EVALUATION OF PRE-STORAGE LEUCOREDUCTION EFFECT ON PRO AND ANTI-TUMOUR CYTOKINES IN WHOLE BLOOD DURING STORAGE

SRLY BINTI SAMAN

UNIVERSITI SAINS MALAYSIA

2024

EVALUATION OF PRE-STORAGE LEUCOREDUCTION EFFECT ON PRO AND ANTI-TUMOUR CYTOKINES IN WHOLE BLOOD DURING STORAGE

by

SRLY BINTI SAMAN

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

August 2024

ACKNOWLEDGEMENT

In the name of Allah, the Most Beneficent, the Most Merciful.

Alhamdulillah, this project was finally completed. First and foremost, I would like to extend my utmost gratitude towards my supervisor, Dr. Sharifah Azdiana Tuan Din, for her guidance, support, and tremendous flexibility. Her patience and countless assistance led to the completion of this study.

I would like to address my sincere thanks to my co-supervisor, Dr Rafeezul Mohamed, for his ideas, advice, help, supervision, support, and trust throughout my master journey. I would also like to acknowledge Dr. Siti Mardhiana Mohamad and Dr. Muzirah Musa for her skillful statistical assistance.

I wish to thank the staff of the Immunology, Haematology and Transfusion Medicine Unit from Clinical Diagnostic Laboratory for their guidance and facilities. Special thanks to Nordiyana Ishak, Halianis Mohd Yusoff, and NurHafizah Hamzah for help, support and sharing their knowledge throughout the entire process of completing my research project.

Last but not least, thanks to my beloved husband and family for their love, aspiration, countless sacrifices, and unconditional support and tolerance.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AABB	American Association Blood Bank
ANOVA	Analysis Of Variance
APC	Allophycocyanin
CMV	Cytomegalovirus
DCs	Dendritic Cells
DF	Donor Filtered
DN	Donor Normal
DNA	Deoxyribosonucleic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme-linked Immunosorbent Assay
EPO	Erytropoietin
FBC	Full Blood Count
FCS	Flow cytometry standard
FDA	Food and Drug Administration
FITC	Fluorescent Isothiocyanate
FL	Fluorescence Intensity
FNHTRs	Febrile Non-Haemolytic Transfusion Reaction
FSC	Forward Light Scatter
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3- Phosphate Dehydrogenase
HBV	Hepatits B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatits C Virus
HLA	Human Leucocyte Antigens
IFN-γ	Interferon Gamma
IL	Interleukin
LR	Leucoreduction

LIST OF APPENDICES

PENILAIAN KESAN PENGURANGAN SEL DARAH PUTIH SEBELUM PENYIMPANAN KE ATAS PARAS SITOKIN PRO DAN ANTI KANSER DI DALAM DARAH UTUH YANG DISIMPAN

ABSTRAK

Kehadiran sel darah putih (SDP) dan protein lain seperti sitokin dalam sel darah merah (SDM) semasa penyimpanan mungkin memberi kesan kepada pesakit kanser yang memerlukan pemindahan darah. Kajian ini bertujuan untuk menentukan baki SDP dalam SDM selepas proses penapisan SDP dan perubahan pada paras sitokin yang berlaku semasa penyimpanan SDM. Sebanyak 31 sukarelawan terlibat di dalam kajian ini. Tiga ratus lima puluh mililiter darah telah diambil daripada setiap sukarelawan tersebut. Separuh daripada isipadu darah (175 mL) telah melalui proses penapisan, manakala separuh lagi (175 mL) tidak ditapis dan digunakan sebagai kawalan. Kiraan darah penuh (KDP) dilakukan menggunakan Sysmex KX-21, Jepun dan baki subset SDP diukur menggunakan FACSCanto™ II, Beckton Dickinson, USA. Paras sitokin daripada beg plasma kawalan dan beg darah ditapis pada hari ke-0, 10, 20, dan 30 diukur dengan menggunakan kaedah ELISA. Subkumpulan limfosit T-helper (Th) dan ekspresi gen dalam sampel kawalan dianalisis menggunakan tindak balas rantai polimerase masa nyata. Terdapat perbezaan yang ketara di dalam min semua parameter KDP dan baki subset SDP antara kedua-dua kumpulan ini ($p < 0.05$). Paras IL-2 dan IL-6 tidak dikesan di dalam kedua-dua sampel. Terdapat perbezaan yang ketara pada paras TGF-β dan IFN-γ di antara sampel kawalan dan juga sampel darah ditapis pada hari 0 dan juga hari ke 10. Paras TGF-β menunjukkan peningkatan sehingga hari ke 30. Ekspresi gen Tbet, GATA3 dan Fox3p juga dapat dikesan dalam sampel kawalan. Proses penapisan menunjukkan kesan terhadap parameter darah, tahap kandungan

sitokin dan ekpresi gen di mana ia menunjukkan kepentingannya di dalam darah utuh semasa tempoh penyimpanan.

EVALUATION OF PRE-STORAGE LEUCOREDUCTION EFFECT ON PRO AND ANTI-TUMOUR CYTOKINES IN WHOLE BLOOD DURING STORAGE

ABSTRACT

The presence of white blood cells (WBCs) and cytokines in whole blood (WB) during storage may have effect on cancer patients requiring blood transfusions. This study aims to determine the residual of WBCs in WB following leucoreduction and changes in cytokine levels which occur during storage of WB. A total of 31 donors were recruited in this study. Three hundred fifty millilitre of whole blood (WB) was withdrawn from each donor. Half of the blood volume (175 mL) were leucoreduced (LR), while the other half (175 mL) were not leucoreduced and used as a control. Full blood count (FBC) was done using Sysmex KX-21, Japan and residual WBCs subsets were measured using FACSCanto™ II Beckton Dickinson, USA. Cytokines levels from plasma non LR and LR bags on day 0, 10, 20, and 30 days was measured by ELISA method. T-helper (Th) lymphocyte subgroups and gene expression were analysed in the non-LR samples by real-time polymerase chain reaction, respectively. There was a significant difference in the mean of all FBC parameters and residual WBCs subsets between these two groups ($p < 0.05$). Levels of IL-2 and IL-6 were not detected in both bags during storage. There was a significant difference in TGF-β and IFN-γ between non-LR and LR on day 0 to day 10. TGF-β level showed an increase up to day 30. T-bet, GATA-3, and Foxp3 gene expression were detected in non-LR samples. Leucoreduction demonstrated a significant effect on blood parameters, cytokine levels, and gene expression, emphasizing its importance in WB storage.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Blood transfusion is the infusion of both soluble and cell-associated forms (red blood cells (RBCs), white blood cells (WBCs), and platelets into a recipient (Vamvakas & Blajchman, 2001). In general, it is an acute intervention, implemented to solve life and health-threatening conditions (Tsai *et al.*, 2010). However, a blood transfusion may cause transfusion reactions to the patient, as well as in cancer patient even if serological tests for infection diseases have been performed. The leucocytes in blood components can mediate febrile transfusion reactions (C. C. Chang *et al.*, 2018), induce alloimmunisation of human leucocyte antigen (HLA) in transfusion recipients (Saris *et al.*, 2018), and transmit other cell-associated pathogens such as cytomegalovirus (CMV) (Ainley & Hewitt, 2018). The 2020 Hemovigilance report disclosed that Malaysia experiences a higher incidence of mild allergic reactions, febrile transfusion reactions, and uncommon complications during transfusions are more prevalent in Malaysia (*Pusat Darah Negara Ministry of Health Malaysia*, 2023). Additionally, a study conducted in the northern region suggested the implementation of pre-storage leucoreduction as a preventive measure against adverse transfusion outcomes (Ros *et al.*, 2021). At the same time, the presence of proteins such as cytokines found in the blood component itself may also lead to reactions in the patient undergoing transfusion. During storage, a study has shown elevated levels of cytokines including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta (IL-1β), and IL-8, as well as regulated on activation, normal T cell expressed and secreted (RANTES), in pre-leucoreduction (LR) RBCs compared to post-LR RBCs (Chang et

al., 2018). Furthermore, IL-22 and the transforming growth factor-beta (TGF-β) were detected from day 21 until day 42 in non-LR RBCs, (Bal et al., 2018).

During an inflammatory response, cytokines are released into the affected tissue initiating a cascade of events. Pro-inflammatory cytokines such as TNF-α, IL -1β, and IL-6 promote inflammation by increasing vascular permeability, attracting immune cells to the site of inflammation, and activating these cells to release additional cytokines and inflammatory mediators (Zhang & An, 2007; Sut *et al.*, 2017a).

In cancer, different types of cytokines in the tumor microenvironment can have a significant impact on disease progression. Some cytokines are associated with promoting tumor growth and metastasis, while others have antitumor effects. For example, pro-inflammatory cytokines such as IL-6 and TNF-α have been shown to promote growth by stimulating angiogenesis, suppressing immune responses, and promoting cancer cell survival (Cata *et al.*, 2013; Nguyen *et al.*, 2014). Although transfusion-related immune modulation (TRIM) is well established, its influence on immune competence in the recipient and its effects on cancer recurrence remains unknown (Ramírez *et al.*, 2013).

Furthermore, the effect of cytokine levels in plasma which is not separated from RBCs during the storage period at 4˚C for 3 days, found that no significant degradation to cytokine levels occurred (Vincent *et al.*, 2019). Similarly, there was a study where they discovered that despite using different types of anticoagulants for samples, cytokine levels in plasma remained stable during storage at 4˚C (Hennø *et al.*, 2017). According to a study by Sut et al., the release of cytokines and lipid factors by the donor's leucocytes during RBCs storage can activate neutrophils. To prevent this, LR can reduce the release of metabolites and cellular components into the RBCs product (Sut *et al.*, 2017a). Moreover, the previous studies revealed the cytokines in

WB was higher than the cytokines in buffy coat depleted RBCs during the storage period up to day 21 (Shukla *et al.*, 2015; S. U., 2018).

Adverse transfusion events manifest in the presence of leucocytes, particularly T cells. T-lymphocytes, or T-cells, are integral to cell-mediated immunity, constituting the predominant lymphocyte population such as, in febrile reactions and transfusion-associated graft-versus-host disease (TAGVHD) (Dasararaju & Marques, 2015). The involvement of T cells in both febrile non-hemolytic reactions and transfusion-associated graft-versus-host disease (TAGVHD) stems from their recognition of foreign antigens present in transfused blood. Upon encountering these antigens, recipient T cells trigger an immune response, resulting in the release of inflammatory cytokines such as IL-1, IL-6, and TNF-α (Sut *et al.*, 2017b). In TAGVHD, donor T cells in transfused blood perceive recipient tissues as foreign due to HLA disparities, causing tissue damage and organ dysfunction. (Kleinman & Stassinopoulos, 2018) . Thus, the activation of T cells is pivotal in both mediating febrile reactions and contributing to the pathogenesis of TAGVHD.

Additionally, T-cells also are pivotal in cancer, impacting its onset, advancement, and response to therapies, notably immunotherapy. CD4+ T-cells assist other immune cells and are divided into subsets like helper and regulatory T-cells, while CD8+ T-cells target harmful cells including cancer cells (DeNardo *et al.*, 2009) (Lee *et al.*, 2022). Furthermore, transcription factors like T-bet and GATA-3 regulate the development and function of T-cells. T-bet facilitates CD4+ T-cells to adopt characteristics akin to helper T-cells, whereas GATA-3 facilitates their transition towards regulatory T-cells (Palmer *et al.*, 2006).

Several approaches have been considered to prevent adverse reactions associated with transfusions. One such approach involves the transfusion of leucocyte-

reduced RBCs concentrates, as highlighted by (Sharma *et al.*, 2017; Eldesouky *et al.*, 2019); where LR leaves a leucocyte count of < 5 x 10^6 per unit (99.9%, or a 3 log, reduction)(Hart *et al.*, 2015). Additionally, several studies, have emphasized the importance of pre-storage LR blood components in preventing cytokine accumulation, thereby reducing the incidence of febrile non-haemolytic transfusion reaction (FNHTRs), HLA alloimmunisation, TRIM, and the transmission of WBC-associated viruses (Sut *et al.*, 2017b; S. U., 2018).

In Malaysia, there is an increasing demand for LR blood components, particularly among patients needing multiple transfusions mainly thalassaemia patients (*TRANSFUSION PRACTICE GUIDELINES for Clinical and Laboratory Personnel*, 2016). These patients are potentially at risk of acquiring alloimmunisation and transfusion-transmitted infection through transfusion. The Hemovigilance report in 2017 from the National Transfusion Medicine Service in Malaysia suggested the use of leucocyte-reduced blood components to reduce adverse transfusion events associated with WBCs. However, there is a recommendation for further research on the cost-effectiveness of incorporating this practice into routine transfusions (HAEMOVIGILANCE_REPORT_2016-2017_PDN). On the contrary, pre-storage LR is mandatory in Canada and in most of Europe but only recommended by the FDA in the USA due to cost constraints (FDA,2012).

Furthermore, there is a lack of research on gender-specific subsets of WBCs reduction on leucoreduction methods in our country. The existing study focused solely on T cell subtype differences between genders (Chin *et al.*, 1993) (Al-nuri & Noh, 2020). Therefore, looking at the existing reports, this study was conducted to look at the gender differences in LR among donors and these differences will provide information on the efficiency of the LR based on gender. Additionally, this study also

assesses how the removal of buffy coat affects the efficiency of LR blood and the percentage of haemolysis in WB. Moreover, it examines the residual WBCs levels and cytokine concentrations in both non-LR and LR blood bags. Furthermore, this study analyzes the impact of cytokines on extended storage duration, aligning with recommendations by AABB (American Association Blood Banks) and Malaysia's guidelines (*TRANSFUSION PRACTICE GUIDELINES for Clinical and Laboratory Personnel*, 2016) , a 35-day storage period for WB. The purpose is to prevent transfusion reactions in the recipient because of these cytokines being present in the WB.

1.2 Problem Statement

The impact of LR on blood components has been studied, encompassing its effects on WB during storage, with aims to remove the WBCs to prevent a transfusion reaction among the recipient. In Malaysia, only selective patient received LR blood, for example patients who require multiple blood transfusions such as transfusion dependent thalassaemia patients (*Pusat Darah Negara Ministry of Health Malaysia*, 2023). Studies have shown that side effects due to blood transfusion such as febrile non-haemolytic reactions, allergic responses, and infections can occur in cancer patients when transfused with unfiltered blood components due to the presence of WBC-produced cytokines (C. C. Chang *et al.*, 2018). This cytokine has the potential to stimulate normal cells such as tumor-associated macrophages (TAM) and endothelial cells, prompting them to generate additional cytokines that facilitate the progression of malignancy (Wang et al., 2019). Moreover, cytokines circulating in the bloodstream play a pivotal role in regulating the growth and spread of cancer. Cancer cells also have the capability to secrete cytokines, serving as autocrine signals or

paracrine signals on supporting tissues such as fibroblasts and blood vessels, thus creating a favourable environment for cancer progression.

At present, there is no available data on adverse transfusion outcomes for cancer patients, regardless of whether they receive non - LR or LR blood, in Malaysia. Moreover, there is a lack of study examining gender-based differences in the reduction of WBCs subsets in WB within the country. The existing study only offers insights into T cell subtypes based on gender (Al-nuri & Noh, 2020). Additionally, a comprehensive analysis to examine cytokine levels across various blood components in Malaysia has yet to be conducted. Therefore, this study is designed to identify the level of cytokines in stored blood and to determine whether non-LR and LR WB has a significant association with duration of WB storage. The results of this study may help improve clinical transfusion practices among cancer patients by determining whether they should receive LR blood or non-LR blood.

1.3 Research Hypothesis

1.3.1 Null Hypothesis (Ho)

Ho: There are no differences in the quality indicator variables before and after filtration of WB in the full blood count and haemolysis percentage, residual of WBCs and the level of cytokines in a different storage days of LR and non-LR WB, and corelations between gene expression of WBCs in non-LR samples.

1.3.2 Study Hypothesis (Ha)

Ha: There are differences in the quality indicator variables before and after filtration of WB in the cell blood count and haemolysis percentage, residual of WBCs and the level of cytokines in a different storage days of LR and non-

LR WB, and corelations between gene expression of WBCs in non-LR samples.

1.4 Research Objectives

General:

To evaluate the quality indicator variables such as FBC, WBCs subtypes and level of haemolysis, before and after filtration of WB and the level of cytokines in a different storage days of LR and non-LR WB.

Specific:

i. To compare the haemoglobin level, total WBCs count, platelet count, and percentage of haemolysis in pre and post LR stored WB
ii. To compare the level of cytokines between LR and non-LR stored WB (Day 0, Day 10, Day 20, Day 30).
iii. To compare gene expression of T cell subtypes between gender in non-LR WB.

CHAPTER 2

LITERATURE REVIEW

2.1 Blood donation

Blood transfusion has emerged as a routine medical procedure widely employed in the care of hospitalized individuals, entailing the intravenous infusion of blood or its components (*Singer et al*., 2015). The transfusion of blood products plays a crucial role in restoring the body's oxygen-carrying capacity and replenishing depleted or deficient blood components. However, blood transfusion does entail certain risks for recipients, including the transmission of transfusion-transmitted infections (TTIs), acute or delayed transfusion reactions, alloimmunization, and immunomodulation (Clevenger & Kelleher, 2014). Moreover, the blood donation is crucial to global healthcare, with over 100 million units donated annually. It supports patients undergoing surgery, trauma care, chronic illness management, and cancer treatment, and can also serve as therapeutic phlebotomy for conditions like hereditary hemochromatosis and polycythemia vera (Drew *et al.*, 2017). The practice evolved significantly from Karl Landsteiner's identification of ABO blood groups in the early 20th century (Crow *et al.*, 2000). Initially hindered by clotting issues and the need for immediate donor-recipient connection, the onset of World War I spurred advancements in blood storage and transfusion methods. Adding citrate to prevent clotting and glucose to preserve red blood cells allowed for the first "blood banking" by Captain Oswald Hope Robertson, enabling stored blood to be used later, thus revolutionizing blood donation and transfusion practices (Tubei, no date) (Rous & Turner, no date).

The blood donation process begins with registration and screening, where donors provide identification and undergo a medical history review to determine eligibility. After pre-donation counselling, donors proceed to the donation area where blood is collected by a trained healthcare professional. Following donation, donors receive refreshments and are observed for potential side effects. All procedures adhere to the guidelines outlined by Malaysia (*TRANSFUSION PRACTICE GUIDELINES for Clinical and Laboratory Personnel*, 2016). In Malaysia, blood donation is centered on voluntary non-remunerated sources (Ling *et al.*, 2018). On this basis, the total number of blood donations from mobile sites, blood collection centers, and hospitals increased by 3.4% from 653,124 to 675,315 cases between 2013–2014. However, despite the increase in the number of blood donors in Malaysia, there is still a severe shortage of blood with decreasing donor population and increasing demand (Wooi Seong *et al.*, 2014). The World Health Organization (2016) stated that blood donation rates are 33.1, 11.7, and 4.6 donations per 1000 people for high, middle and low-income countries respectively, the current Malaysian scenario translates to only 22.5 donations per 1000 people (Ling *et al.*, 2018).

Meanwhile, the autologous donation, where patients donate their own blood ahead of surgery, was prevalent from the 1980s to 1990s due to fears of transfusion-transmitted infections like HIV and hepatitis C (Vassallo *et al.*, 2015). Enhanced safety measures have since reduced these risks, decreasing the popularity of autologous donations. However, it remains useful for patients with common alloantibodies, ensuring compatible blood supply and reducing risks of alloimmunization and allogeneic infections (Vassallo *et al.*, 2015).

2.2 Storage of RBCs

Longer storage time generate storage lesion alterations in RBCs, which may affect recipients. There is no standard definition for fresh or old blood that may be used to clinically distinguish a substantial storage lesion. However, many studies that looked at the impacts of RBCs storage age classified fresh blood or new blood as having a storage age of less than 7 or 14 days (Naeem *et al.*, 2021), while older blood had a storage age of more than 14 or 21 days(D'Alessandro *et al.*, 2017).

During storage, RBCs undergo gradual time-dependent functional and structural changes collectively known as the "storage lesion," including storage-induced changes in RBCs viscoelastic properties (Yoshida *et al.*, 2019). Stored RBCs may also contain pro-inflammatory cytokines such as interleukin IL-1, IL-6, TNF-α, neutrophil-activating factor, and chemoattractant IL-8, residual plasma proteins, immunoglobulins, platelets (PLTs), microparticles, immunomodulators, and procoagulant phospholipids (Grimshaw et al., 2011; Martinez & Fedda, 2019). Furthermore, the degradation in red blood cells (RBCs) significantly contributed to their apparent cancer-promoting effects (Antonelou *et al.*, 2012). It was observed that both autologous and allogeneic blood transfusions accelerated cancer progression if stored for more than nine days. Conversely, fresh blood, whether allogeneic or syngeneic, showed no detrimental effects (Atzil *et al.*, 2008).

An association between RBCs transfusions and improvements in immune systems in individual had documented almost 40 years ago. However, there is evidence that allogeneic RBCs transfusions cause immunomodulation, which can lead to infectious morbidity and unfavourable oncologic outcomes (Deeb *et al.*, 2020). Furthermore, the allogeneic transfusion of RBCs capable to downregulates cellular immunity and dysregulates innate inflammation immunity. This effect occurs by

increased IL-10 and IL-4 cytokine secretion after transfusion by stimulation of T helper 2 cell (Th2) pathway in allogeneic RBCs while the Th1 response is subsequently downregulated by IL-2 (Pandey *et al.*, 2010; Obrador *et al.*, 2015). Transfusion of RBCs also contributes to pro-inflammatory effects by priming neutrophil activity and increasing the release of inflammatory cytokines like IL-6, IL-8, and IL-22 (Muszynski *et al.*, 2017; Remy *et al.*, 2018). The pro-inflammatory and immunosuppressive effects of RBC transfusion may be significant for critically ill and surgical patients if excess inflammation and immune suppression are associated with adverse effects (Muszynski *et al.*, 2017). A study has shown that cytokine accumulation over RBCs storage time is correlated with transfusion-mediated systemic inflammatory reactions and increased bacterial infection probability (Deeb et al., 2020).

2.3 Transfusion related adverse reaction and cancer patient

Transfusion reactions refer to adverse events linked to the transfusion of whole blood or its components, indicating an undesirable response observed in patients, usually happening shortly after blood or its components are administered (Jolee Suddock & Crookston Affiliations, no date). These reactions, often referred to as transfusion-associated adverse reactions (TAARs), manifest within hospital settings and encompass symptoms such as fever, chills, rigors, dyspnoea, hypotension, itching, and urticaria, varying in severity from mild to potentially life-threatening (Sahu *et al.*, 2014). Among these, febrile nonhemolytic transfusion reactions (FNHTRs) are prevalent, characterized by fever (with a body temperature elevation of 1°C or above 38°C, accompanied by chills and rigors, typically resolving without intervention (C.-C. Chang *et al.*, 2018). Studies indicate FNHTRs occurring at a frequency of 0.5–6.8%

across transfused blood units, with leukocytes and cytokines within blood units implicated in their occurrence (C.-C. Chang *et al.*, 2018) (Sahu *et al.*, 2014) . Notably, the prevalence of FNHTRs has been notably diminished in pre-leukoreduced (LR) red blood cell (RBC) transfusions compared to post-LR RBC transfusions, as per prior investigations (Rajesh *et al.*, 2015).

The administration of blood products not only induces immune suppression but also enhances cancer cell proliferation and metastasis through accumulated angiogenic and oncogenic factors during storage (Guo *et al.*, 2014). Despite a recent decrease in perioperative blood transfusions in colorectal and prostate cancer patients, rates remain notably high (Boehm *et al.*, 2015). Studies show that allogeneic blood transfusions in gastrointestinal surgery decrease NK cell count and increase mortality risks (Guo *et al.*, 2014), with similar adverse effects observed in liver resection and ampullary cancer patients (Liu *et al.*, 2013). Meta-analyses reveal heightened mortality and cancer recurrence risks among patients receiving allogeneic RBCs perioperatively, particularly in lung cancer cases (Luan *et al.*, 2014).

2.4 Evolution of leucoreduction techniques

The original leucocyte depletion filter, designed by Diepenhorst and published in 1972, employed sterile cotton wool as a filtering medium to reduce leucocytes in transfusion blood, with a capacity to retain approximately 1 log of WBCs (Diepenhorst & Engelfriet, 1975). Then, the cellulose acetate filters were subsequently identified as more suitable alternatives. The emergence of second-generation filters, facilitated by red cell washing, centrifugation, buffy coat removal, freezing and deglycerolizing of red cells, and blood component collection through apheresis technology, promoted the

retention of approximately 3 log leucocytes, demonstrating verified efficacy in preventing non-haemolytic febrile reactions (NHFR) (Novotny *et al.*, no date).

Currently, third or fourth-generation filters, with pores ranging from 5–50 micrometres, remove more than 99.9–99.99% (>3 log) of leucocytes originally present in donated blood, meeting the current quality standards for haemocomponents (Ferdowsi *et al.*, 2021). In Malaysia there are various commercial leucoreduced including the BioR flex (Fresenius), LeucoLab filter (MacoPharma), In line leucocyte's filter system (JMS), and Leucocyte reduction filter (Puri Blood), are available.

2.5 The leucoreduction (LR) process.

Since last decades, the removal of WBCs from blood components is an effective way in preventing transfusion related adverse reactions such as acute haemolytic, septic (bacterial contamination), transfusion-related acute lung injury (TRALI), and FNTRs. LR or leucofiltered blood is the reduction of WBCs concentration in blood components, namely RBCs, platelet concentrates (PC), and plasma obtained from the fractionation of whole blood or apheresis. Freshly collected RBCs are passed through a filter that uses two mechanisms to achieve LR, named the barrier filtration and cell, and adhesion. It separates cell types based on their sizes using a barrier filter. Platelets and deformable RBCs can pass through modern LR filters, but leucocytes do not. According to research by Schuetz et al (2004), leucocyte retention is also affected by cellular adhesion that occurs between filter medium and leucocytes (Schuetz *et al.*, 2004). An overview of the new application of leucocyte reduction filters (LRFs) is shown in Figure 2.1

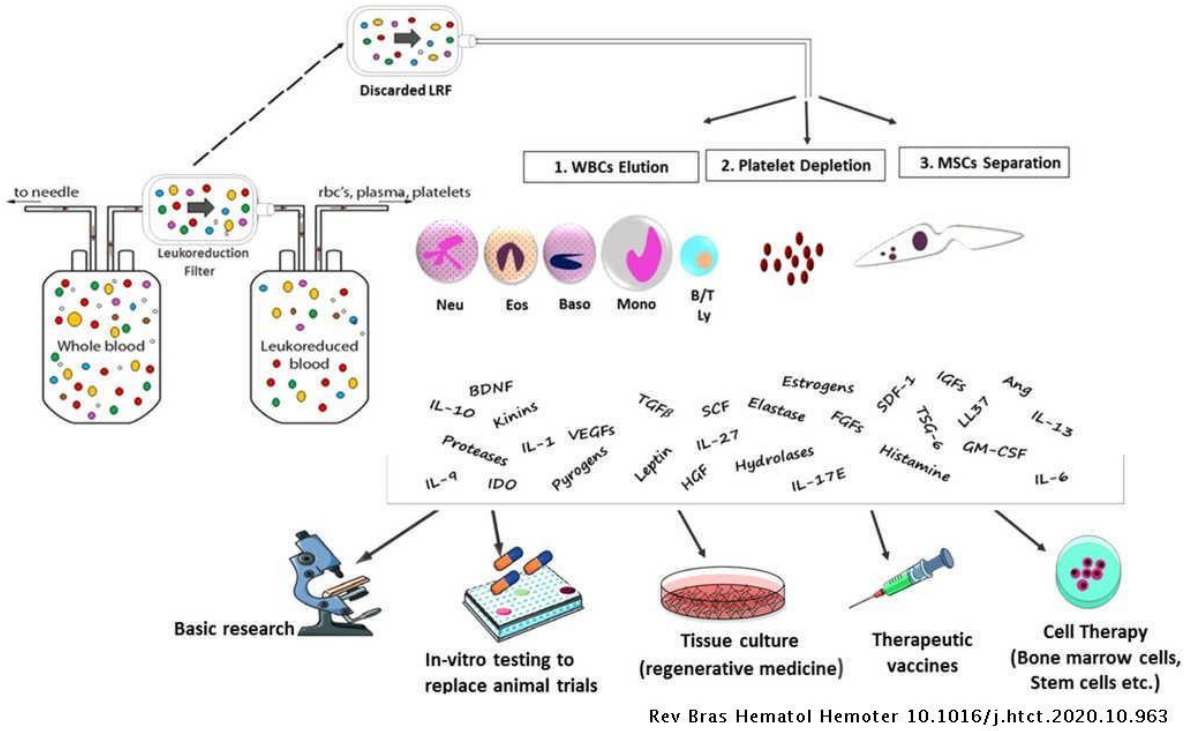

Figure 2.1 Overview of the new application of Leucocyte Reduction Filters (LRFs). Discarded LRFs are an economic source of viable functional cells and bioactive molecules that can play a role in basic research, animal models, cell therapy and, tissue engineering. (Adopted: Rev Bras Hematol Hemoter 10.1016/j.htct.2020.10.963)

B/T Ly: lymphocytes B and T, LRF: leuco-reduction filter, Mono: monocytes, RBCs: red blood cells, WBCs: white blood cells, BDNF; Brain-Derived Neurotrophic Factor, FGFs; Fibroblast Growth Factor, HGF; Hepatocyte Growth Factor, IGFs; Insulin-Like Growth Factor, VEGF Vascular Endothelial Derived Growth Factor, TGF-β, IL-6, IL-10; IL-10, IL-27; IL-27, IL-17E; IL-17, IL-13; IL-13, IL-9; IL-9, IL-1, Ang; Angiopoietin, GM-CSF; Granulocyte-Macrophagee CSF, SCF; Stem Cell Factor, SDF-1; Stromal Cell-Derived Factor 1, TSG-6; (TNF)-Stimulated Gene-6.

During the LR process, the entire blood bag is gently and thoroughly mixed before being hung upside down on a drip stand. The hang-up system is set up so that the LR RBCs bag is beneath the bag filter shown in Figure 2.2.

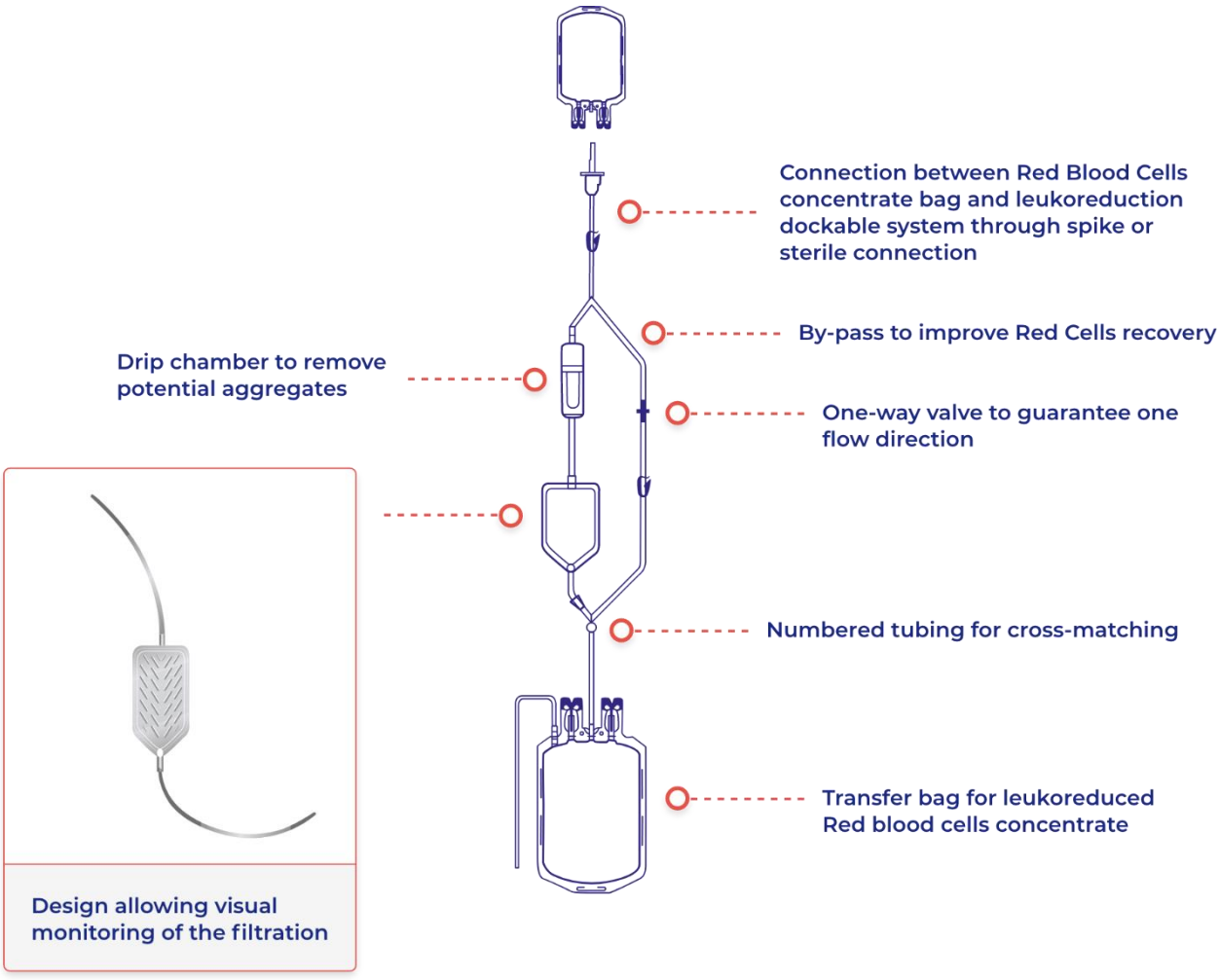

Figure 2.2 The process of filtration using Marcopharm leucofilter. Adopted from: www.marcopharm.com

In the filtration process, RBCs are allowed to flow into the transfer bag by gravity until there is no more blood dripping in the drip chamber. The WBCs are trapped by the LR filter, which is then discarded. RBCs and other essential blood components are collected in a new bag (at the bottom). More than 99.99 percent of donor leucocytes can be removed with today's technology. There are many LR methods, but the process is currently carried out using selective LR filters, which allows less than 1×10^6 WBCs residuals to be obtained in an RBCs or PC unit. Suggested pre-storage blood leucocyte count should be $< 5 \times 10^6$ per unit (99.9 percent, or 3 logs, reduction) (Hart *et al.*, 2015).

Evaluating the leucocytes content in blood products is crucial to verify the completion of the leucoreduction (LR) process. There are several methods to measure the efficacy of this technique, one of them is to use a flow cytometer analyser. Flow cytometry is an effective method in medical research because of its ability to analyse thousands of particles with multiple physical features of a single cell as they flow in a fluid stream through a beam of light in a short period without causing physical damage to the cells (Villas, 1998). Flow cytometry can be used to measure WBCs residual in WB and identify WBCs subtypes in WB using monoclonal antibodies against the surface antigens of the WBCs (Dzik & Moroff, 2000; Bashir & Cardigan, 2003). Monoclonal antibodies are identical immunoglobulins formed from a single clone of B-cells. These antibodies can recognise unique epitopes on a single antigen, or binding site (Akagi *et al.*, 2018). To detect a unique marker on a cell, monoclonal antibodies conjugated with one or more fluorescent dyes (fluorochromes) are used such as fluorescent isothiocyanate (FITC), phycoerythrin (PE), or pyridine chlorophyll protein (PerCP), which absorbs energy from the laser. Lasers are used as light sources in flow cytometers, producing both scattered and fluorescent light signals that are read by

detectors such as photodiodes or photomultiplier tubes. These signals are converted into electronic signals, which are then analyse and written to a data file known as flow cytometry software (FCS).

Multiparameter data corresponding to forward light scatter (FSC), side scatter (SSC), and relative fluorescence intensity (FL) are obtained from flow cytometric analysis of a single cell suspension and are set for the optimal detection of FITC, PE, PerCP, and allophycocyanin (APC). FSC and SSC parameters can be used to categorize the cells shown in Figure 2.3.

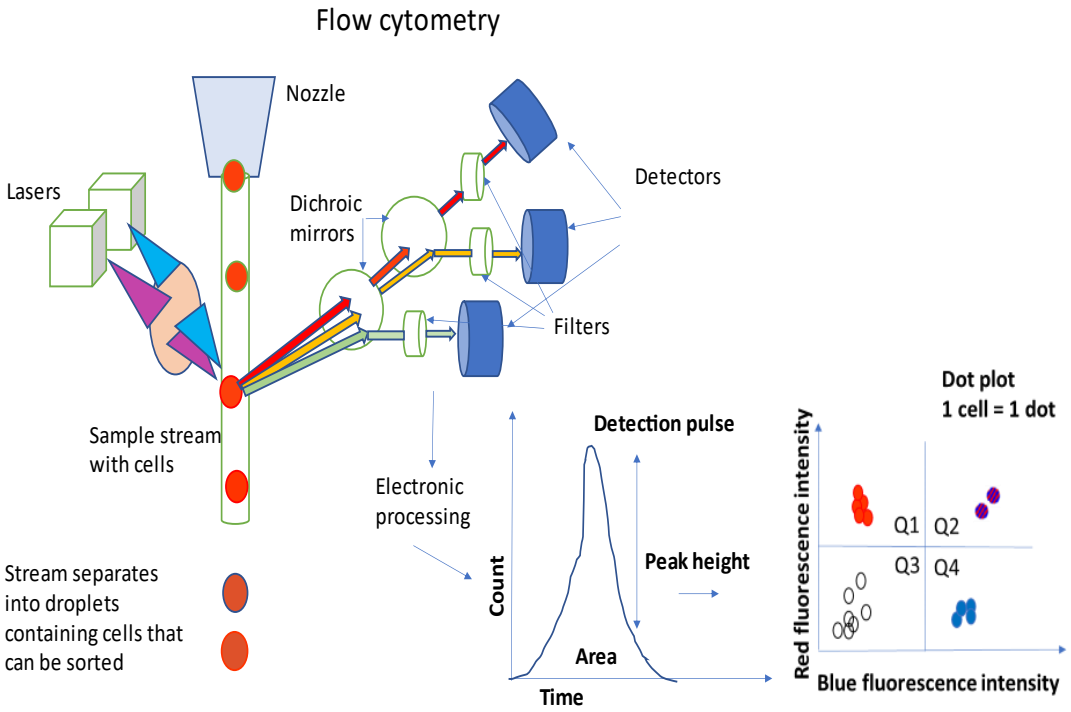

Figure 2.3 Basic principles of flow cytometry. Scattered light will be collected from multiple angles and at right angles to the laser beam axis by the laser beam. (Adapted from Introduction to Flow cytometry, Becton Dickinson, BD 2008)

The FSC is related to the cell size while the SSC is proportional to the granularity and complexity of the cells. FSC and SSC measurements that are correlated allow cell types to be distinguished in a heterogeneous cell population shown in Figure 2.4.

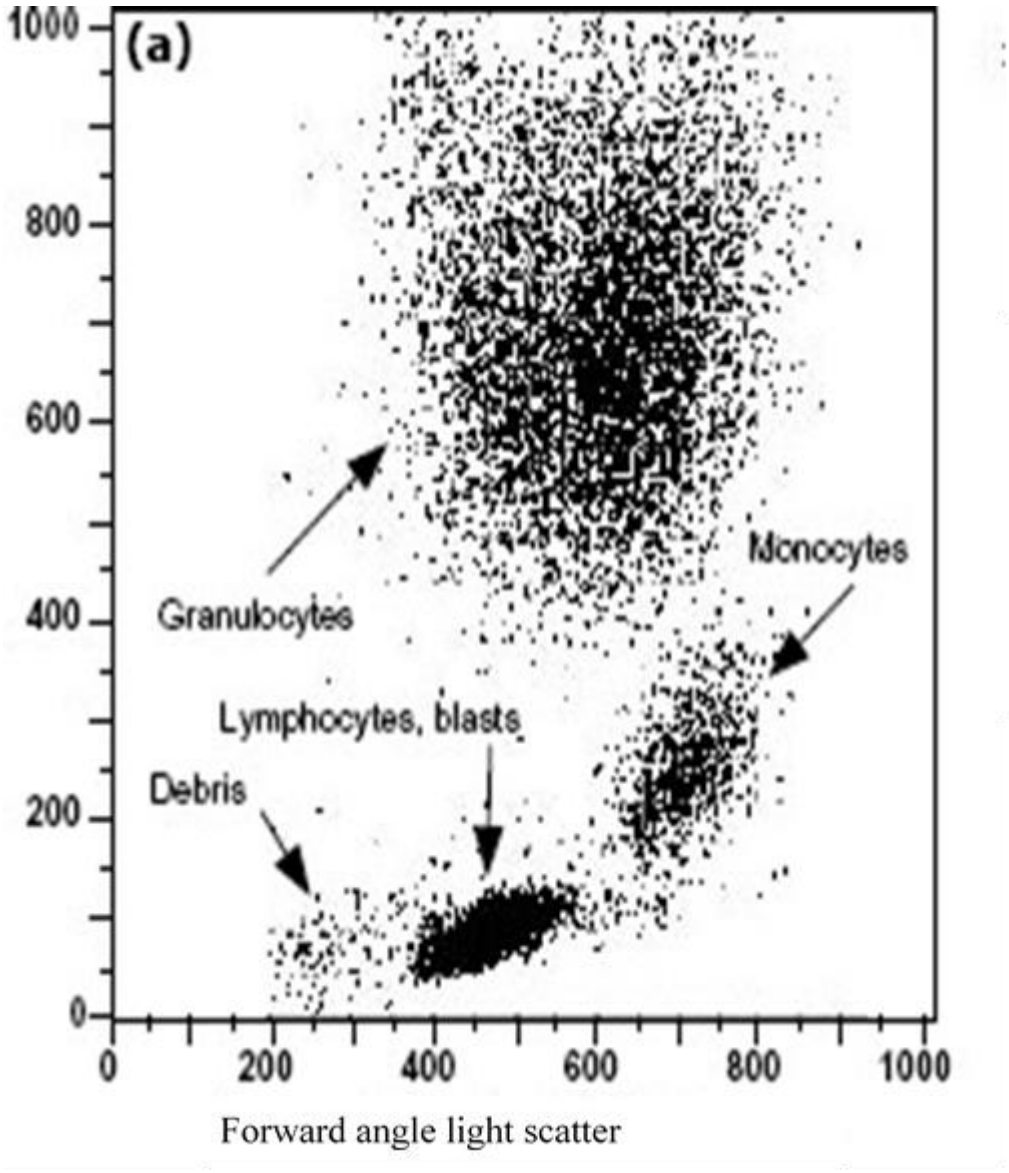

Figure 2.4 Dot plot of forwarding light scatter (FSC) on the X-axis and side scatter (SSC) on the Y-axis. These two physical parameters plot the size and density of the analysed cells on the X- and Y-axis, respectively. (Adapted from Introduction to Flow cytometry, Becton Dickinson, BD 2008)

2.6 Overview of WBCs

WBCs, also known as leukocytes or leucocytes, are immune system cells that help to protect the body from both infectious disease and foreign intruders. Leucocytes can be found in all parts of the body, including the blood and lymphatic systems. The WBCs has nucleus distinguishes them from anucleated red blood cells and platelets. The leucocytes are classified according to their structure (granulocytes or agranulocytes) or cell lineage (myeloid cells or lymphoid cells). These categories are further subdivided into five types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes based on their physical and functional characteristics (LaFleur-Brooks, M., 2008). WBCs have a lifespan of 13 to 20 days before being destroyed in the lymphatic system.

Granulocytes represent approximately 65% of all WBCs and is derived from the enormous number of granules found in their cytoplasm. Basophils (0.5-1% of granulocytes) have granules with intense blue staining; eosinophils (3-5%) have granules with red stain, and neutrophils (90-95%) have granules that are unstained. Granulocytes circulate in the blood and migrate into tissues, especially during inflammatory conditions. While monocytes make up about 5% to 10% of all circulating WBCs and have a limited lifespan of about 24 hours in the blood, they have a single nucleus and abundant granular cytoplasm and are bigger than neutrophils and lymphocytes.

Although it was previously thought that monocytes and lymphocytes were the primary sources of cytokines in blood cells, cytokines also can be produced by a variety of other cell types as well. Since neutrophils are the first cell type to encounter and interact with the etiologic agent in an inflammatory context, a stimulus-specific neutrophil response in terms of cytokine production may aid in certain types of

inflammatory reactions. Recent studies revealed neutrophils can produce many types of cytokines as shown in Figure 2.5, where neutrophils can have a role in physiological processes including haematopoiesis, angiogenesis, and wound healing (Coffelt *et al.*, 2016; Ley *et al.*, 2018), as well as pathological conditions like inflammatory, viral, autoimmune, and neoplastic illnesses (Tecchio *et al.*, 2013; Tamassia *et al.*, 2018).

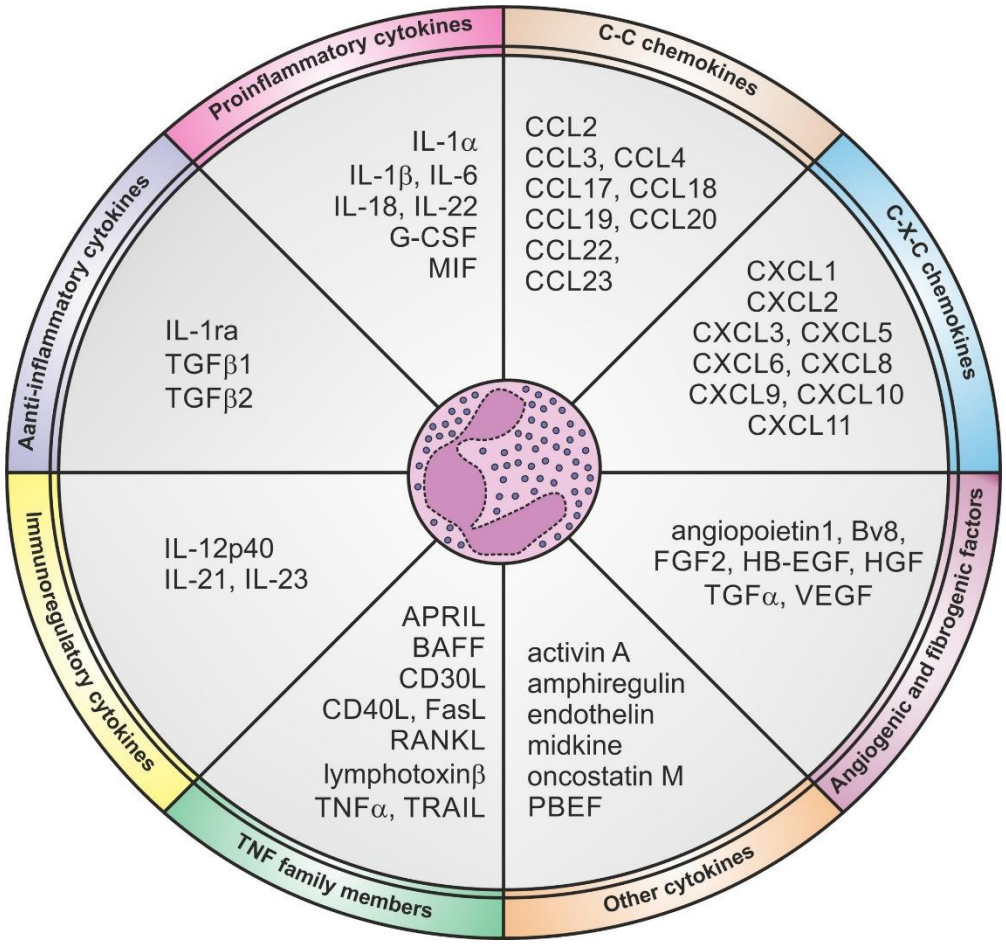

Figure 2.5 Human neutrophils have been found to express and release a variety of pro-and anti-inflammatory cytokines in vitro and in vivo, especially when stimulated appropriately. It demonstrates that neutrophils have a role in a range of physiological and pathological processes, including hematopoiesis, angiogenesis, wound healing, autoimmune and neoplastic illnesses, and, of course, acute inflammatory diseases (Tamassia *et al.*, 2018).

Lymphocytes represent 25-35% of WBCs and their name derived from their close relationship with the lymphatic system. The lymphocytes are classified into two types: B and T, which are around in proportions of 1:5 in the blood. The T lymphocytes can be divided into two groups based on the expression of lymphocyte-specific cell surface markers: CD4+ and CD8+. Normal T cells in the peripheral blood express either CD4 or CD8; hence, T lymphocytes in the peripheral blood can be classified as CD3+CD4+ or CD3+ CD8+ leucocytes. Because monocytes also express low amounts of CD4 and some NK cells express CD8, the presence of CD4 or CD8 by itself is insufficient to classify a peripheral blood leukocyte as a T lymphocyte. For the immunophenotypic enumeration of T cells, co-expression of CD3/CD4 or CD3/CD8 is required. Furthermore, CD4+ cells are also known as T helper cells. When activated, they divide quickly and secrete cytokines that regulate or aid the immune response (Dong, 2021). These cells can differentiate into one of several subtypes, Th1, Th2, T follicular helper cells (Tfh), T helper 17 cells (Th17), regulatory T cells (Treg) each with a distinct function (Zhu *et al.*, 2010; Kirchenbaum *et al.*, 2019).

Monocytes are a heterogeneous population of cells that circulate in the blood for up to 3 days before differentiating into tissue macrophages or myeloid dendritic cells upon recruitment to various human tissues (Boyette *et al.*, 2017). These monocytes can be further classified into three distinct subsets based on their expression of phenotypic markers: classical (CD14+CD16−), inflammatory or intermediate (CD14+CD16+) monocytes (Zawada *et al.*, 2011). CD14, a crucial glycosylphosphatidylinositol (GPI)-anchored protein, plays a vital role in the innate immune response by recognizing and binding to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) from gram-negative bacteria (Wu

et al., 2019). In gastric cancer, CD14 is implicated in increasing tumor invasiveness through the activation of E-cadherin (Li *et al.*, 2013).

Meanwhile, CD66b, also known as CEACAM8, is a glycoprotein belonging to the carcinoembryonic antigen (CEA) family and serves as an activation marker for human granulocytes, notably eosinophils and neutrophils, with prominent expression on human peripheral blood eosinophils (Yoon *et al.*, 2007). This glycoprotein is localized in specific granules, acting as a surface marker for their exocytosis, indicating its pivotal role in granulocyte activation and function (Schmidt *et al.*, 2015). Its expression level is heightened during infections and inflammatory conditions like bacterial sepsis and acute dengue virus infection, underscoring its significance in the immune response and granulocyte activation (Opasawatchai *et al.*, 2019). Notably, CD66b-positive tumor-infiltrating neutrophils (TINs) are implicated in the tumor microenvironment, with their association with immune checkpoints suggesting a role in the prognosis of lung cancers (Shen *et al.*, 2021). Figure 2.6 illustrates what happens when bacteria invade the body. The innate immune system, depicted in yellow, springs into action right away. Cells like macrophages and neutrophils detect the bacteria and gobble them up in a process called phagocytosis. They also release signals called cytokines (shown as triangles) to call other immune cells to the infection site. Complement proteins are also activated to help destroy the bacteria. Meanwhile, in the green area, special T cells with innate-like qualities get activated. These cells, like γδ T cells or natural killer T cells, spot specific patterns linked to bacterial infections. They pitch in by producing cytokines and directly killing infected cells, acting as a bridge between the innate and adaptive immune responses. As the infection continues, the adaptive immune system enters in B cells in the orange area make antibodies that latch onto the bacteria's surface proteins, flagging them for destruction by other

immune cells and complement proteins. Additionally, various T cells, such as helper T cells and cytotoxic T cells, get activated to help organize the immune response and eliminate the bacteria-infected cells (Kirchenbaum *et al.*, 2019).

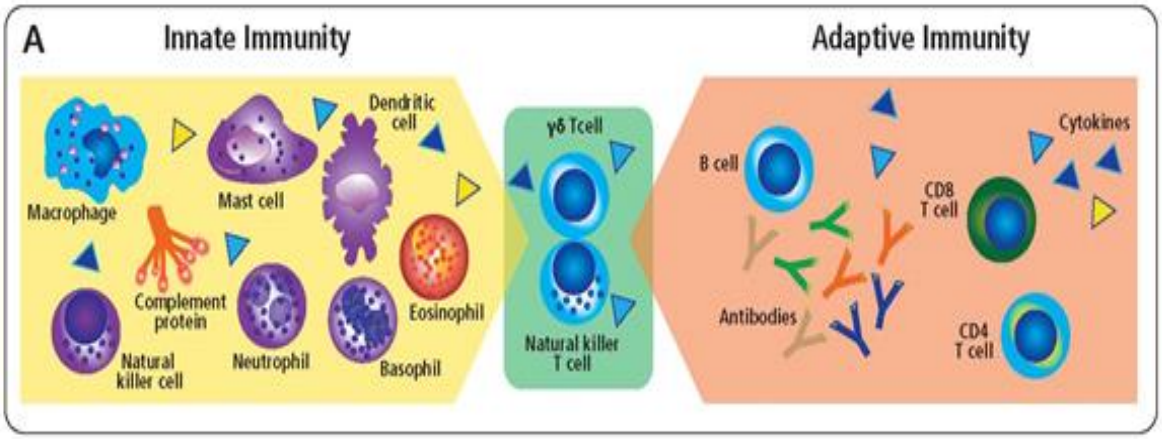

Figure 2.6 The human immune system has two parts: innate and adaptive. Innate cells and molecules are in yellow, adaptive cells and molecules are in orange, and in green, there are special T cells that link the two parts together (Kirchenbaum *et al.*, 2019).

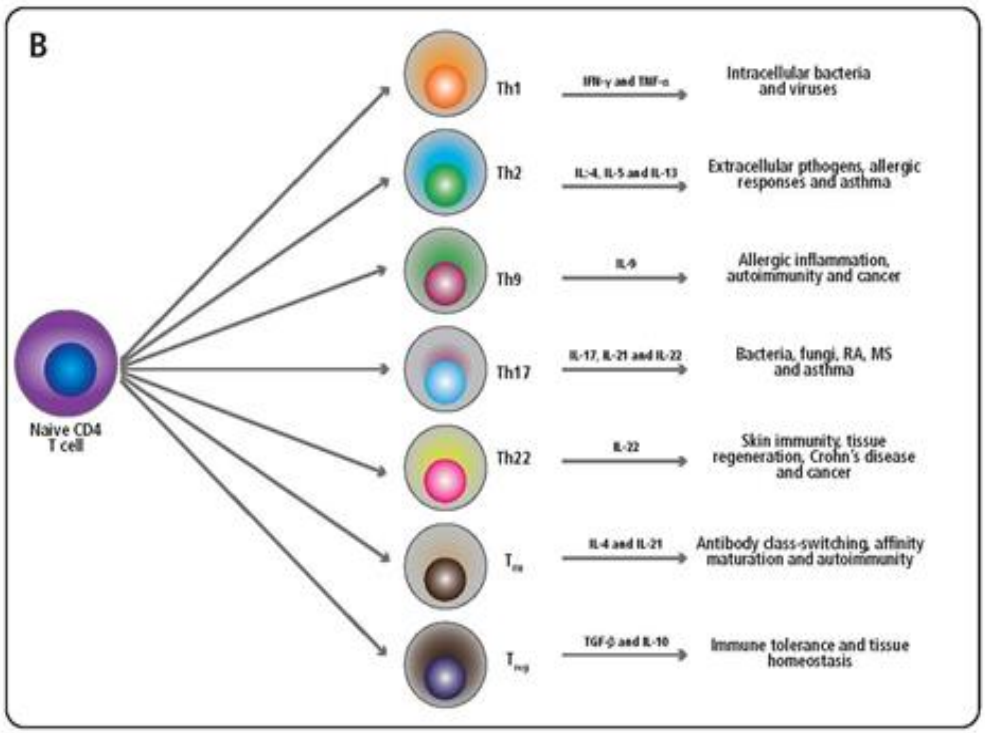

Figure 2.7 Schematic of CD4+ T cell subsets, including their principal cytokine signature and association with specific immune responses (Kirchenbaum *et al.*, 2019)

Figure 2.7 shows that T cells are directed into specific subtypes by cytokines. T lymphocytes play an important role in human defense against infectious organisms and some cancers. They are responsible for the elimination of nascent malignancies as well as intracellular pathogens such as viruses and bacteria, as well as the regulation of adaptive immune responses. T lymphocytes break down the cancerous cells or infected cells, trigger inflammatory reactions, produce, and secrete cytokines, which are soluble intercellular mediators. Th1 responses can regulate anti-tumor mechanisms that limit cancer progression, both directly and indirectly where Th1 cells can directly kill tumor cells by releasing high levels of IFN-γ, TNF-α, and cytolytic granules. Furthermore, the induction of tumor-immune surveillance programs by Th1 cells, Treg, and Th2 cells have the capacity to induce alternative activation states of