RBC to PBL coculture [7].

CHAPTER III. PLATELET CONTAMINATION

Platelets are anucleate cells derived from megakaryocytes that play a crucial role in hemostasis and thrombosis. Just 1 ml of human blood can contain as many as 450 million platelets [17]**.** Akin to RBCs, platelets are beginning to be recognized as immunoregulatory cells [18, 19]. They contain and release multiple signaling cytokines and adjust their secretory behavior based on environmental cues [18, 20, 21]. Platelets have a significant impact on T-cell function and proliferation *in vitro*, modulating the release of Th1/Th17 cytokines and phenotype-specific cell activation [20, 21].

In addition to regulating immune function through cytokine signaling, platelets are also known to bind to lymphocytes on occasion [22]. Lymphocytes with bound platelets have been shown to have reduced TCR-induced proliferation and reduced cytokine production [22]. While only about 3% of circulating lymphocytes have bound platelets, both platelet activation and lymphocyte activation *in vitro* increase binding rates, up to as many as 15% of cytotoxic lymphocytes [22].

Not only do platelets themselves suppress T-cell proliferation, but the molecules contained within platelets are known to suppress T-cell proliferation and modulate their function [11, 23, 24]. Platelet factor-4 (PF-4) and TGF-β are major molecules carried by platelets and have a significant suppressive effect on T-cell proliferation [20, 21, 23, 24]. Platelet factor-4 also modulates Th1/Th17 and regulatory T-cell (Treg) activation and function [23, 24**].**

Effect on T-cell proliferation

In general, platelets have a suppressive effect on T-cell proliferation [11, 20, 21**].** In a pure CD4+ cell 72-hour culture, a platelet to CD4+ cell ratio of 250:1 induced a >50% reduction in 3H-thymidine incorporation [20]. The activation of platelets seems to play an important role in T cell suppression, as activated platelets release PF-4 and TGF-β [20]. Platelet factor-4 inhibits proliferation of anti-CD3 activated pure T-cells in a dose-dependent manner from 1.25 ug/mL up to 29 ug/mL [24]. Peripheral blood mononuclear cells activated by either a purified protein derivative of tuberculin or tetanus toxoid saw a massive decrease in proliferation after 96 hours of culture with 29 ug/ml of PF-4 as determined by 3H-thymidine uptake [23]. Proliferation suppression was seen with as little as 3.5 ug/ml of PF-4 and increased in a dose-dependent manner up to 29 ug/ml PF-4 where proliferation was suppressed by 60% [23]. This effect is apparently not dependent on accessory cells, as pure T-cells cultured with 29 ug/ml PF-4 and activated with anti-CD3 + anti-CD28 saw a 64% decrease in proliferation after 96 hours of culture [23]. This suppression may be a result of inhibited IL-2 production, as these cells saw an 84% decrease in IL-2 secretion and a major inhibition in the induction of IL-2 mRNA [23]. Moreover, the addition of exogenous IL-2 was able to almost completely rescue the T-cells from the suppressive effect of PF-4, giving further evidence to an IL-2 mediated mechanism of suppression [23]. In a culture with a PLT to CD4+ cell ratio of 250:1 the number of spontaneously activated platelets nearly doubled from 17.6% to 29.6% after a 24-hour period, triggering the release of 13 ng/ml of PF-4 by 48 hours [20]. While this concentration is not nearly enough to suppress T-cell proliferation, activated platelets

from 1 ml of whole blood rapidly release PF-4 levels in the range of 10 ug/ml [25]. While few cell cultures use whole blood, it is important to note that significant concentrations of PF-4 may be released prior to separation, or during a separation method that induces significant platelet activation.

Effect on T-cell phenotype

Platelets exert a biphasic effect on the activation of CD4+CD25- cells [21]. Over the course of a 5-day coculture of 250:1 platelet to CD4+ cells activated with anti-CD3 + anti-CD28, the Th1 cell percentage saw a moderate increase by 48 hours compared to cultures without platelets [21]. However, past this 48-hour mark the Th1 percentage began to see a steep decline, ending significantly lower on day 5 than the control culture [21]. Meanwhile, in the same culture, Tregs immediately saw a significant increase in activation that persisted throughout the 5 days [21]. When a TGF-β neutralizing antibody was added to these cultures, the Th1 proliferation was restored to control levels, while Treg proliferation continued to be bolstered by platelet contamination regardless of the antibody's presence [21]. Since platelets release about 50 times more TGF-β than Tregs, they can be held as the main suspect responsible for the Th1 suppression seen in platelet-CD4+ cocultures [21].

PF-4 has also been shown to suppress CD4+CD25- cell proliferation during stimulation by PHA, anti-CD3, and anti-CD3 + anti-CD28 [24]. Consistent with Fleischer's group, CD4+CD25- cells had their proliferative capabilities restored when exogenous IL-2 was added [24]. On the other hand, Tregs continue have their proliferation significantly enhance by PF-4 for all combinations of anti-CD3 with anti-CD28 and IL-2 [24]. At a starting ratio of 1:1 CD4+CD25- to Treg with 5 ug/mL PF-

4, there were enough bolstered Tregs to overcome the CD4+CD25- cell suppression and result in a net increase in cells when activated with anti-CD3 for 72 hours [24]. This enhancement of Tregs and simultaneous suppression of CD4+CD25- cells past the 48-hour mark is consistent with the effect of platelets themselves, as seen by Zhu's group**.** While Tregs typically do not respond to anti-CD3 activation alone, the presence of PF-4 causes them to proliferate in response to this stimulus [24]. Additionally, the presence of PF-4 seems to interfere with their ability to suppress CD4+CD25- cells as these cells had higher proliferation when cocultured with Tregs and PF-4 than when cultured with PF-4 alone [24]. It seems PF-4 contamination and platelet contamination both independently suppress CD4+CD25- proliferation, platelets primarily through TGF-β release as they do not often release enough PF-4 to be significant. Platelet factor-4 on the other hand, exerts its effect directly on CD4+ cells.

Effect on T-cell function

Cocultures of platelets and CD4+ cells produce significantly more IFN-γ, IL-10, IL-17, than cultures of pure CD4+ cells [20]. When activated with anti-CD3 + anti-CD28, cocultures of CD4+ cells and platelets saw a dose-dependent increase in IFN-γ production ranging from 15:1 platelet to CD4+ cells, up to 250:1 platelet to CD4+ cells, resulting in more than a 2-fold increase in IFN-γ [20]. This IFN-γ upregulation can be somewhat suppressed by the removal of platelet-to-T-cell contact and by the addition of blocking antibodies against P-selectin, CD40L, and GPIIb/IIIa [20]. Platelets also regulate Th1 cytokines IL-2 and TNF-α. CD4+ cells experienced more than a 3-fold increase in IL-2 and TNF-α production when activated with anti-

CD3 + anti-CD28 and cultured at a platelet to CD4+ cell ratio of 250:1 for 48 hours [20].

Platelets regulate Th17, Th1, and Treg differentiation. The percentage of Th1, Th17, and Treg cells all saw a significant increase when activated with CD3/CD28 and cultured at a platelet to CD4+ cell ratio of 250:1 for 48 hours [20, 21]. Corresponding cytokines IL-2, TNF-α, IL-17, and IL-10 also saw a very significant increase in these cultures, and to a lesser extent IFN-γ [20, 21]. This increase in IL-2 is in marked contrast to the IL-2 suppression from PF-4 seem by Fleischer's and Liu's groups. When a PF4-neutralising antibody was added to these cultures reversed platelet-mediated enhancement of anti-CD3 + anti-CD28 induced production of TNF-α and IL-10, causing these cytokines to drop below control levels [20]. Furthermore, the addition of 1ug/ml recombinant PF-4 to anti-CD3 + anti-CD28 stimulated CD4+ cells mimicked the effects of platelets on both TNF-α and IL-10 production [20]. Platelet factor-4 blockading by a neutralizing antibody also reduced IL-2, IFN-γ, and IL-17 production in anti-CD3 + anti-CD28 stimulated CD4+ T-cell-platelet cocultures to below control levels [20]. These effects seem to be caused by the PF-4 released by activated platelets, although it has been shown that higher levels of PF-4 have the opposite effect [23, 24]. If high levels (>1.25ug/ml) of PF-4 suppress CD4+CD25- proliferation and function, it may be that low levels (<1ug/ml) of PF-4, such as those release by a platelet to CD4+ cell ratio of 250:1, have the opposite effect. Of note, TGF-β neutralization did not influence platelet-enhanced TNF-α or IL-10 production in anti-CD3 + anti-CD28 stimulated cocultures [20]. A summary of platelet can PF-4 contamination can be found below in Table 1.

Table 1. Platelet and PF-4 Contamination Effects on CD4+ Cells in Culture

	Proliferation	IFN-γ	TNF-a	IL-2	IL-10	IL-17	Il-4	IL-5	TGF-B
Platelets[1]	--	++	+++	+++	++	+	No effect	No effect	No data
PF-4[2]	--	---	--	---	---	No data	--	--	-

1 – When cocultured at a ratio of 250:1 platelet to CD4+ cell, and activated with anti-CD3 + anti-CD28

2 – When cultured at a PF-4 concentration of 5 ug/ml, and activated with anti-CD3 + anti-CD28

CHAPTER IV. CONSIDERATIONS FOR CELL SEPARATION METHODS

Naturally, different cell separation methods come with their own advantages and disadvantages. The most important properties to consider when evaluating any cell separation method are purity, recovery, viability, throughput, and cost. Purity is of particular importance in this context as the level of RBC and platelet contamination will be used to inform the evaluation of each separation method as is relates to T-cell culture and its applications. Recovery refers to the percentage of T-cells that remain from the original sample after separation. This recovery rate is important since methods with a low recovery rate can require significantly more blood to reach satisfactory amounts of T-cells, and in many clinical settings only about 2.4 mL/kg of blood can be extracted from a patient every 24 hours [26]. Cell viability is the percentage of living cells after separation. Throughput refers to the volume of blood or the number of cells that can be separated per unit time. In this section we will review representative examples of common and emerging T-cell isolation techniques and evaluate them based on these typical criteria and new criteria derived from the information about RBC and PLT contamination. All representative methods mentioned here have high resulting viabilities (>85%) and will be evaluated based on the remaining parameters.

Density gradient separation

By far the most popular separation method, density gradient separation involves the use of a centrifuge to fractionate cells based on their densities. Spinning whole blood in a centrifuge causes blood cells to accumulate at the bottom of the tube in order from highest density to lowest density. To separate specific leukocytes such as PBMC, one or more artificial reagents of a specific density can be used to further isolate a leukocyte sub-population that has a density within a known range. The most common and cheapest reagent used for this purpose is Ficoll-Paque, particularly the version with a density of 1.077 g/m. After density gradient isolation, PBMC recovery is typically about 50 – 70% [27]. Despite the cheap cost, Ficoll is expensive in time and effort. Separation often takes more than 1 hour and requires an experienced technician for the best results [27, 28]. To cut down on time and complexity Ficoll can be used in conjunction with a cell-preparation tube (CPT). This is a blood collection tube that contains an inert gel barrier, allowing separation to be completed in a single centrifugation step without requiring multiple pipetting manipulations from a technician.

A slightly more expensive alternative to Ficoll is Lymphoprep. As opposed to Ficoll, Lymphoprep does not contain a chelating agent and is often used in conjunction with SepMate, a specialized tube meant to reduce separation time. Similar to the CPT, the SepMate tube contains an inert barrier that enhances cell recovery and reduces separation time**.** Both CPT and Lymphoprep with SepMate have increased recovery compared to traditional Ficoll [28]. The advantages of SepMate being that it has a much larger volume per tube at 50 ml and does not require blood to be drawn

straight from a donor into the tube. Up to 5% of the total recovered cells by density gradient methods may be RBCs [29].

Density gradient separation is highly dependent on the skill and experience of the technician [27, 28]. An experienced technician can recovery up to 61%, 36%, and 42% more PBMC for CPT, Ficoll, and Lymphoprep with SepMate respectively [28]. Donor-to-donor differences also account for a significant portion of variance in cell yield and recovery [27]. Leukocyte distribution post-separation is similar between the three discussed methods. All methods produce a composition of 65-70% T-cells, 5-22% B-cells, 5-14% NK cells, and 4-12% monocytes [28]. Cell viability after these methods is regularly above 90% and there is no significant difference between Ficoll, CPT, and Lymphoprep [27, 28].

Antibody-based separation

Magnetic-activated cell sorting (MACS) utilizes tiny magnetic beads coated with cell-specific antibodies that bind to the antigens on desired or undesired cells [30]. MACS has two main approaches, positive and negative selection. Binding the magnetic beads to the desired cells and using a magnet to hold them in place while all other undesired cells are removed is called positive selection [31]. Negative selection is the opposite. The beads are bound to all undesired cells and held in place with the magnetic while the desired cells are removed [31]. Magnetic-activated cell sorting can be easily automated with the use of the AutoMACS separator sold by Miltenyi Biotec. AutoMACS separation can perform negative selection, positive selection, or both simultaneously, commonly producing lymphocyte purities over 90% with negative selection and over 99% with positive selection [32]. Another advantage to MACS is

the ability to use the magnetic beads to both separate and activate T-cells, essentially in on step. While MACS has a number of advantages, it is one of the more expensive options, requiring an initial investment for the magnetic columns and a continual cost for the beads.

Another antibody-based cell separation method is fluorescence-activated cell sorting (FACS). In essence, FACS uses flow-cytometry to identify cells of interest via bound fluorescent antibodies, and alters their path through the machine using an electric pulse, resulting in one container with the cells of interest and one container with all other cells. Like flow cytometry, FACS sorts cells one at a time, resulting in extreme specificity at the cost of time and money [31]. This results in a low throughput of 1e7 cells/hr at a huge initial investment typically over $250,000 [31]. Due to the low throughput, and high purity, FACS is typically reserved for research with rare cell types such as Tregs.

Microfluidic separation

Microfluidics is an emerging and promising cell separation approach that utilizes micro-scale flow channels and features to filter cells [33]**.** In addition to traditional microfluidic filtering techniques, many researchers have been exploring supplemental separation measures like the use of magnetism, acoustics, vibrations, preferential lysis, oscillating flows, and more to try to enhance device performance [34]. While such approaches will surely find important roles or niche's in the future, the most developed and effective microfluidic devices are currently those that utilize Dean drag forces and inertial forces [35]. For simplicity, only a few of the most promising devices designs for lymphocyte isolation will be evaluated for comparison.

Some of the latest inertial microfluidic designs are able to achieve a white blood cell (WBC) recovery rate of 93.2% while removing 99.9% of erythrocytes at a whole blood flowrate of 15 ml/min [36]. A huge advantage of microfluidic devices is their ability to be operated in parallel, enhancing their through-put dramatically while maintaining high purity and recovery. An array of parallel devices created by Multu et al. was able to separate whole blood with a WBC recovery of 96.6% and a removal of 99.99% of erythrocytes at a flowrate of 3 ml/min with cell viability over 94% [37]. Some devices are even able to further separate WBCs into subpopulations. Ramachandraiah's group was able to segregate whole blood into granulocytes, monocytes, and lymphocytes with purities of 86%, 43%, and 91% respectively, at a flowrate of 1 ml/min [38]. Through a cell-activation study with lipopolysaccharide**,** it was found that this device does not activate cells or introduce artifacts that could affect a stimulated blood sample [38]. In fact, microfluidic devices can even be beneficial to T-cell culture, as PBMCs separated by a microfluidic device was able to produce a 2-fold higher cell yield compared to PBMCs separated with density gradient methods [39]. A spiral design from Chiu's group was able to produce a 97% pure lymphocyte sample with >95% viability from pre-lysed blood, at a 1.7 ml/min flowrate [40]. This combination of extreme recovery, high purity, and excellent throughput places microfluidic devices in a strong position to revolutionize the blood separation world.

Density gradient methods and MACS currently represent the two most commonly used separation methods, while microfluidic is an emerging and promising technology. Density gradient offers the highest throughput and lowest price at the cost

of sample purity. Magnetic-activated cell sorting offers high purity at a moderate throughput for a significant price disadvantage. Microfluidics often have a lower throughput but have the capability of achieving excellent recovery and purities for little investment. Typical performance values for these three methods can be found below in Table 2.

Table 2. Comparison of Common Separation Methods

Method	Throughput	Lymphocyte Recovery	Lymphocyte Purity
Density Gradient[27, 28, 31]	>1e11 cells/hr	~50-70%	~65-70%
MACS[31, 32]	1e9 cells/hr	~62%	~90%
Microfluidics[37, 40]	7.8e6 cells/hr	up to 96.6%	up to 97%

CHAPTER V. CONSIDERATIONS FOR CULTURE APPILCATIONS

T-cell culture has a variety of applications for both research and clinical purposes. Naturally, one must isolate T-cells to some extent before a culture can be started. For research, the priorities between purity, throughput, recovery, and cost are highly dependent on the given experiment. For example, a study looking at the alterations to RBC's immunoregulatory effects in certain diseases may use Ficoll to save money, as it is concerned with the RBCs rather than specific T-cell subsets [13]. Meanwhile, an experiment concerned with the function of Tregs in certain culture conditions may do well to use magnetic beads for separation, as Tregs are a rare cell type. Thus, in light of the presented RBC and platelet immunoregulatory effects, it is up to each individual researcher to weigh the strengths and weaknesses of each isolation method, and chose based on their own circumstances and goals.

Chimeric antigen receptor T-cell therapy considerations

One clinical application of particular salience is CAR therapy. Chimeric antigen receptor therapy is a powerful cancer immunotherapy achieving high remission rates in hematological malignancies and showing potential for treating other tumor types [41, 42]. In this novel therapy T-cells are genetically engineered to produce an artificial T-cell receptor, often CD19, which allows them to recognize and eliminate tumor cells [42]. However, one major drawback of CAR-T therapy is it's complex and variable manufacturing methods [42, 43]. The production of CAR T-cells requires the completion of the following steps: leukapheresis, washing, activation,

viral transduction, expansion, and lastly the formulation of the final product**.** While the overall set of steps remains the same across the board, the exact products and techniques used to complete these steps are often specific to a given institution, and can results in significantly different CAR-T products [42]. This difficulty is exacerbated by the uncertainties in optimal dose size, transgene delivery methods, and optimal costimulatory domains [40].

Manufacturing CAR-T cells is sensitive process with many variables affecting the expansion and function of these cells. Due to the personalized nature, not all patient's cells produce a therapeutic dose or persist and expand *in vivo* [41, 42]. To achieve sufficient expansion *in vitro* certain culture conditions must be met, the first of which is the starting sample composition. A high level of lymphocytes is an obvious necessity but what number of other cells in the sample will affect the expansion process? From the previously presented information it is clear that RBC and platelets have a significant role in T-cell proliferation, function, and phenotype and their presence should be accounted for. Currently, density gradient or filter-based leukapheresis is the most common method of initially isolating a patient's T-cells [42]. Leukapheresis is a process in which leukocytes are separated from a patient's blood for use in CAR-T cell production and the remaining blood is transfused back into the patient after washing and removal of anticoagulants [42]. Density gradient separation is commonly used in leukapheresis to isolate WBCs, either manually or in an automated closed system such as the Spectra Optia [42, 44]. Due to the extensive steps and manipulations involved in CAR T-cell production, automated closed systems are extremely attractive. Here we will compare three representative separation methods

that are either currently in use as a closed system for CAR T-cell manufacturing or have the potential to be integrated into one. These methods are the Spectra Optia MNC, CliniMacs Prodigy, and the microfluidic device created by Ramchandraiah's group. This selection is meant to represent the overall separation approaches of density gradient, magnetic separation, and microfluidic separation respectively. A comparison of performance metrics between these three methods can be found below in Table 3.

Table 3. Comparison of T-cell Isolation Methods for CAR T-cell Production

Method	Throughput	Recovery %	T-cell Purity	RBC contamination	Platelet contamination
Spectra Optia MNC[44, 45]	43 ml/min	~55%	~78%	12 to 1	44 to 1
CliniMacs[46, 47]	2.75 ml/min	~55%	~89%	915 to 1^1, or 12 to 1^2	50 to 1^3
Microfluidics[38]	1 mL/min	~53%	~91%	0.19 to 1^4	450 to 1

Red blood cell and platelet contamination values are estimates of the worst-case scenarios when specific data is not provided. CliniMacs can have variable RBC contamination depending on if the leukapheresis sample was isolated using density gradient separation or a leukoreduction filter respectively (1,2). The platelet contamination produced by these two methods is similar (3). The RBC contamination for the microfluidic device is estimated based on 9% of the expected remaining RBCs after lysis (4).

Indeed, many attempts at optimizing the CAR T-cell manufacturing process have already been made [48 - 52]. It has been shown that PBMC activated with anti-CD3 + anti-28 and supplemented with IL-2 exhibited a high proliferative capacity and bolstered long-term in-vivo persistence when cultured in human platelet lysate (HPL) [48]. However, it seems that sample composition has been severely neglected in regards to CAR-T production optimizations. Recently a retrospective analysis of initial

sample composition has revealed many interesting relationships between starting sample composition and the resulting CAR product [53].

Red blood cell breakpoints for CAR-T expansion

To help evaluate the best method of separation for the production of CAR T-cells it is helpful to determine the highest number of allowable RBCs in the starting sample. The number of RBCs allowable in the starting sample for CAR T-cell manufacturing depends largely on the intended separation, activation, and culture methods. In fact, depending on these variables it may actually be beneficially to have some amount of RBC contamination. By far the most common activation method is the use of paramagnetic beads coated with anti-CD3 and anti-CD28 antibodies, accounting for roughly 65% of CAR products used in trials from 2002 to September 2017 [42]. Another 29% used anti-CD3 and anti-CD28 mAbs in conjunction with IL-2 [42]. The most important factor in relation to RBC contamination is whether the cells will be used immediately or if they will be frozen for later use, as this may cause RBCs to lose their beneficial bioactivities. Considering the latter scenario, it has been shown that RBCs enhance anti-CD3 + anti-CD28 mediated T-cell proliferation and survival up to at least 100 RBCs per PBL [6]. Thus, a ratio of up to 100 RBCs per T-cell is acceptable and even preferable when activating CAR-T cells with either of the above-mentioned methods. Of note, RBCs survival enhancement is nearly equal to that provided by IL-2 and/or IL-15 [5]. As such, it may be possible to simply replace the use of exogenous IL-2 or IL-15 with the presence of RBCs thereby reducing reagent cost.

In the case of a frozen blood product, ideally all RBCs should be removed and no more than 1.6 RBC per PBMC should be present to avoid any suppressive effects [15]. This applies even to cultures with exogenous IL-2 as blood banked RBCs do suppress IL-2 production but the addition of IL-2 does not rescue T-cell from the RBC mediated proliferation suppression [14, 15]. The effect of contamination on the resulting CD4+:CD8+ ratio should also be considered as a CAR T-cell CD4+:CD8+ ratio of 1:1 has shown enhanced effectiveness and reduced side-effects compared to studies with undefined ratios [54]. Typically, healthy humans have a CD4+:CD8+ ratio greater than 1 [55]. Elavia's group has shown that the resulting CD4+:CD8+ ratio of anti-CD3 + IL-2 activated CAR products is positively correlated with the RBC to CD3+ ratio up to 80:1 [54]. However, the analysis in this study uses both fresh blood and cryopreserved blood without differentiating between the two, so the effects of RBCs may not be accurately represented. Thus, some fresh blood RBC contamination may be useful to reduce the typical CD4+:CD8+ ratio towards the 1:1 standard.

For the use of a cryopreserved blood product the Optia MNC can expect significant proliferation reduction as it produces about 12:1 RBC to lymphocyte ratio [44]. For fresh blood the Optia MNC will see optimal proliferation and survival benefits from its amount of RBC contamination. CliniMacs Prodigy uses a leukapheresis product similar to the Optia MNC, but with the additional step of magnetic selection of T-cells, which further removes RBCs and platelets while enriching target T-cells. CliniMacs Prodigy is unaffected by the decision to store RBCs or use them immediately, as it has too few RBCs in its starting sample to see either the negative effects of thawed RBCs or the positive effects of fresh RBCs.

CliniMacs Prodigy also has the special advantage of specific cell selection, allowing it to consistently produce products of a defined CD4+:CD8+ ratio as demonstrated by Turtle et al**.** [54]**.** This makes CliniMacs Prodigy a good choice for when the flexibility to use frozen or fresh blood is needed or when a defined CD4:CD8 is preferred. As Ramachandraiah's device uses pre-lysed blood diluted with a resulting lymphocyte purity of 90%, it can be expected to have much lower than a 1.6 RBC to T-cell ratio, thus avoiding any negative RBC-mediated effects from a frozen blood product. Additionally, Ramachandraiah's device can be used with varying dilutions of whole blood, modulating the RBC contamination to potentially take advantage of its immunoregulatory effects, at the cost of separation efficiency. There is also the possibility of the addition of immunomagnetic beads for CD4+ and CD8+ isolation during microfluidic filtration as shown by Lin et al, potentially allowing it to produce a defined CD4+:CD8+ ratio [56].

Platelet breakpoints for CAR-T expansion

Another often overlooked variable in CAR-T cell production is the number of platelets in the starting culture sample. As explained previously, low concentrations of PF-4 can have some beneficial effects for T-cell cytokine secretion but quickly turns sour when the concentration exceeds 1.25 ug/ml [20, 24]. As such the potential concentration of PF-4 released by platelets should be considered when determining the maximum acceptable number of platelets in the starting sample. It may also be important to consider platelet activation before or during separation as this has the potential to release PF-4 that might persist after these platelets are removed. A coculture of 250:1 platelet to CD4+ cells can expect about a 30% platelet activation

rate and a release of 13 ng/ml PF-4 in 48 hours. Thus, if 13 ng/ml PF-4 is released by 75 activated platelets per T-cell, then about 7,200 platelets per T-cell would be needed to reach the 1.25 ug/ml threshold. This is more than 10x the ratio of platelets to lymphocytes commonly seen in whole blood [17]. Thus, no separation method will produce nearly enough PF-4 to suppress T-cell proliferation or function.

However, there are still concerns with platelet contamination, as 250:1 platelet to CD4+ cells are still enough to suppress proliferation significantly, although not through PF-4 effects. While this concentration decreases proliferation, it also increases Th1 differentiation, Th17 differentiation, Treg activation, IL-2, IL-10, IL-17, TNF-α, and IFN-γ. It is not known if this increase in Th1/Th17 activation would persist past 48 hours, or if it would significantly regress as in the case of high PF-4 contamination. Some increase in cytokines can be seen with as few as 15 platelets per CD4+ cell, as IFN-γ still saw a significant increase at level of contamination. If this number of platelets can affect cytokine production, they may also be able to affect T-cell proliferation. While it isn't clear at what ratio platelets start to reduce T-cell proliferation, more than 250:1 platelet to lymphocyte ratio should be absolutely avoided, and less than 15:1 should be preferred. As a cautionary measure, exogenous IL-2 should be added to any cultures as it will preemptively counteract any residual PF-4 contamination and provide beneficial survival and stimulatory effects.

Optia MNC produces a platelet to lymphocyte ratio of about 44:1, which is well below the 250:1 threshold but still enough to possible see some minor modulation to cytokine production and T-cell proliferation. As with RBCs, CliniMacs Prodigy's additional magnetic separation step further reduces platelet contamination to

significantly below that of the Optia MNC. This avoidance of platelet-mediated immunoregulatory effects further solidifies the CliniMacs Prodigy's position as the reliable method to produce consistent CAR T-cells. However, since both Optia MNC and CliniMacs use a traditional leukapheresis product, they may be susceptible to left over PF-4 released by platelets activated during leukapheresis. Microfluidic devices have the unique advantage of potentially creating their own leukapheresis product that might activate fewer platelets. Ramachandraiah et al. did not specify how many platelets remain after separation in their device. Since this device separates cells based on size, and platelets are smaller than lymphocytes, it can be reasonably assumed that many platelets will be contaminating the lymphocyte sample. However, Shevkoplyas et al. have demonstrated a microfluidic device design capable of removing more than 86% of platelets with a 1 ml/min flowrate [57]. This corresponds to a maximum platelet to lymphocyte ratio of roughly 60 to 1. Simply running these two devices in series will produce a cell sample with similar platelet contamination to the Optia MNC.

Cell isolation methods during CAR T-cell manufacturing

Overall, there are many factors that need consideration when deciding on how to isolate lymphocytes for CAR T-cell manufacturing. Common automated leukapheresis methods that use density gradient separation, such as the Optia MNC, represent a cheaper option that may have more variability in its product, as it is more susceptible to the effects of RBC and platelet contamination. Methods that employ additional separation after leukapheresis, such as the CliniMacs Prodigy, represent a resource heavy top-notch option, that will produce more consistent and defined

products. Microfluidic devices, represent a possible direction for the automation of leukapheresis for CAR T-cell production, being both cheap and adaptable to a desired outcome. If these microfluidic devices can reach closed-system commercialization, they may become the top candidate for automated separation and manufacturing of CAR T-cells. However, as it stands now, closed systems like the CliniMacs Prodigy are the most effective way to automate this manufacturing process, while typical leukapheresis is the go-to when resources or circumstances do not allow for the expensive option.

CHAPTER VI. FUTURE CONSIDERATIONS

A fundamental base of information has been uncovered about the immunoregulatory effects of RBCs and platelets, but there are still many aspects that warrant further investigation. While some form of anti-CD3/anti-CD28 is by far the most popular activation method, most RBC contamination studies use PHA for activation, making the results difficult to apply to common situations. Additionally, no studies present data with more than 100 RBC per PBMC, thus it is unknown at what ratio past 100:1 will RBC's benefits reverse or even become a hindrance. This information would give a clearer breakpoint for the maximum allowable number of RBCs in culture, at least for fresh blood. For cryopreserved blood, we know that suppression begins at 1.6 RBC per PBMC, but the exact mechanism that causes RBCs to lose their beneficial bioactivities is still unclear. Furthermore, much focus has been given to RBC's effects on proliferation and survival, yet their modulation of T-cell cytokine production has been mostly overlooked [7]. On the other hand, the immunoregulatory effects of platelet and PF-4 contamination has focus primarily on CD4+ cells while leaving a major lack of data on CD8+ cells. The exact relationship between platelet and PF-4 contamination remains to be elucidated, as somewhat contrary evidence has been reported on their effects. If these problems can be solved there will be a clear understanding of the effect RBC and platelet contamination on T-cell culture.

In relation to RBC and PLT contamination, the future of blood separation points to microfluidic devices, and their amazingly adaptable design that allows for precise tuning of the sample composition. This will allow researchers and clinicians to

take full advantage of the immunoregulatory effects of RBC and platelet contamination has on T-cell culture. With the current and possible future knowledge of these effects researchers will be able to choose a microfluidic device tuned to their specific requirements. With the addition of magnetic cell selection during microfluidic separation, it is easy to envision a microfluidic-based closed system that can produce a consistent and defined CAR T-cell product while utilizing RBCs and platelets to potentially bolster cell expansion and function.

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