ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

CLONING, PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF)

M.Sc. THESIS

Cansın KIRMAÇOĞLU

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JANUARY 2021

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

CLONING, PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF)

M.Sc. THESIS

Cansın KIRMAÇOĞLU
(521171104)

Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Prof. Dr. Gizem DİNLER DOĞANAY

JANUARY 2021

ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

GRANÜLOSİT-KOLONİ UYARICI FAKTÖRÜ'NÜN (G-CSF) KLONLANMASI, ÜRETİMİ, SAFLAŞTIRMA VE KARAKTERİZASYONU

YÜKSEK LİSANS TEZİ

Cansın KIRMAÇOĞLU
(521171104)

Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı
Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı

Tez Danışmanı: Prof. Dr. Gizem DİNLER DOĞANAY

OCAK 2021

Cansın KIRMAÇOĞLU, an **M.Sc**. student of **İTU Graduate School** student ID 521171104, successfully defended the thesis/dissertation entitled "**CLONING, PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF)**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor :	**Prof. Dr. Gizem DINLER DOĞANAY** İstanbul Technical University	.....
Jury Members :	**Assoc. Prof. Halil BAYRAKTAR** İstanbul Technical University	.....
	Assoc. Prof. Demet CANSARAN DUMAN Ankara University	.....

Date of Submission : 26.11.2020

Date of Defense : 20.01.2021

To my family…

FOREWORD

First and foremost, I would like to express my appreciation and gratitude to my supervisor Prof. Dr. Gizem DİNLER DOĞANAY for giving me the opportunity of being a member of her research team. I am grateful for her tremendous support, inspiring guidance, and motivation during my thesis.

Secondly, I would like to thank all of my previous and current GD Lab members for their help, support and patience. As much as I have gained so many decent friendships, I also have improved my scientific knowledge and perspective in this research team.

Additionally, I would like to thank TUBITAK (The Scientific and Technical Research Council of Turkey) for financially supporting me from the 1007 project, number 115G078.

I would like to also thank my dear friend Sera ZAFER for her cheer, support, and friendship not only during my thesis but also throughout my entire life. I am so lucky to have a friend who I can call as sister. I am also, really grateful to Derya YAVUZ for her friendship. She always supported me during this thesis.

Last but not least, I have to extend my greatest thanks and love to my family, my mother Neşe KIRMAÇOĞLU and my father Hakkı İlhan KIRMAÇOĞLU, for their unconditional love, constant support and patience. I would not have done anything without their support and encouragement.

January 2021 Cansın KIRMAÇOĞLU

TABLE OF CONTENT

Pages

ABBREVIATIONS

ANC : Absolute Number of Neutrophils in the Circulation
APS : Ammonium persulfate
cDNA : Complement Deoxyribonucleic Acid
CD : Circular Dichroism
CLP : Common Lymphoid Progenitor
CMP : Common Myeloid Progenitor
CRH : Cytokine Receptor Homology
CSF : Colony Stimulating Factors
CSF3 : Granulocyte Colony Stimulating Factor gene
DC : Dendritic Cells
DMEM : Dulbecco's Modified Eagle Medium
DNA : Deoxyribonucleic Acid
G-CSF : Granulocyte Colony Stimulating Factor
G-CSF-R : Granulocyte Colony Stimulating Factor Receptor
GM-CSF : Granulocyte-Macrophage Colony Stimulating Factor
h-G-CSF : Human Granulocyte Colony Stimulating Factor
HCS : Haemopoietic Stem Cell
HGF : Hematopoietic Growth Factors
HPLC : High Performance Liquid Chromatography
IL : Interleukin
IPTG : Isopropyl-β-D-Thiogalactopyranoside
JAK : Janus Kinases
kDa : Kilodalton
LB : Luria-Bertani medium
LPS : Lipopolysaccharide
M-CSF : Macrophage Colony Stimulating Factor
MPO : Myeloperoxidase
mRNA : Messenger Ribonucleic Acid
NET : Neutrophil Extracellular Traps
NFκB : Nuclear Factor-κB

NKC : Natural Killer Cells
NSC : Neural Stem Cell
PBS : Phosphate Buffered Saline
PCR : Polymerase Chain Reaction
pH : Power of Hydrogen
RNA : Ribonucleic Acid
rRNA : Ribosomal Ribonucleic Acid
SCN : Severe Congenital Neutropenia
SDS : Sodium Dodecyl Sulfate
SDS-PAGE : Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEC-HPLC : Size Exclusion-High Performance Liquid Chromatography
SOCS3 : Suppressor of Cytokine Signaling 3
STAT : Signal Transducers and Activators of Transcription
TBS : Tris-Buffer Saline
TBS-T : Tris Buffer Saline-Tween 20
TEMED : Tetramethylethylenediamine
TNFα : Tumor Necrosis Factor-α
T_{REG} : Regulatory T-cells
UV : Ultraviolet
U87-MG : Uppsala 87 Malignant Glioma
VEGF : Vascular Endothelial Growth Factor
WT : Wildtype

SYMBOLS

°C	**:** Celcius
bp	**:** Base Pair
kDa	**:** Kilodalton
L	**:** Liter
M	**:** Molar
mg	**:** Milligram
min	**:** Minute
ml	**:** Milliliter
mM	**:** Millimolar
OD	**:** Optical Density
rpm	**:** Revolutions per minutes
µg	**:** Microgram
µL	**:** Microliter
µM	**:** Micromolar

LIST OF TABLES

Page

LIST OF FIGURES

Page

CLONING, PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF GRANULOCYTE-COLONY STIMULATING FACTOR (G-CSF)

SUMMARY

Hematopoiesis is a crucial process for producing white blood cells in the body. Hemopoietic Stem Cells (HSC) differentiate into a variety of blood cells. The common myeloid progenitor cells, which are destined from HSC, differentiate into mast cells as well as granule carrying granulocytes called an eosinophil, basophil, and neutrophil. The differentiation process of HSC to a variety of blood cells is strictly regulated by many small proteins called cytokines; such as interferon, interleukin, and growth factors. Generation of different cells lines at different rates is depending on the cellular demand and homeostasis. Therefore, each cell has some unique cytokine that maintain its homeostasis; for instance, Colony Stimulating Factors (CSF): Macrophage CSF, Granulocyte-Macrophage CSF, Granulocyte CSF, and Multi potential-CSF, and IL-3.

Neutrophils, the most abundant type of leukocytes in the circulation, are the first cells that arrive at the inflamed or the infected tissue and fight against the invaders such as bacteria, viruses, and fungi. These phagocytic cells have unique morphology: they have lobulated nuclei and granules that carry many anti-bacterial enzymes, proteases, proteins to assist movement of the neutrophils. They phagocyte the foreign cells and destroy them by either using those granules or forming superoxide radicals. Besides, neutrophils destroy them by exocytosing them or their proteins such as DNA proteins granules to the environment. Furthermore, granules and these proteins may form Neutrophil Extracellular Traps (NETs) to trap invaders. Therefore, they are significantly essential in innate immunity.

Differentiation of neutrophils from HSCs is strongly modulated by a number of cytokines, yet one of the most important of these cytokines is Granulocyte Colony Stimulating Factor (G-CSF). It is a small mature protein composed of 174 amino-acids with 4 α-helixes and 2 disulfide bonds and harbors an O-glycosylation site. Each of the disulfide bonds are required for the biological activity of G-CSF. G-CSF dimerizes with a cytokine receptor, G-CSF receptor (G-CSFR), with a stoichiometry of 2:2 (ligand-receptor). When G-CSF dimerizes with its receptor, a number of signaling pathways including JNK/STAT, PI-3K/AKT, and Ras/MAPK are activated. By those pathways, survival and proliferation of neutrophils is increased, development of neutrophils from progenitor cells are induced, and their migration towards blood circulation from the bone marrow is stimulated. On the other hand, suppressor of cytokine signaling 3, SOC3, is a negative regulator of the G-CSF signaling pathway. In addition to neutrophils, G-CSF has an impact on immune system cells such as T-cells, dendritic cells, monocytes. In addition to this, they also have an impact on nervous system cells such as neurons, astrocytes on various processes, as in the case of regulating T_{REG} cell and Type II dendritic cell production, cytokine production, and neuropoiesis, respectively.

Although there is a strictly controlled homeostasis of neutrophils, their levels in blood might be low in particular conditions. This abnormally low number of neutrophils in the body has been clinically named as neutropenia. Neutropenia can be the main disease, such as severe congenital neutropenia, or may develop as a secondary outcome due to a disease such as drug-induced neutropenia and chemotherapy-induced neutropenia. Moreover, sometimes neutropenia can be seen with elevated fever, in other words, febrile neutropenia. Either way, neutropenia causes failure of innate immunity when the body faces an infection or inflammation since there are not sufficient neutrophils to destroy invaders.

After, scientists have cloned recombinant human growth factors and elucidated their significant roles for the production of blood cells from progenitors, growth factors started to be used in treatment of hemopoietic diseases. One of those growth factors, which has been used as a therapy since 1991 by the approval of FDA, is G-CSF or in its well-known name, filgrastim in the US. This treatment was firstly used for the patients receiving chemotherapy and then developed neutropenia, which is a disease that could affect the duration and severity of chemotherapy of the patients. Therefore, filgrastim is one of the main biologic medicines which has many biosimilars globally

In line with the above explanations, this thesis aimed to obtain active human G-CSF from human brain glial U87-MG cell line and construct the pET-30a(+)::hG-CSF plasmid for prokaryotic systems. The production of hG-CSF using a *Escherichia coli* strain, BL21 (DE3) and purification of it from inclusion bodies by a simple method in ÄKTA Avant chromatography systems were other goals. We also intended to confirm and compare the identity and purity of G-CSF with the reference product by peptide mapping, intact mass analysis, capillary electrophoresis, SEC-HPLC, circular dichroism and host cell protein assay methods.

For this purpose, the G-CSF gene *CSF3* has been cloned from the brain glioblastoma U87-MG cells, placed into the pET-30a(+) cloning vector and in conclusion created a pET-30a(+) ::hG-CSF plasmid. *E. coli* BL21(DE3) strain has selected as the host for the production with IPTG induction. The best growth conditions for this cell strain with pET-30a(+) ::hG-CSF plasmid has been determined.

This experiment has also shown that hG-CSF stays at the inclusion bodies in the cell lysate. Thereafter, inclusion bodies have been washed with wash buffer, solubilized with solubilization buffer and refolded with step-wise dialysis afterwards. The purification of target protein from protein lysate is achieved by cation exchange chromatography while analyzed by sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and the detection of the target protein is analyzed by immunoblotting techniques. The molecular weight estimation and comparison with the reference product is achieved with intact mass analysis via LC/MS. According to this analysis, both monomer recombinant hG-CSF is similar to reference product.

The primary structure of the purified target protein is achieved by peptide mapping analysis on LC-MS/MS. As a result, *SV-8* GLU-C and digested h-GCSF and reference has same primary structure. On the other hand, secondary structure of both reference protein and purified hG-CSF was analyzed by Circular Dichroism (CD). The two spectra appeared to be the same and represented an alpha-helix rich structure.

Then, oligomeric state of both reference protein and G-CSF was detected by high-performance liquid size exclusion chromatography (HPLC-SEC) and indicated that both proteins exist as monomer in solution. On the other hand, HPLC SEC is used for concentration determination, which is estimated as 0.1 mg/ml per growth. CE-SDS

showed that our G-CSF has >95% p Host cell protein ELISA shows that, there is no significant host cell protein remained in the final G-CSF product.

The biological function of our G-CSF is tested via determination of activation of one of the proteins on the MAPK pathway, Extracellular signal-regulated kinase 1/2 (ERK), by phosphorylation on human monocytic cell, THP-1. As a result of this experiment, G-CSF product, activates the MAPK and it is an active protein.

As a result of these experiments, the cloning and purification used for hG-CSF, the main ingredient of the filgrastim drug, was successful. It was shown that the final product retained its folded form and its similarity with the reference product was demonstrated by characterization technique. Also, its biological function was tested.

GRANÜLOSİT-KOLONİ UYARICI FAKTÖRÜ (G-CSF) KLONLANMASI, ÜRETİMİ, SAFLAŞTIRMA VE KARAKTERİZASYONU

ÖZET

Hemopoez, vücudun kırmızı ve beyaz kan hücreleri üretmesi sürecidir. Uzun süre canlı kalan Hemopoietik Kök Hücreleri (HKH) çeşitli kan hücrelerine farklılaşabileme yetenekleri olan özel hücrelerdir. HKH'dan farklılaşan ortak miyeloid progenitör hücreler, mast hücrelerine veya eozinofil, bazofil ve nötrofil adı verilen granülleri taşıyan granülosit hücrelerine farklılaşırlar. HKH'nın çeşitli kan hücrelerine farklılaşma süreci, interferon, interlökin ve büyüme faktörleri gibi sitokin adı verilen birçok küçük protein tarafından sıkı bir şekilde düzenlenmektedir. Talep ve homeostaza bağlı olarak, farklı oranlarda farklı hücre hatları üretilir. Bu nedenle, her hücre tipinin kendilerini düzenleyen bir ya da birkaç sitokini vardır. Bu sitokinlere örnek olarak Koloni Uyarıcı Faktörler (CSF) olan Makrofaj CSF, Granülosit-Makrofaj CSF, Granülosit CSF ve Çoklu potansiyel-CSF verilebilir.

Kan dolaşımındaki en bol lökositlerden olan nötrofiller, iltihaplanmaş ya da enfekte olmuş dokularda bakteri, virüs ve mantar gibi istilacılara karşı savaşan ilk savunma hücreleridir. Bu fagositoz kabiliyetleri olan hücreler benzersiz bir morfolojiye sahiptir. Çok-loblu çekirdekleri vardır ve birçok anti-bakteriyel enzimi, proteazları, nötrofillerin hareketine yönelik yardımcı proteinleri ve benzeri proteinleri taşıyan granüllere sahiptir. Yabancı hücreleri fagosit hücre içine alıp bu granüllerdeki enzimler ile veya süperoksit oluşumunu fagosom ile birleştirerek yabancı hücreleri yok ederler. Ayrıca, nötrofiller granülleri ve çevreye ekositoz yapabilirler. Üstelik, DNA'daki histon proteinleri benzeri nükleik asit proteinlerini ve DNA'nın kendisini ekositoz yaparak Nötrofil Hücre Tuzakları, NET'ler oluşturup; kendileri ölürken istilacıları NET'e tuzağa düşürüp başka hücreler tarafından yok edilmesini kolaylaştırırlar. Bu nedenle, doğal bağışıklık sisteminde rol alan son derecede önemli hücrelerden birisidir.

Nötrofillerin HSC'lerden farklılaşması bir dizi sitokin tarafından güçlü bir şekilde modüle edilir, ancak bu sitokinlerin en önemlilerinden biri Granülosit Koloni Uyarıcı Faktördür (G-CSF). 4 α-sarmallı ve 2 disülfit bağı olan G-CSF, 174 amino asitten oluşan ve bir O-glikosilasyona bölgesi barındıran küçük bir proteindir. Disülfit bağlarının her biri G-CSF'in biyolojik aktivitesi için önemlidir ancak Thr^{133} bölgesindeki glikolizlenmenin bu aktivite için önemi yokturç G-CSF bir sitokin reseptörü olan G-CSF reseptörü (G-CSFR) ile 2: 2 (ligand-reseptörü) stokiyometrisi ile dimerize olurç G-CSF reseptörü ile dimerleştiğinde, JNK / STAT, PI-3K / AKT ve Ras / MAPK gibi çeşitli sinyal yolakları aktive edilir. Bu yollarla, nötrofillerin hayatta kalması ve proliferasyonu artar, nötrofillerin progenitör hücrelerden gelişimi indüklenir ve kemik iliğinden kan dolaşımına göçleri uyarılır. Nötrofillerin yanı sıra, G-CSF'nün T hücreleri, dendritik hücreler, monositler gibi bağışıklık sistemi hücreleri ile nöronlar ve astronitler gibi sinir sistemi hücrelerinde, bazı hücrelerin üretimini destekleme, sitokin üretimini destekleme nöropoezi destekleme gibi çeşitli süreçler üzerinde bir rolü bulunmaktadır.

Homeostaza göre sıkı bir şekilde kontrol edilmesine rağmen, nötrofillerin miktarları belirli koşullar nedeniyle normal durumdan daha düşük olabilir. Vücuttaki bu anormal düşük sayıdaki nötrofil durumu klinik olarak nötropeni olarak adlandırılmıştır. Nötropeni ağır konjenital nötropeni (AKN) gibi ana hastalık olabilir veya ilaca bağlı nötropeni ve kemoterapiye bağlı nötropeni gibi asıl bir hastalıktan sonra ikincil bir hastalık olarak geliştirilebilir. Üstelik bazen nötropeni yüksek ateş ile de görülebilir. Her durumda, nötropeni, vücut bir enflamasyon ya da enfeksiyon ile karşı karşıya kaldığında anlık koruma sağlayan doğuştan gelen bağışıklık sisteminde bozukluklara neden olur çünkü işgalcileri yok etmek için yeterli fagositotik nötrofil hücreleri vücutta bulunmamaktadır.

Hematopoetik büyüme faktörlerinin olgunlaşmış kan hücrelerinde ve hematopoietik kök hücrelerdeki etkileri rekombinant olarak izole edilip açıklanmaya başlayınca bilim insanları onları tedavi olarak kullanmaya da başladılar. Bu klonlama ile tedavi olarak günümüzde de kullanılan büyüme faktörlerinden biri de 1991 yılında FDA tarafından onaylanan ve Amerika Birleşik Devletleri'nde Filgrastrim adıyla bilinen G-CSF'tir. Bu tedavi ilk olarak kemoterapi alan ve daha sonra nötropeni geliştiren hastalar için kullanılmış ve günümüzde azalan nötrofil ile ilgili birçok hastalığa karşı önemli bir tedavi haline gelmiştir. Bu nedenle filgrastim, küresel olarak birçok biyobenzerine sahip olan ana biyolojik ilaçlardan biridir.

Yukarıdaki açıklamalar doğrultusunda, bu tez insan beyni glial U87-MG hücre hattından insan G-CSF sekansını elde etmeyi ve prokaryotik sistemler için pET-30a (+):: hG-CSF plazmidini oluşturmayı amaçlamıştır. Bir *E. coli* suşu, BL21 (DE3) kullanılarak hG-CSF'nin üretimi ve ÄKTA Avant kromatografi sistemlerinde basit bir yöntemle inklüzyon cisimciklerinden saflaştırılması diğer hedeflerdir. Ayrıca peptit haritalama, dekonvolut kütle analizi, kapiler elektroforez, SEC-HPLC, sirküler dikroizm ve konak hücre protein tahlil yöntemleri ile G-CSF'nin kimliğini ve saflığını referans ürünle doğrulamayı ve karşılaştırmak amaçlanmıştır.

Bu amaçla, ilk önce U87-MG hücrelerinin RNA izole edilmiş ve başarıyla izole edilen RNA'lar cDNA'ya çevrilmiştir. Bu cDNA'lerden elde edilen hG-CSF geni pET30a (+) vektörünün içine başarıyla klonlanmış, sonuç olarak bakteri sistemlerinde kullanılacak bir bir pET-30a (+)::hG-CSF plazmidi oluşturulmuştur. *E. coli* BL21 (DE3) suşu, IPTG indüksiyonlu üretim için konakçı olarak seçilmiş ve transforme edilmiştir. pET-30a (+)::hG-CSF plazmidi içeren hücreler için en iyi büyüme koşulları belirlenmiştir.

Bu deneyler ayrıca hG-CSF'nin hücre lizatındaki inklüzyon cisimciklerinde kaldığını da göstermiştir. Bu nedenle, daha sonra pellette kalan inklüzyon cisimcikleri yıkama tamponu ile yıkanmış, çözündürme tamponuyla çözünmüş ve daha sonra diyalizle yeniden katlanmıştır. Hedef proteinin protein lizatından saflaştırılması katyon değişim kromatografisi ile yapılmış, analizi sodyum dodesil sülfat jel elektroforezi (SDS-PAGE) ve hedef proteinin tespiti immünoblotlama teknikleriyle yapılmıştır.

Saflaştırılan hedef proteinin moleküler ağırlık tahmini ve referans ürünle moleküler ağırlık karşılaştırması LC -MS yoluyla intakt kütle analizi metodu ile gerçekleştirilmiştir. Bu analize göre, hG-CSF ve referans ürün 18800 Da moleküler ağırlığa sahip monomer proteinlerdir.

Saflaştırılmış hedef proteinin birincil yapısı, LC-MS / MS üzerinde peptid haritalama analizi ile elde edilmiştir. Sonuç olarak, *SV-8* Glu-C enzim ile kesilen hGCSF ve referansın aynı amino asit sekansina sahip olduğu görülmüştür. Öte yandan, hem

referans proteinin hem de saflaştırılmış hG-CSF'nin ikincil yapısı sirküler dikroizm (CD) ile far-UV (<260 nm) aralığınd analiz edilmiştir. Her iki örneğin spektrumu benzer ve alfa-sarmal açısından baskın bir olduğu gözlemlenmiştir.

Monomer olması gereken G-CSF'nin oligomerik durumu, yüksek performanslı sıvı boyutu dışlama kromatografisi (HPLC-SEC) ile saptanmış ve refereans ile karşılaştırılmıştır. Öte yandan, HPLC SEC konsantrasyon tayini için kullanılmış ve konsantrasyonu 1litre bakteri kültüründen 0.109 mg/ml olarak hesaplanmıştır. CE-SDS ise G-CSF ürünümüzün >% 95 saflığa sahip olduğunu göstermiştir. Her iki proteinin, çözelti içinde monomer olarak var olduğunu gösterilmiştir. *E. coli* konak hücre proteini ELISA tayini ile G-CSF ürününde önemli bir konakçı hücre proteini kalmadığı gösterilmiştir.

G-CSF'mizin biyolojik işlevi, insan monositik hücresi THP-1 üzerinde fosforilasyon ile MAPK yolağındaki proteinlerden biri olan Hücre Dışı sinyalle düzenlenen kinaz 1/2 (ERK) aktivasyonunun belirlenmesi yoluyla test edilir. Bu deney sonucunda G-CSF ürünü, MAPK'yı aktive eder ve aktif bir proteindir.

Karakterizasyonu tamamlanan G-CSF ürününün biyolojik işlevi, insan monositik hücresi THP-1 üzerinde, MAPK yolağındaki proteinlerden biri olan ekstraselüler sinyalle düzenlenen kinaz 1/2 (ERK) fosforilasyonla aktivasyonunun belirlenmesi yoluyla test edilmiştir. Bu deney sonucunda G-CSF ürünümüzün stimülasyonu ile THP-1 hücrelerindeki p-ERK miktarı artmıştır. Sonuç olarak kendisinin aktif bir protein olduğu gözlemlenmiştir.

Bu deneylerin sonucunda, filgrastim ilacının ana maddesi olan hG-CSF için kullanılan klonma ve saflaştırma başarılı olmuş, son ürünün katlanmış halini koruduğu ve referans ürün ile olan benzerliği karakterizasyon teknikleri ile gösterilmiştir, biyolojik fonksiyonu test edilmiştir.

1. INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis or sometimes referred to as Haematopoiesis is a term that describes blood cell formation. This name was coined by merging the two Greek words; Hemato, which means the “blood” and poiesis, which refers to “formation”. This formation involves the commitment and differentiation processes of the stem cells into different blood cell types. Hematopoiesis predominantly occurs in the bone marrow; yet, it also minorly occurs in the liver, thymus, and spleen tissues.

The development of blood cells begins initially with multipotent Hematopoietic Stem Cells, HSCs. These long-lasting self-renewal progenitor cells have a regulated quiescence and a capacity to rebuild the entire adult hematopoietic system. Yet, HSCs are rare cells, approximately one in a million cells within the bone marrow (Birbrair & Frenette, 2017; Rankin & Sakamoto, 2018). They give rise to all types of blood cells in the bone marrow; differentiate into one of the two hematopoietic progenitors, either (i) a common lymphoid progenitor cell, CLP, or (ii) a common myeloid progenitor cell, CMP. The CLP cells will differentiate into either natural killer cells (NKC) or lymphoblasts with special genetic and functional profiles. When lymphoblasts depart from the bone marrow to the bloodstream, those cells will become either naïve B-cell, naïve T-cells, or dendritic cells, DC.

Approximately two-thirds of the hematopoiesis is dedicated to myelopoiesis which is a process where common myeloid progenitor cells differentiate into the cells that circulate in the blood. (Birbrair et.al., 2017; Borregaard, 2010). Common myeloid progenitor cells, destined from HSCs, differentiate to myeloblast, and eventually, they enter either the granulocyte linage or the promonocyte linage. This granule containing granulocyte linage gives rise to either neutrophilic band cells, eosinophilic band cells, or basophilic band cells. Once band cells enter the blood circulation, they maturate and become neutrophils, eosinophils, basophils, respectively. Meanwhile, the promonocyte developed from myeloblast becomes a circulating macrophage precursor monocyte. When monocyte migrates to a tissue, it becomes tissue-resident and is

named as a macrophage. Aside from myeloblast, the common myeloid progenitor cells also differentiate either erythrocyte, mast cells, or megakaryocytes (Birbrair & Frenette, 2017; Borregaard, 2010).

Different cell lines are generated at different rates depending on the demand. In the case of inflammation or infection, the blood cell formation process increases immensely. Yet, this process is strictly regulated by many factors such as Hematopoietic Growth Factors, HGF. For instance, Erythropoietin stimulates erythrocyte production; GM-CSF acts on myeloblast to increase the number and the function of mature eosinophils, and monocytes, and neutrophils; G-CSF, stimulates the differentiation of granulocytes towards the neutrophil linage and maturation of the neutrophils (Hauke & Tarantolo, 2000). Since these factors are significant in the production of fighting cells against infection or inflammation, some of those growth factors have already been cloned and produced for clinical use by recombinant technology (Hauke & Tarantolo, 2000; Ketley & Newland, 1997).

1.1.1 Neutrophils

Neutrophils, also identified as heterophil, neutrocytes, or polymorphonuclear cells, are the first innate immune cells that have recruited and localized into the tissue from the bloodstream in response to an inflammation or infection by bacteria, fungi, and viruses. In humans, the extremely abundant leukocytes rolling in the circulation, approximately 50-70% of the leukocytes, are neutrophils (Doeing et. al., 2003). They are generated from myeloid precursors cells located in the bone marrow through several stages mentioned in Section 1.1. Although more than 10^{11} human neutrophils are being generated each day in a healthy human; they are short-lived cells, whereby following their migration to the circulation, they live approximately 1.5 hours in mice and 8 hours in humans (Borregaard, 2010). Nevertheless, a study, which was published by Pillay et. al. in 2010, suggested that the lifespan of human neutrophils is approximately 5.4 days (Pillay et al., 2010).

Even if their lifespan has some debates, once postmitotic neutrophils are produced, they are stored at the bone marrow, where this is termed as bone marrow reserve (Birbrair, 2020; Rankin, 2010). Approximately 1-2% of the neutrophils are circulating while others reside in the bone marrow reserve under normal homeostatic conditions. This release from reserve to bone marrow is strictly regulated via two CXC chemokine

receptors; CXCR2 and CXCR4. CXCR4 interacting partner, CXCL12, which is expressed by bone marrow cells are produced while CXCR2 ligands are downregulated to keep neutrophils in the bone marrow (Köhler et al., 2011; Summers et al., 2010). To release them into the circulation, CXCR4 interaction with CXCL12 is disrupted, CXCR4 production is downregulated and CXCR2 ligand on bone marrow cells is upregulated (Rosales, 2018).

Since neutrophils exhibit a rapid homeostatic turnover, there is a delicate balance between their production, storage, death, and migration to the blood circulation (Figure 1.1). Granulopoiesis, the development of neutrophils, is regulated via various transcription factors such as and growth factors. The main growth factor that influences neutrophil development is G-CSF. Under inflammation or infection, these cells that remain at storage are mobilized into the blood and the number of neutrophils increases vastly (Summers et al., 2010). This process is called emergency granulopoiesis. IL-1, tumor necrosis factor (TNF), and colony-stimulating factors stimulate the granulopoiesis and their movement to blood circulation in emergency granulopoiesis. Even-though homeostatic granulopoiesis takes place in bone marrow, emergency granulopoiesis can be supported by spleen and liver (Nauseef & Borregaard, 2014).

Along with other members of granulocytes, neutrophils have an atypical morphology. Their nucleus has segmented to multi-lobulated nuclei as they diapedesis through the endothelial cells to the tissue when the tissue has infected (Niemiec et al., 2015). Furthermore, it carries three to five lobulated nuclei and carries granules that are the key elements for fighting against pathogens. There are three forms of granules discovered in neutrophils: primary granules, secondary granules, and tertiary granules. Primary granules, which are well-known as azurophilic or peroxidase-positive granules, are the first granules that emerge during development and they are the major storage sites for proteases and toxic compounds such as cathepsins, serine proteases (neutrophil elastase) and myeloperoxidase (MPO), respectively (Witko-Sarsat et. al., 2000). It is the major microbicidal compartment; yet, they are not considered as lysosomes because they do not contain lysosome-associated membrane proteins (LAMP) on their membrane surface (Cieutat et al., 1998). Secondary granules, so-called special granules, contain antimicrobial molecules, complement activators, and enzymes (such as cathelicidin, lactoferrin, collagen, and gelatinase B). Similarly,

tertiary granules contain lactoferrin and matrix metalloprotease 9 (gelatinase B) yet they also carry proteins for supporting movement through connective tissues. In addition to granules, there are easily mobilized secretory vesicles present in the neutrophils. Upon neutrophil activation, cell adhesion proteins carried by those vesicles are transported to assist neutrophils for adhering to endothelial cells (Häger et.al., 2010; Kolaczkowska & Kubes, 2013; Lacy, 2006; Witko-Sarsat et al., 2000).

When the foreigner microorganisms intrude on physical barriers, macrophages and the resident in the tissues and endothelium itself generate signals named chemoattractants such as chemokines for endothelial cells at the infected zone to the secrete the adhesion proteins on their surfaces (Witko-Sarsat et. al., 2000). At this time, neutrophils, which are rolling in the circulation, are attached and captured by the endothelial cells by these adhesion proteins. 3 types of protein groups have a role in this attachment and capture: selectins, Intracellular Adhesion Molecules (ICAM) on endothelial cells surfaces, and leukocyte integrins. Thereafter, neutrophils are extravasated into the infected tissue through endothelial cells (Borregaard, 2010; Witko-Sarsat et al., 2000). In this tissue infiltration, for example, accumulation of the excessive amount of foreigner substances in the tissue activated neutrophils are seen in many diseases such as rheumatoid arthritis (RA), vasculitis, cystic fibrosis (CF), inflammatory bowel disease and chronic lung disease (Eyles et. al., 2006).

When neutrophils are introduced with pathogens, they can eliminate them by several antimicrobial actions (Figure 1.1). The first action is the phagocytosis of the pathogens. They are endocytosed into a formed membrane named phagosome, then those phagosomes are destroyed by either activating the reactive oxygen mechanism or using anti-bacterial proteases, such as cathepsins, lactoferrins found in their granules. Phagocytosis reduces the life-span of neutrophils because it gains plenty of toxic compounds from pathogens.

Secondly, those granules and superoxide can be secreted into the extracellular environment; therefore, proteases and toxic compounds inside the granules can execute extracellular intruders (Kolaczkowska & Kubes, 2013; Witko-Sarsat et al., 2000). This process is termed as degranulation. Moreover, these granules carry vasoactive peptides, such as histamine, which activates the endothelial cells to become leakier (Kenne et al., 2019). Therefore, this exocytosis leads to a rise in the extravasation of some immune cells.

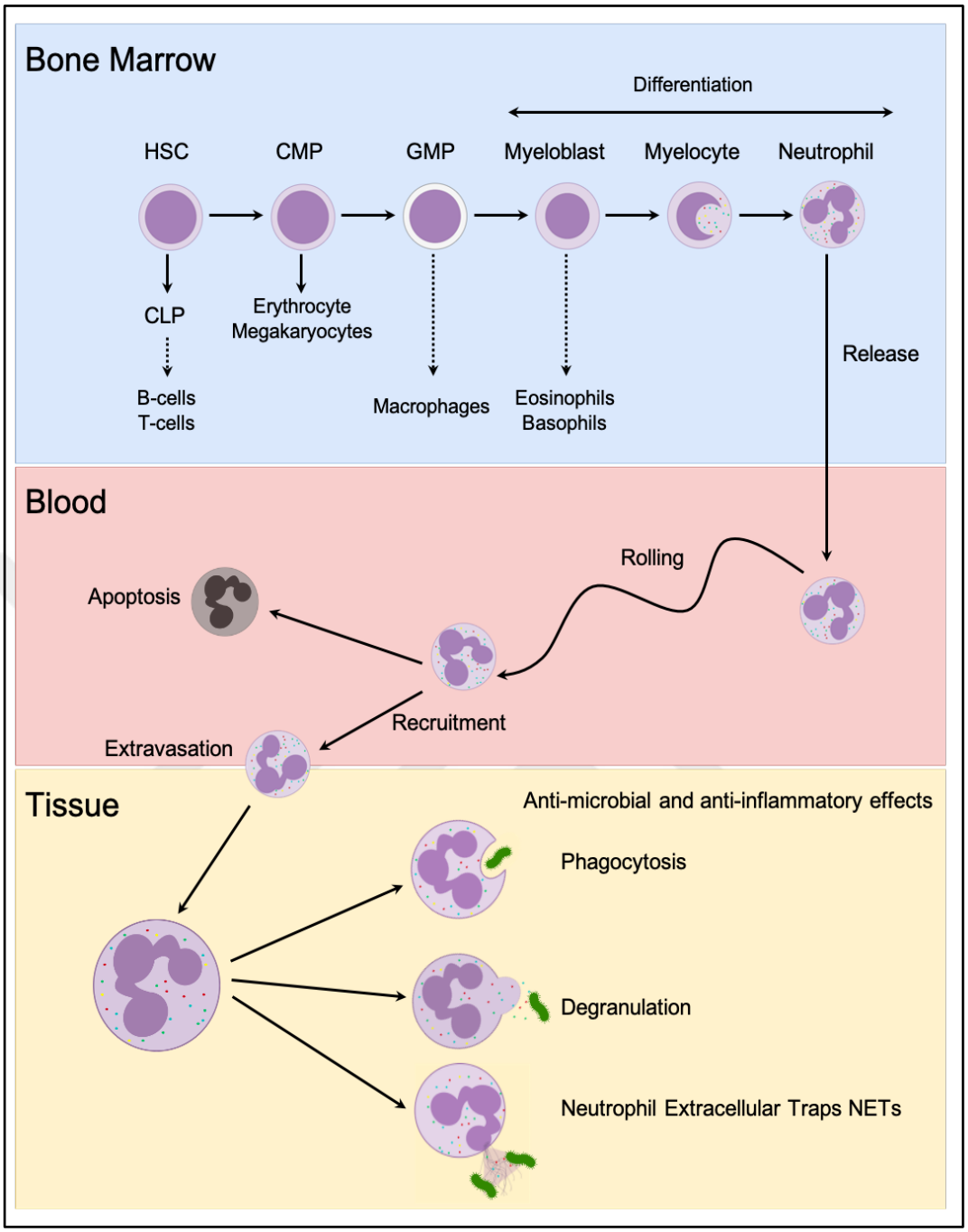

Figure 1.1: Development, recruitment, and activation of neutrophils from stem cells to itself. They differentiate and maturate in bone marrow, then released into circulation. Under inflammation or infection, it is attached to endothelial cells and sequestered into inflammation or infection zone. It destroys invaders by either phagocytosis, superoxide production, or degranulation, NETs. Abbreviations: HSC, Hemopoietic stem cell; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor. (Adapted from Eyles et. al, 2006).

The third way of neutrophils to cope with pathogens by releasing neutrophil extracellular traps (NETs), which is a also cell death type named NETosis (Brinkmann et al., 2004). These traps are made of decondensed chromatin, core DNA proteins (i.e.

histones), proteases (i.e. cathepsins), and enzymes (i.e. MPO). It was mentioned that both ROS generation and autophagy are required for chromatin formation therefore NEtosis (Remijsen et al., 2011). NETs trap pathogens and immobilize them; hence, pathogens cannot spread more and become a facile target for phagocytosis (Brinkmann et al., 2004; Kolaczkowska & Kubes, 2013; Papayannopoulos & Zychlinsky, 2009). Interestingly, recently NETs have been mentioned to have a role in some pathophysiological processes such as vascular diseases (ie. atherosclerosis and venous thrombosis) and inflammatory pathologies, (ie. gout and pancreatitis)(Rosales, 2018). In the absence of infection or inflammation, maturated neutrophils roll in the circulation and they undergo programmed cell death, apoptosis, naturally. Apoptosis is important for keeping neutrophil homeostasis in balance. It has been stated that apoptotic neutrophils are cleaned-up from circulation through the liver, spleen, and bone marrow (Bratton & Henson, 2011). In these places, they are engulfed by macrophages. As neutrophils age, the expression of CXCL4 protein on the cell surface rises. This expression is presented to be the reason why they go back to their home, bone marrow. (Bratton & Henson, 2011; Summers et al., 2010). The phagocytosis of apoptotic neutrophils in the spleen, bone marrow and liver by macrophages triggers the production of G-CSF in return and maintain neutrophil homeostasis (Bratton & Henson, 2011; Summers et al., 2010).

On the other hand, in the case of inflammation, inflammatory tissue-resident macrophages engulf the apoptotic neutrophils. This phagocytosis cause macrophages to suppress pro-inflammatory expressions and stimulate anti-inflammatory expressions. Consequently, this action slows down inflammation. Interestingly, this engulfment via peripheral macrophages decreases the expression of Interleukin-23 (IL-23), which is one of the regulators of G-CSF. Thus, G-CSF production reduces, and subsequently, granulopoiesis and neutrophil released from bone marrow declines (Rankin, 2010; Witko-Sarsat et. al, 2011). As a note, if the phagocytosis system is overwhelmed, neutrophils undergo secondary necrosis. Their cell cytotoxic molecules are engulfed by macrophages, of which induces the pro-inflammatory cytokines.

Recently, it has presented by many papers that, neutrophil counts increase vastly in the blood of cancer patients, even-though how does is occurs is still blur. This high number of neutrophils in blood is associated with a poor outcome (ie. Aggressive or advanced level of cancer, poor patient survival). Therefore, neutrophil to lymphocyte ratio

(NLR) could be used for a simple indicator of many types of cancer. Yet, some clinical authorities do not accept it (Rosales, 2018). NLR is still a controversial problem.

1.1.2 Neutropenia

The term neutropenia has been described for the condition where there is an abnormal amount of low levels of neutrophil found in the human body (Hsieh et.al, 2007). When the neutrophil number decreases, the first line of the immune system starts to rupture; thus, the human body becomes prone to invaders (i.e. bacteria, viruses, and fungi). There is four broad range of categories of problems that may cause the reduction of neutrophilic cells in the body. These are: (i) low production of neutrophils in bone marrow, (ii) problems in the development of the neutrophils, (iii) increased destruction of neutrophils, and (iv) problems in the neutrophils shifting to tissue pools.

Neutrophil amounts in the body can fluctuate nonrandomly by the physiological changes, such as milieu and host conditions. Hence, neutropenia should be confirmed by three samples taken over weeks (Boxer, 2012; Horwitz et. al, 2013). In certain populations or ethnicities, the normal level of the neutrophil amount is observed lower than the standard level. This phenomenon is called Benign ethnic neutropenia and these involve some Middle Easterns as well as African Americans (Haddy et. al., 1999). African Americans inherit these low numbers of neutrophils due to a gene called, Duffy Antigen Receptor for Chemokine (*DARC*) (Haddy et al., 1999).

The severity of the neutropenia has been classified into three classes. These classes are based on the absolute number of neutrophils found in the blood (ANC). It is calculated by multiplying the total number of leukocytes by the percentage of neutrophils and band neutrophils in the blood (Boxer, 2012). In a healthy adult human being, the reference range for the ANC is between 1500 to 8000 cells per microliter (µl) of blood. In a child younger than 12 months, the lower limit of ANC is 1000 cells/µl, to be count as healthy. When ANC is lower than 1500 (ANC<1500) cells/µl, this is clinically defined as neutropenia. If ANC is in range of 1000-1500 cells/µl, it is referred to as mild neutropenia; when it is between the range of 500-1000 cells/µl, it is suggested as moderate neutropenia and when it is lower than 500 (ANC<500) cells/µl, it is termed as severe neutropenia (Boxer, 2012).

Neutropenia can be divided into two as per duration. When neutrophil reserves are used rapidly and the low amount of production in the bone marrow lasts for a few

hours to days, it is termed as acute (or transit) neutropenia. This is usually seen after viral infections (such as HIV, malaria), chronic bacterial infections, and some inflammatory of autoimmune diseases (such as rheumatoid arthritis and sarcoidosis) (Newburger & Dale, 2013). Meanwhile, neutropenia would be diagnosed as chronic if its development lasts for at least 3 months (Boxer, 2012; Schwartzberg, 2006). Chronic neutropenia can begin due to extrinsic and acquired causes such as nutritional deficiency, auto-immune diseases, Myelodysplastic syndromes (MDS), and acute myeloid leukemia (AML) (Newburger & Dale, 2013).

On the other hand, neutropenia can be classified into two, depending on the causes. A person can have neutropenia since birth (congenital neutropenia), or it can develop later (acquired neutropenia) (Boxer, 2012; Schwartzberg, 2006). The rarely faced congenital neutropenia is divided into cyclic neutropenia and severe congenital neutropenia (SCN) (Boxer, 2012; Dale et al., 2018). Both of them originate from excessive apoptosis of myeloid progenitor cells during development. This results in the ineffective production of neutrophils (Newburger & Dale, 2013; Skokowa, Dale, Touw, Zeidler, & Welte, 2018). SCN is inherited as autosomal dominant due to some mutations which occur in the gene *ELANE*. This gene encodes for a serine protease named neutrophil elastase found in the primary granules (Skokowa et al., 2018). SCN could also be in the form of autosomal recessive, which is the case when some mutations in the gene *HAX1* develops. *HAX1* is a protein that binds to an adaptor protein required in downstream interactions in G-CSF-R activation (Skokowa et al., 2012). On a further note, this recessive inherited SCN causes Kostmann syndrome (Newburger & Dale, 2013).

Acquired neutropenia is usually due to secondary findings in a patient with another disease. It contains broad processes such as primary or secondary immune-associated neutropenia, chronic idiopathic neutropenia, infection-related neutropenia, drug-induced neutropenia, nutrition-related neutropenia, and chemotherapy-induced neutropenia (Boxer, 2012; Schwartzberg, 2006).

Chemotherapeutic agents target rapidly dividing cells like myoblast precursors (Bagnyukova et al., 2010). Thus, one of the hematologic side effects of chemotherapy is acute neutropenia. Chemotherapy receivers with malignant tumors often display neutropenia accompanied by high fever, over 38°C or 100.3°F. This is clinically termed as febrile neutropenia and it has a potential risk of life-threatening

complications for cancer patients as well as leads to a decrease of the dose or delay in chemotherapy (Weycker et al., 2016). Bacteria and fungi infections are the most common complications seen mostly in the oral cavity, mucous membranes, and skin (Schwartzberg, 2006).

1.2 Colony Stimulating Factors

As mentioned above, white blood cells are important for defending against the foreigner cells right after the invasion and maintain the homeostasis. So, since most of them are short-lived cells, they are constantly manufactured within the bone marrow. However, this production is tightly regulated through many soluble hematopoietic regulators. The most of well-studied regulators is a consortium of small glycoproteins termed Colony Stimulating Factors, CSF. CSFs are members of type I cytokine superfamily, which is a hemopoietic growth factor that mediates the hematopoietic stem cells to divide, proliferate, and differentiate into the mature cell. CSF is not only restricted to help the growth or differentiation of the cell, but it also enhances cell survival by suppressing apoptosis and activates the functions of cells such as chemotaxis, degranulation, adhesion of mature blood cells (Feldmann, 1998; Schrader, 1998). The overall functions of CSF are presented in Figure 1.2.

Several kinds of cell types are produce CSF, i.e, macrophages, stromal cells, endothelial cells, epithelial cells, fibroblasts, and T lymphocytes. (Schrader, 1998) Even-though CSFs are reported to be mainly secreted in response to cell stress; in some cases, they are released upon normal conditions. Endotoxins, LPS, and microbial nucleic acids are some of the microbial products that stimulate the CSF production. (Schrader, 1998).

Four kinds of CSF have already identified: Macrophage CSF, M-CSF, or widely used as CSF-1; Granulocyte-Macrophage CSF, GM-CSF; Granulocyte CSF, G-CSF, and Multi potential-CSF, widely named as Interleukin-3, IL-3 (Schrader, 1998).

1.2.1 Granulocyte Colony Stimulating Factor

The human Granulocyte Colony Stimulating Factor gene, *CSF3,* is positioned on chromosome 17q11.2-q12 and encodes for G-CSF or known as CSF-3 protein. It contains five exons that are interrupted by four introns, shown in Figure 1.3 (Nagata et.al., 1986). The messenger RNA (mRNA) of G-CSF is short-lived, the half-life is

less than 15 minutes (Koeffler, Gasson, & Tobler, 1988). Its mRNA possesses poly-AUUUA sequences in the 3' untranslated region (UTR) and it is thought to be associated with mRNA instability (Demetri & Griffin, 1991).

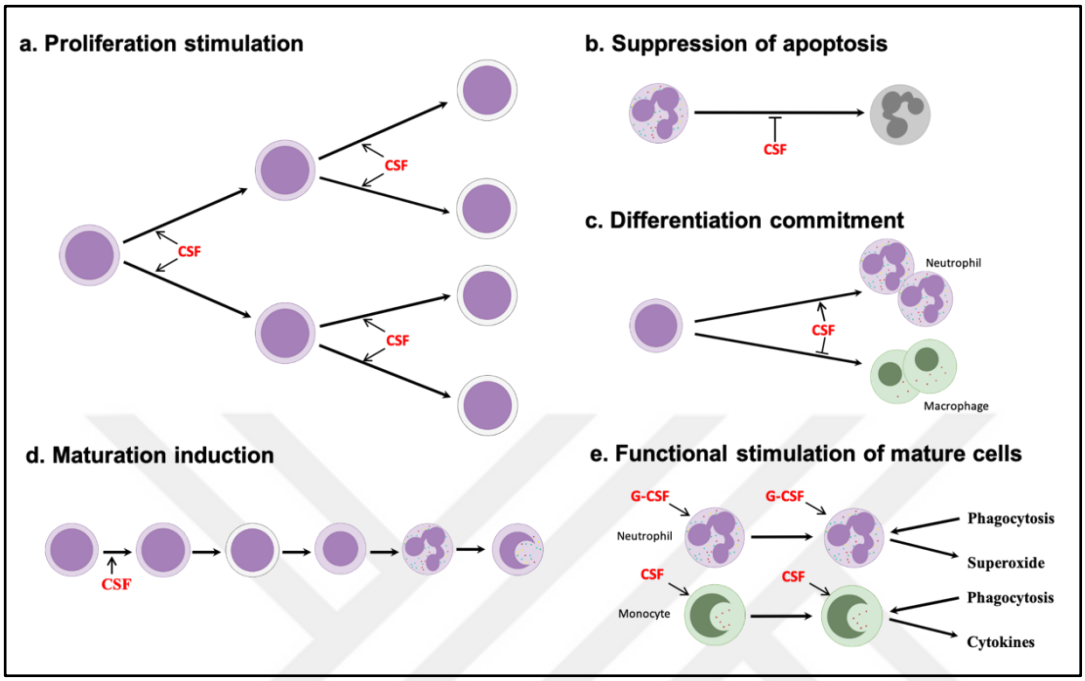

Figure 1.2: The scheme of multiple actions of colony stimulating factors (CSF) on the hematopoietic cells as well as mature immune cells (Adapted from Metcalf, 2010).

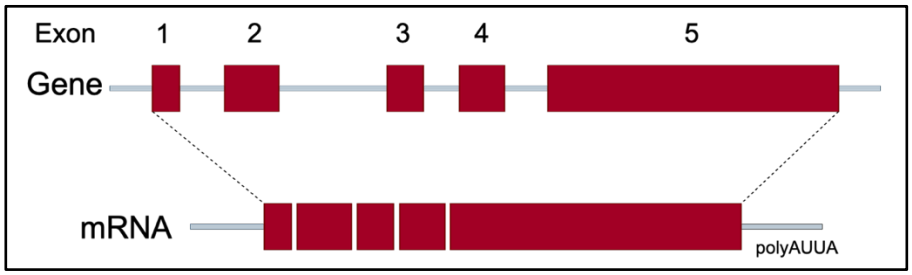

Figure 1.3: The structure of human G-CSF gene and mRNA. It has 5 introns which are interrupted by 4 exons (Adapted from Kato, 2016).

Until now, there are plentiful orthologs of human G-CSF that have been identified in mammals, fish, and birds. Human G-CSF shares 99% similarity with chimpanzees, 74% similarity with mice, 36% similarity with chickens, and 14% similarity with rockfishes. Besides, G-CSF shares scant sequence similarity with IL-6 (39%) and IL-23 (41%) (Molineux, Foote, & Arvedson, 2012). G-CSF has produced by endothelial cells, activated fibroblasts, T lymphocytes, and several immune cells such as monocytes, macrophages (Dawn et. al., 2012). Moreover, some tumorigenic cells have

presented to be producing G-CSF as well (Schneider et. al., 2005). For example in melanoma, non-small cell lung cancer, bladder cancer, prostate cancer, and brain cancers (Wang et al., 2012). In the absence of inflammation or infection, the serum levels of G-CSF are undetectable, lower than 30 pg/ml; however, with stress conditions like infection or inflammation, it increases rapidly and it may even exceed 2000 pg/ml (Watari et al., 1989).

CSF3 encodes for 204 amino-acid long, ~18 kDa molecular weighted glycoprotein. The first 30 amino-acid from the N-terminus is the signal sequence and the rest of 174 amino acids are the mature protein itself. The mature G-CSF has one O-linked glycosyl at Thr^{133} and two intramolecular evolutionary conserved disulfide bridges between Cys^{36}- Cys^{42} and Cys^{64}-Cys^{74}. (Demetri & Griffin, 1991; Kato, 2016). According to the research conducted by Hill et al., the disruption of disulfide bonds between and Cys^{64}-Cys^{74} have resulted in the formation of only half of the native helices. The other disulfide bonds between Cys^{36}- Cys^{42} have been mentioned as the major part of neutralizing antibody epitope (Hill et. al., 1993). Hence, it can be concluded that disulfide bonds are essential for biological activity. On the other hand, O-linked glycosylation at Thr^{133} is reported to be curial for neither biological activity nor receptor binding (Hill et al., 1993; Metcalf, 2010). Nevertheless, it has been reported as critical for protecting the molecule against the aggregation (Hill et al., 1993). There is also another variant of G-CSF due to the 5' splice donor at the second exon. This 177 amino-acid long variant has a three amino acid insert, Val-Ser-Glu, between Leu^{35}- Cys^{36} (Nagata et al., 1986; Nagata et al., 1986).

The G-CSF protein has 4 α-helix in an up-up-down-down orientation. These helix bundles are named as helicases A-D, and their connecting loops are called loop AB, BC, and CD. Moreover, there is a 3_{10}-helix loop between A and B helicases, named as AB loop (Hill et al., 1993; Molineux et al., 2012). Its tertiary structure model is drawn with the PyMOL software (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC) in Figure 1.4 (See: Appendix B for the PDB information of G-CSF).

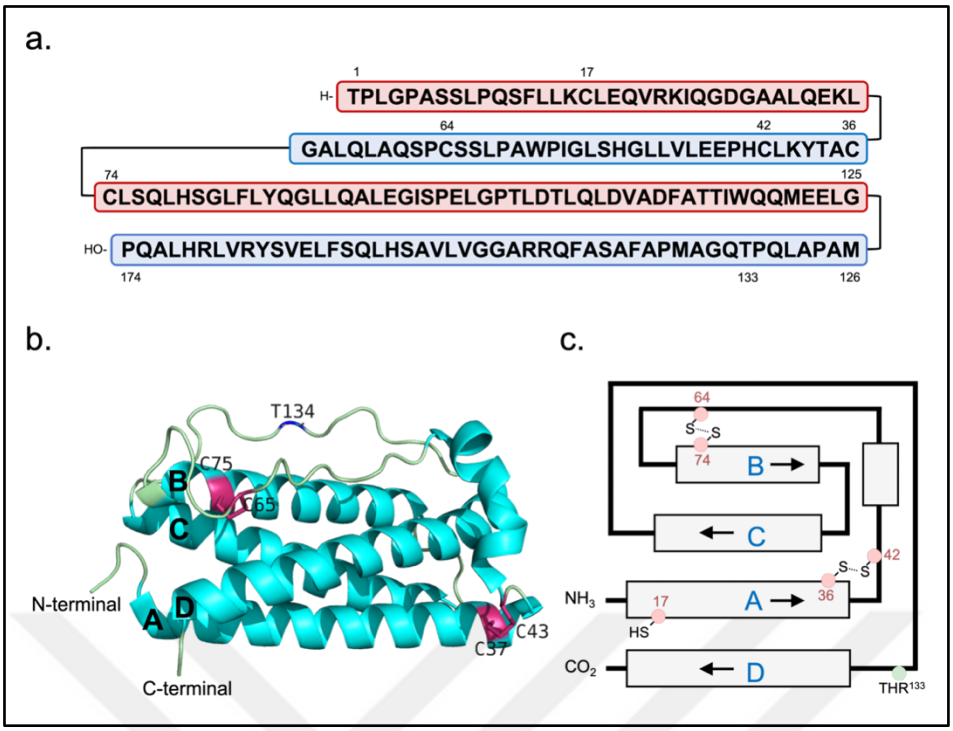

Figure 1.4: The structure of G-CSF: (a) Linear representation of G-CSF (Met+ 174 amino acid). (b) and (c) are representation of tertiary structure of G-CSF with helix names and directions. ((a) and (b) was adapted from Roberts et al., 2015). PyMOL software was used to draw (b), whose PDB details were mentioned in Appendix B.

The regulation of G-CSF is accomplished by a combination of transcriptional and post-transcriptional mechanisms. The evolutionary conserved regulatory site of the G-CSF is located at 300 bp upstream of the gene initiation site of G-CSF (Demetri & Griffin, 1991). Some important transcription factors that bind to the regulatory site of the G-CSF are nuclear factor-κB (NFκB) and nuclear factor-Interleukin 6 (IL-6). Also, inflammatory mediators such as IL-1, tumor necrosis factor-α (TNF), microbial components such as lipopolysaccharide (LPS), T-cell produced cytokine IL-17 and IL-17's regulator IL-23 are the key molecules in the regulation of G-CSF production (Andrew et. al., 2010).

Lieschke et. al. has published a paper, which suggests that G-CSF deficiency in mice causes the reduction of neutrophil numbers as well as impaired neutrophil mobilization. Thus, G-CSF deficient mice were incapable of controlling the *Listeria monocytogenes* infection, even more, a higher mortality rate was observed. When G-CSF was given to them artificially, they were indistinguishable with wild type G-CSF mice (Lieschke et al., 1994). As in the case of this paper, the main role of G-CSF is a prototypic neutrophil growth factor for the formation and development of progenitor

cells to neutrophil in the bone marrow and the transition of stored neutrophils in the bone marrow to circulation. This transition is performed by upregulating CXCR2 ligand production in other immune cells in bone marrow, abortion of CXCR4-CXCL12 interaction between neutrophil and bone marrow cells, and downregulating CXCR4 production on neutrophils (Köhler et al., 2011; Rosales, 2018). On the other hand, its absence leads to HSC retention in the bone marrow (Birbrair & Frenette, 2017).

Many scientists have shown that multiple actions of G-CSF are not restricted to neutrophils. It also has several influences on the other immune cells in both innate and adaptive immune systems, such as T-cells, DC, and monocytes. G-CSF suppresses T-cells activity against allogeneic reactions meanwhile increases the regulatory T-cells, T_{REG} (Xiao, Lu, & Link, 2007). On the other hand, G-CSF increases the tolerogenic DC (type II DC) in peripheral blood. DC are immune cells that have no function in killing the pathogen, but they collect and present antigens to cells of the adaptive immune system. Tolerogenic DC has immuno-suppressive properties that lead the immune system into the tolerogenic state, which regulates the T_{REG} as well (Švajger & Rožman, 2018; Xiao et al., 2007). G-CSF has a modulatory role on other important immune system cells, monocytes. It alters the cytokine production by monocytes via increasing the anti-inflammatory cytokine while decreasing the pro-inflammatory cytokine (Figure 1.5).

Except for the immune system, G-CSF has a significant role in the nervous system, such as on the central nervous system (CNS), neural stem cells (NSC), and some neurological conditions (Schneider, Krüger, et al., 2005; Schneider, Kuhn, et al., 2005). It is reported that G-CSF could be one of the stimulators of proliferation and differentiation of NSC (Jung et al., 2006). On a further note, the cytokine receptor G-CSF-R is present in neurons and glial cells. In acute stroke models, the endogenous G-CSF is secreted in upregulated neurons under ischemia conditions. By this, G-CSF protects neurons to go under apoptosis and increases their activity via its receptor (Schneider, Krüger, et al., 2005). Furthermore, G-CSF triggers the HSC cells to mobilize the ischemic brain and thus repair the lesions (Xiao et al., 2007). Astrocytes, a glial cell in CNS, are reported to be stimulated by G-CSF for secreting Vascular endothelial growth factor (VEGF) thus promoting angiogenesis (Xiao et al., 2007). STAT 3 is one of the upregulated proteins via JNK upon G-CSF binding to G-CSF-R

and causes a pro-proliferation effect. It is also referred to as one of the pathways altered in human glioblastoma (Wang et al., 2012).

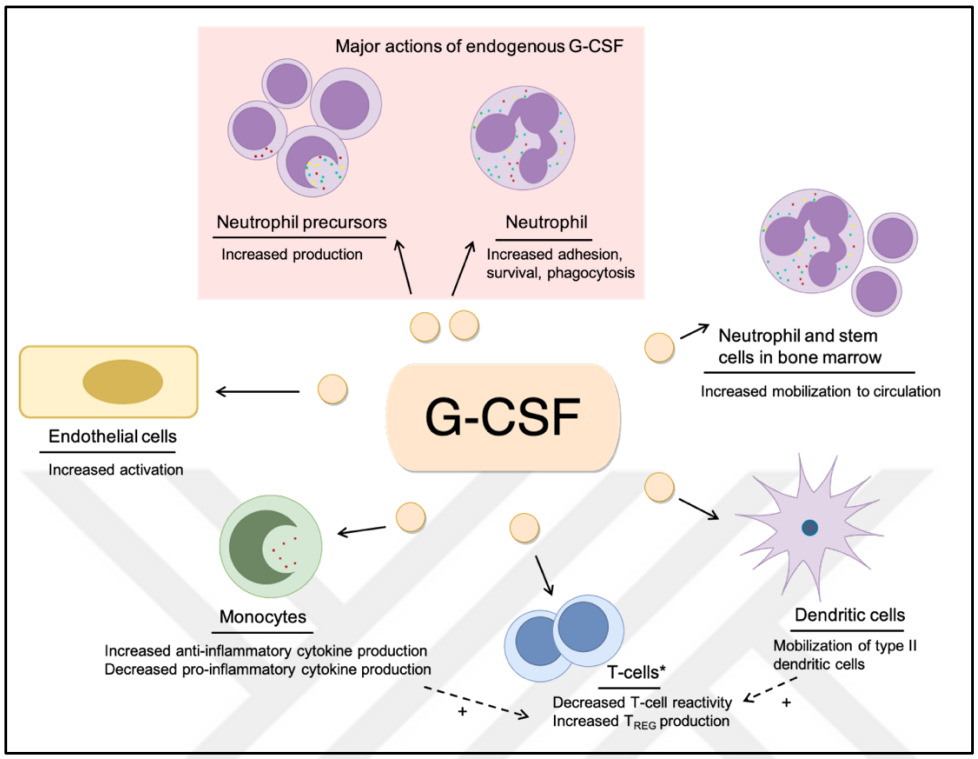

Figure 1.5: Presentation of biological activity of endogenous G-CSF. Its major action is presented in a soft pink box. *Administered G-CSF might act on T-cell via directly of indirect through monocytes and dendritic cells. T_{REG}, Regulatory T-cell (Adapted from Eyles et. al, 2006)**.**

To sum up, endogenous G-CSF is a critical cytokine for basal level neutrophil development from progenitor cells, neutrophil proliferation, survival, and mobilization to blood from bone marrow. Aside from neutrophil, they are important for neuropoiesis and have effects on T-cells, DC, and monocytes.

1.2.1.1 Action mechanism of G-CSF

G-CSF interacts with a specific cell surface receptor, Granulocyte Colony Stimulating Factor Receptor, G-CSF-R, in a 2:2 ratio to form oligomeric structure. This receptor is also well known by the name of Cluster of Differentiation, CD114. (Layton & Hall, 2006). The typical cytokine receptor G-CSF-R is expressed in hematopoietic progenitors, monocytes, platelets, neurons, (Adusumilli & Krothapalli, 2012). Remarkably, it has been reported that endothelial cells and lung cancer cells additionally carry these receptors (Schneider, Kuhn, et al., 2005).

The transmembrane protein G-CSF-R is a type I membrane protein that members of the hematopoietic cytokine receptor superfamily, along with erythropoietin receptors, some interleukin receptors, and pituitary growth hormones receptors. They share particular motif similarities in the region at the extracellular domain; however, upon heterodimerization with its ligand, this dimerized structure resembles the cytokine gp130 receptor with its ligand IL-6 or leukemia inhibitory factor (LIF) (Molineux et al., 2012).

G-CSF-R protein has 3 domains; the extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular portion of G-CSF-R contains the immunoglobulin-like domain (Ig-like domain), cytokine receptor homology (CRH domain), and three fibronectin type-II domains. G-CSF-R binds its ligand via CRH and Ig-like extracellular domains (Aritomi et al., 1999; Touw & Van De Geijn, 2007). G-CSF-R has 3 box regions, Box1, Box2, and Box3; at its cytoplasmic tail of the intracellular domain. G-CSF-R does not possess an intracellular tyrosine kinase activity; thus, it relies on the cytoplasmic enzymes for signaling cascades. (Pankaj & Greis, 2017; E. B. Rankin & Sakamoto, 2018).

The activation of the G-CSF-R by the G-CSF binding leads to JAK/STAT pathway action and causes the phosphorylation cascade in the downstream. Activation triggers G-CSF-R to change their conformation and brings two G-CSF-R closer. By this change, Janus Kinases (JAK) comes closer as well. The Janus Kinases trans-phosphorylate each other as well as the Tyr residues located at the C-terminal of the receptor straightaway. These phosphorylated Tyr residues become a docking site for several proteins that contain SH2 domains. Different types of proteins with SH2 domain can dock with the Tyr residues on the G-CSF-R simultaneously. One of the well-known protein of them is the Signal Transducers and Activators of Transcription protein (STAT). Different types of STAT isomers are recruited during this signal transduction. For instance, STAT3 has a pro-differentiation role while STAT5 has a proliferation and survival role. Once STAT proteins dock with the phospho-tyrosine residue of the receptor either at Tyr^{704}, Tyr^{744} or Tyr^{729}, the STAT will be phosphorylated and homodimerize with itself or heterodimerize with one of its isomers. Dimerized STAT will migrate into the nucleus to express γ-activated sequence, GAS, elements (Pankaj & Greis, 2017). Its downstream cascades are presented in a scheme in Figure 1.6.

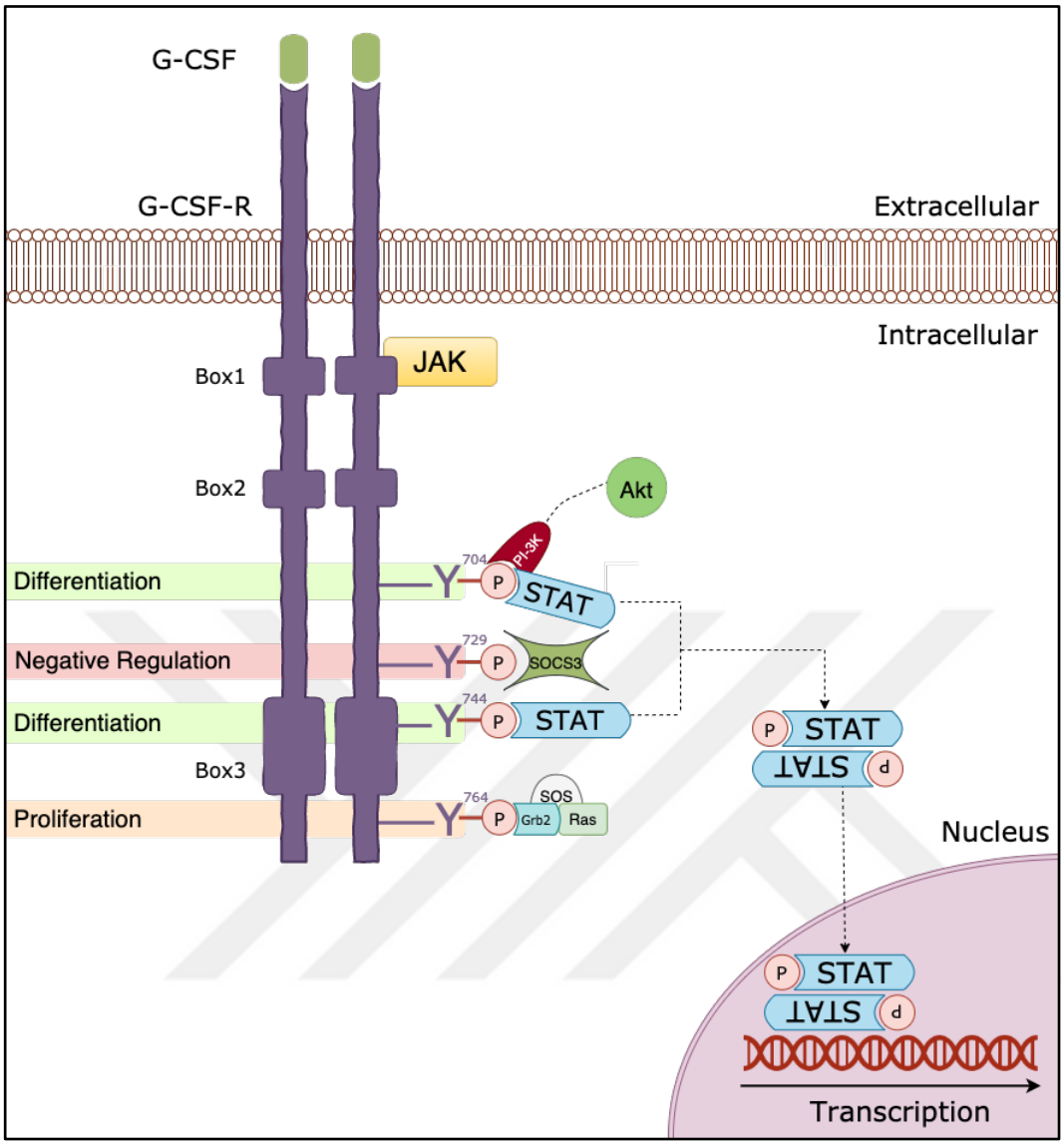

Figure 1.6: Schematic map of the cascades activated upon G-CSF activation on G-CSF-R. Upon binding to the its receptor, G-CSF may activate the JAK/STAT, PI-3K/Akt and Ras/MAPK pathways. Abbrevations: Y, Lysine; P, Phosphorylation (Adapted from Eyles et. al, 2006).

In addition to development induction via JAK/STAT pathway, G-CSF-R activation by G-CSF induces proliferation and cell survival via p21Ras/MAP kinase and PI-3K/ AKT pathways. Even though it is not clear whether the activation of PI-3K/AKT pathways is dependent or independent from JAK, PI-3K has a role in cell proliferation and cellular survival by inhibition of the pro-apoptotic protein Bad and anti-apoptotic protein Bcl-2 interaction (Pankaj & Greis, 2017). Meanwhile, Grb2 of p21/Ras mediated MAPK pathway is activated via phosphorylated Tyr^{764} on the receptor of G-CSF (Demetri & Griffin, 1991; Layton & Hall, 2006).

It is also presented that, G-CSF induces a T-cell regulator called suppressor of cytokine signaling 3, SOCS3 protein on the DC (Figure 1.6). Yet, it suppresses the G-CSF-R mediated signaling pathway by binding the G-CSF-R at the Tyr^{729} position and inhibits the activation of STAT3 *in vitro* (Xiao, Lu, & Link, 2007). In the neutrophils with SOCS3 knockout mice have presented that activation of STAT3 *in vitro* (Croker et al., 2004).

1.2.2 Clinical use of G-CSF

Due to significant proliferation, development, and activation effects on the progenitor neutrophil cell, G-CSF has been widely used to treat severe congenital neutropenia and hematopoietic recovery following chemotherapy (Rankin, 2010). The most recognized medical use of recombinant methionyl human G-CSF is Filgrastim (Neupogen®, Amgen Inc.), which is a drug approved by the FDA in 1991 in the United States (Gascon, 2012).

Filgrastim has recombinantly been engineered between 1984-1986 and clinically approved in 1991. It was firstly used in lung cancer patients receiving chemotherapy because they were having neutropenia as a side effect (Welte, et al, 1996). As a result of this treatment used on patients, there was a significant decrease in the duration of neutropenia, infection, and hospital admissions (Schwartzberg, 2006; Welte et al., 1996). Filgrastim is not only used for chemotherapy receiver patients. In the first 10 years, filgrastim has been approved in 70 countries including Europe and Australia for treating diseases such as myelosuppression after bone marrow transplantation, SCN, AIDS-associated neutropenia, acute leukemia, aplastic anemia(AA), myelodysplastic syndromes (MDS), non-Hodgkin's lymphoma (NHL), mobilization of peripheral blood progenitor cells (PBPCs) for transplantation (Arvedson et. al., 2015; Renwick et. al., 2009; Welte et al., 1996).

Filgrastim reduces the maturation time of neutrophils from 5 days to 1 day and affects both the progenitor and mature neutrophils and enhances neutrophils chemotaxis and superoxide formation (Welte et al., 1996). At the beginning of using G-CSF for the treatment of SCN for a long time, it is suspected that its administration would cause the development of leukemia as patients are more susceptible to develop acute myeloid leukemia (AML) since these cells carry G-CSF-R (Frampton et. al., 1994). However,

until now, the G-CSF's role in developing AML in SCN patients, who are taking G-CSF for treatment, has not been proven.

Depending on the use, filgrastim is given to the patient by either under the skin, by injecting into a vein. It has a short circulating half-life (3.5 hours); thus, its daily injection is needed until recovery (Arvedson et al., 2015). Moreover, it requires body weight-based dosing while frequent monitoring of neutrophil counts.

The side effects of filgrastim have mainly been reported as mild to moderate bone pain in approximately 10% to 30% of the patients. The severity and the ratio of the pain depend on the dose that has been taken (Root & Dale, 1999). In addition to bone pain, mild to moderate elevation in serum uric acid, lactate dehydrogenase, alkaline phosphatase, and leucocyte alkaline phosphatase was also observed in patients. This increase reflects neutrophil production (Frampton et al., 1994). On the other hand, allergic reactions and local reactions such as pain, swelling, and redness at the injection site were rarely reported.

G-CSF is predominantly cleaned-up from the body mainly via renal mediated filtration. The strategy to increase the reaming of this short half-life drug in the circulation is reducing renal clearance significantly. This could happen if the proteins' weight is increased, made more elongated, and more negatively charged. This strategy has been practiced when G-CSF had conjugated with PEG molecules, repeating ethylene oxide units (Pegfilgrastim) and has been in use globally.

All in all, biosimilar has become a new generation in the pharmaceutical industry by the mid-nineteens. They are unique in the pharmaceutical industry because they are produced from living organisms. Those biopharmaceuticals are extremely similar to a reference molecule; therefore, it is called Biosimilar or follow-on-protein products" by the European Medicines Agency (EMA) and the United States Food and Drug Administration (FDA) (Gascon, 2012). According to the FDA, there are no significant differences between the biosimilar and the reference in terms of safety, purity, and potency (U.S. Food and Drug Administration, 2017). FDA approval process is similar to EMA and Health Canada.

Filgrastim is one of the highest penetrations and a strong track record in marketing of small peptide biosimilars. Its first biosimilar in the US, *Zarxio* (filgrastim-sndz, Sandoz), was approved by the FDA in 2015 (Jarrett & Dingermann, 2015). After that,

filgrastim had at least 8 biosimilars approved by EMA until 2016. Meanwhile, its biosimilars have been on the market in Europe since 2009. Its major market countries in the European continent are Germany, France, Italy, Spain, and the UK. (Araújo, Gonçalves, & Fonseca, 2016). In between second quarter of 2015 and second quarter of 2020, the uptake of Filgrastim reference Neupogen has dropped 28% while its biosimilars Zarxio and Nivestym uptake has increased 48% and 4% respectively (Amgen, 2020).

On the other point of view, biosimilar availability has some positive effect on the affordability of costly drugs. For example, the change from filgrastim to a biosimilar is reported to be associated with saving £1 million annually in London (G-CSF purchasing cost has developed from £3.3 million to £2.3 million in 2011)(Gascón et al., 2013). In addition to the London region, the change from filgrastim to any of its biosimilar has also led to annual savings of €85 million in 17 European countries (Gascón et al., 2013).

Table 1.1: Some of the Filgrastim derived G-CSF biosimilars available in the market. * are not available in the Turkish market.

Brand Name	Producer	Active Ingredient	Description
Neupogen	Amgen	Filgrastim	Human recombinant G-CSF
Zarxio	Sandoz		Human recombinant G-CSF
Fraven	Arven		Human recombinant G-CSF
Ratiograstim*	Ratiopharm		Human recombinant G-CSF
TevaGrastim*	Teva Pharm		Human recombinant G-CSF
Zarzio*	Novartis		Human recombinant G-CSF
Nivestim*	Hospira		Human recombinant G-CSF
Leucostim	Dong-A		Human recombinant G-CSF
Granocyte	Chugai	Lenograstim	Glycosylated form of human recombinant G-CSF
Neulastim	Amgen	PEG-Filgrastim	PEG molecule conjugated human recombinant G-CSF
	Roche		PEG molecule conjugated human recombinant G-CSF
Fulphila*	Mylan/Biocon		PEG molecule conjugated human recombinant G-CSF
Udenyca*	Coherus BioSciences		PEG molecule conjugated human recombinant G-CSF

1.3 AIM

Neutrophils are one of the main defense system cells that mobilizes themselves to the area of infection or inflammation and fight against invaders. Therefore, its reduction makes the body available for infections. Since the granulocyte colony-stimulating factor has undeniable stimulating effects on neutrophil formation, development, survival, mobilization, and action properties; it has been widely used as a treatment reagent for neutropenia developed in response to diseases such as cancer patients who are taking chemotherapy.

In this thesis, the main aim is to perform the cloning, production and purification of human G-CSF protein, which can be used for biopharmaceutical purposes. Cloning the target gene from the glial U87-MG cell line by recombinant DNA technology is one of the first goals of this thesis. After the target protein is expressed and its expression is optimized in the bacteria host, it will be purified by chromatographic methods using Äkta Avant chromatography systems. Finally, the qualitive and quantitative analysis of the active recombinant hG-CSF and its comparison with the reference product is performed by many characterization methods such as HPLC-SEC, circular dichroism (CD), peptide mapping, intact mass and capillary electrophoresis.

2. MATERIAL and METHODS

2.1 Material

2.1.1 Equipment

The equipment used in the experiments is shown in Appendix A.

2.1.2 Chemicals and Enzymes

The chemicals and enzymes used in the experiments are shown in Appendix A.

2.1.3 Commercial kits

The commercial kits used in the experiments are shown in Appendix A.

2.1.4 Cell Strains

2.1.4.1 Bacterial Strains

In this study, several bacterial strains have been used. These were *Escherichia coli* BL21 (DE3) strain [B F– ompT gal dcm lon hsdSB(rB–mB–) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB+]K-12(λS)], and commercially available *HST08* Stellar Chemically competent™ strain [F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ-].

2.1.4.2 Mammalian Strains

In this study, U-87 MG (ATCC® HTB-14™) brain glioblastoma cells were used for capturing the CSF3 mRNA; THP-1 (ATCC® TIB-202™) human monocytic cell line derived from an acute monocytic leukemia patientwere used for detecting recombinant hG-CSF activity.

2.1.5 Buffers and solutions

DMEM Full cell medium and RPMI-1640 that used in mammalian cell culture were prepared according to Table 2.1 and Table 2.2 below.

Table 2.1: The components of the DMEM Full medium.

Components	Amounts
DMEM medium	450 ml
Fetal Bovine Serum	50 ml
Penicillin/Streptomycin	5 ml

Table 2.2: The components of the RPMI-1640 Full medium.

Components	Amounts
RPMI-1640 medium	450 ml
Fetal Bovine Serum	50 ml
MEM Non-Essential Amino Acids Solution	5 ml
Penicillin/Streptomycin	5 ml

Luria Bertani (LB) media used in bacteria culture was prepared according to Table 2.3 below. After the preparation of the LB medium, it was sterilized by autoclaving it at 121ºC for 15 minutes. Then, appropriate antibiotics, Kanamycin were added with appropriate quantity.

Table 2.3: The components of Luria Bertani (LB) medium.

Components	Amounts
Yeast Extract	5 g
Bacterial Peptone	10 g
NaCl	10 g
dH_2O	Fill to 1 L

Luria Bertani (LB) agar that used in bacteria culture was prepared according to Table 2.4. After the preparation of the LB agar, it was sterilized by autoclaving it at 121ºC for 15 minutes. Then, appropriate antibiotics, Kanamycin were added and left to freeze at room temperature for 15 minutes approximately under the sterile condition.

Table 2.4: The components of the Luria Bertani (LB) agar medium.

Components	Amounts
Yeast Extract	5 g
Bacterial Peptone	10 g
NaCl	10 g
Agar	15 g
dH_2O	Fill to 1 L

The components needed for the preparation of 50X TAE Buffer for agarose gel electrophoresis was mentioned in Table 2.5.

Table 2.5: The components of the 50X TAE buffer.

Components	50X TAE
0.5 M EDTA pH 8.0	100 ml
2 M Tris Base	242 g
1 M Acetic Acid	57.1 ml
dH_2O	Fill to 1 L

Preparation of SDS-polyacrylamide separating (12% and 15%) and stacking (5%) for 2 gels were mentioned in Table 2.6 and Table 2.7 respectively.

Table 2.6: The components of 12% and 15% polyacrylamide separating gels.

Components	Amounts	
	12% Gel	15% Gel
dH_2O	3.86 ml	3.54 ml
40% Acrylamide/Bis-acrylamide	3.05 ml	3.75 ml
1.5 M Tris-HCL pH 8.8	3 ml	2.5 ml
10% SDS	120 µl	100 µl
10% Ammonium Persulfate	120 µl	100 µl
Temed (100%)	12 µl	10 µl

Table 2.7: The components of 4% SDS polyacrylamide stacking gel.

Components	Amounts
dH_2O	2.92 ml
40% Acrylamide/Bis-acrylamide	540 µl
1.5 M Tris-HCL pH 6.8	500 µl
10% SDS	40 µl
10% Ammonium Persulfate	40 µl
Temed (100%)	4 µl

The buffers used in SDS-PAGE were listed in Table 2.8-Table 2.14.

Table 2.8: The components of 4X Laemmli Buffer.

5X Laemlli Buffer
0.3 M Tris-HCL pH 6.8
8% SDS
50% Glycerol
4% ß-Mercaptoethanol
0.08% Bromophenol Blue

Table 2.9: The components of Running Buffer.

Components	Amounts
dH_2O	1 L
Tris base	30.3 g
Glycine	144 g
SDS	10 g

Table 2.10: The components of 10X Transfer Buffer.

Components	Amounts
dH_2O	1 L
Tris base	30.3 g
Glycine	144 g
SDS	10 g

Table 2.11: The components of the Transfer Buffer solution.

Components	Amounts
10X Transfer Buffer	100 ml
Methanol	200 ml
dH_2O	700 ml

Table 2.12: The components of the Coomassie Brilliant Blue stain solution.

Components	Amounts
Coomassie Brilliant Blue	1 g
Methanol	400 ml
Acetic Acid	100 ml
dH_2O	Fill to 1 L

Table 2.13: The components of Destaining solution.

Components	Amounts
Methanol	400 ml
Acetic Acid	100 ml
dH_2O	Fill to 1 L

Table 2.14: The components of 10X TBS.

Components	Amounts
dH_2O	1 L
Tris base	12.22 g
NaCl	87.66 g

1X TBS was prepared by diluting 100ml 10X TBS in 900 ml dH_2O. 1X TBS-T was prepared by adding 1 ml Tween-20 to 1L 1X TBS. 5% non-fat dry milk was prepared by weighing 1.5 g non-fat dry milk and dissolving it in 30 ml 1X TBS-T. 5% BSA was prepared by weighting 30 ml 1X TBS-T.

Stock 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was prepared by dissolving 1.19 g IPTG in 10 ml distilled water. Then, it was sterilized with a 0.22 μm syringe filter and aliquoted for storing at -20°C.

Stock 50 mg/ml Kanamycin was prepared by dissolving 0.5 g Kanamycin in 10 ml distilled water. Then it was sterilized with a 0.22 μm syringe filter and aliquoted for storing at -20°C.

The cell lysis buffer used to dissolve bacteria before sonication contains 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, and 1 mM PMSF.

The pellet washing buffer (WB) used to wash inclusion bodies sonication contains 50 mM Tris-HCl pH 8.0, 1 mM EDTA, 50 mM NaCl, 1 mM PMSF, 2M Urea and 0.5% Triton-X. The pellet solubilization buffer (SB) used to solubilize inclusion bodies contains 50 mM Tris-HCl pH 8.0, 8 M Urea and 5 mM β-Mercaptoethanol, 5 mM EDTA and 1 mM PMSF.

Formulation Buffer (FB) used as final buffer of G-CSF that contains 10 mM sodium acetate, pH 4.5, 5% sorbitol, 0.004% Tween 80.

The buffers used for cation exchange are listed below:

- **Binding Buffer:** 25 mM Sodium Acetate pH 4.5.
- **Elution Buffer:** 25 mM Sodium Acetate, 1 M NaCl pH 4.5.

2.1.6 Antibodies

The antibodies used in this thesis and they were prepared according to Table 2.15.

Table 2.15: The antibodies and their dilution amounts.

Antibody	Type	Dilution
G-CSF (Santa Cruz, 3D1)	Monoclonal Mouse	1:1000 (BSA)
p-ERK 1/2(CST, 9101S)	Monoclonal Rabbit	1:1000 (BSA)
Anti-mouse IgG (CST, 7076)	HRP-linked Polyclonal	1:3000 (5% milk)

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Mammalian cell culture

U-87 MG (ATCC® HTB-14) human primary glioblastoma cells were maintained in T-75 flasks with high glucose DMEM medium (Gibco, 41966029) with 10% fetal

bovine serum, FBS (Thermo Scientific, 10500064) and 100 U/100 $mg.ml^{-1}$ penicillin/streptomycin (Gibco, 15140122) at 37°C incubator, 5% CO^2 humidified air. When cells had 90% confluency, they were washed with 3 ml 1X Phosphate Buffered Saline (PBS). Then, they were split with 0.25% Trypsin- EDTA (Gibco™).

THP-1 (ATCC® TIB-202™), human monocytic cell line derived from an acute monocytic leukemia patient, were maintained in T-75 flasks with RPMI-1640 medium (Lonza, 21875034) with 10% fetal bovine serum, FBS (Thermo Scientific, 10500064), 1% MEM Non-Essential Amino Acids Solution (Thermo Scientific, 11140050) and 100 U/100 $mg.ml^{-1}$ penicillin/streptomycin (Gibco, 15140122) at 37°C incubator, 5% CO^2 humidified air. $2x10^5$ cells/ml are seeded into flasks and they are maintained by addition of fresh full RPMI-1640 medium. When the cell density reaches $1x10^6$ cells/ml, they are subcultured into new T-74 flask. To preserve THP-1 cells in -80°C, $3x10^6$ cells in are centrifuged at 300xg for 5 minutes. Then, pellet was dissolved in 6% DMSO in FBS, and kept in -80°C.

2.2.1.2 Bacterial cell culture

Component cell preparation

The competent cells, who are able to take up foreigner DNA or plasmid from the surrounding environment, are made by the protocol mentioned below.

1. The cells are taken from -80°C frozen stock. They are thawed and spread on LB Agar plate, which is then incubated at 37°C for overnight.
2. Then, a colony was selected on the agar plate. That colony was transferred to a 5 ml LB medium and incubated at 37°C overnight.
3. The overnight culture was put in 100 ml LB medium in 1:50 dilution and left to grow at 37°C.
4. Its OD_{600} had been monitored until it reaches 0.6. When it was 0.6, it was harvested by centrifugation at 4000 rpm for 10 minutes. Supernatants are discarded.
5. Next, the pellets were resuspended with 60 ml cold Solution A (1 M $MgCl_2$, 1 M $CaCl_2$, dH_2O) and the tubes were centrifuged at 4000 rpm for 10 minutes again. Supernatants are discarded.

Finally, pellets were resuspended with 2 ml cold Solution B (Glycerol, 1 M $CaCl_2$, dH_2O) and aliquoted by 50 µl samples for long term storage at -80°.

Heat-shock transformation

Stellar competent cells (Takara, 636763), BL21 (DE3), Rosetta, and Rosetta-Gami cells were transformed according to the protocol mentioned below.

1. 50 µl of competent were thawed on ice. 100 ng plasmid or 2 µl ligation product was added on them and waited on ice for 30 minutes.
2. Cells were heat-shocked at 42°C for 60 seconds exactly in the water bath and kept in ice for 3 minutes.
3. Cells were added into 450 µl LB-broth, incubated at 37°C for 1 hour with shaking 225 rpm.
4. Then, 2/5 of the cells are spread onto an LB Agar with Kanamycin. The rest of the cells were centrifuged in 4000 rpm for 5 minutes.
5. 400 µl of supernatant was discarded and the pellet was dissolved in the rest of 100 µl LB. All of them are plated into another LB Agar as concentrated.
6. These transformed cell containing plates are incubated at 37°C with 200 rpm shaking for 16 hours.

Bacterial Growth

Before each growth that will be induced with IPTG, a pre-culture has been grown in in Luria Bertani (LB) medium overnight at 37°C with 200 rpm shaking. Then, it is inoculated from that culture to 1:50 ratio into fresh Luria Bertani (LB) medium at 37°C and 200 rpm.

The ideal inoculation culture conditions such as induction time, induction duration (2 hours, 4 hours and 6 hours), growth temperature (37°C, 30°C and 18°C) and IPTG concentration (0.25 mM, 0.5 mM, 0.75 mM and 1 mM) after induction was determined.

2.2.2 Nucleic acid isolation

2.2.2.1 RNA isolation

Human primary glioblastoma cells U-87 MG cells' RNA were isolated by using the NucleoSpin® RNA Midi Kit (MACHEREY-NAGEL, 740955.50). For this purpose, $2x10^6$ cells were cultured in T-25 flasks. Then, the manufacturer guideline was followed for RNA isolation.

2.2.2.2 Plasmid isolation

Plasmid DNAs from bacteria were isolated by using the NucleoBond Xtra Maxi kit for transfection-grade plasmid DNA Kit. For this purpose, 100 ml LB growth cultured for overnight was used. The manufacturer guideline was followed.

2.2.3 cDNA library construction

cDNA libraries were constructed by using isolated RNA's. RNA was translated into cDNA by using 5X All-In-One RT MasterMix (abm®, G485). A reaction mixture was prepared by using Table 2.16 while tubes were kept in ice. After they had been prepared, the tubes were exposed to several temperatures in Table 2.17. Synthesized cDNA libraries were kept at -20°C for the short term, at -80°C for the long term.

Table 2.16: Components of 5X All-In-One RT MasterMix cDNA synthesis reaction mixture for 20 µl reaction.

Components	Amount
Total RNA	2 pg - 2 µg/20 µl rxn
5X All-In-One RT MasterMix	4 µl
Nuclease-free H_2O	To 20 µl

Table 2.17: Temperature and time for of 5X All-In-One RT MasterMix cDNA synthesis.

Temperature	Time
65°C	5 min
25°C	10 min
42°C	50 min
85°C	5 min

2.2.4 Vector and candidate oligonucleotide primers design

Firstly, specific primers were targeted and designed for and amplification of the *CSF3* gene from the U87-MG cDNA library. NCBI/Primer-BLAST and In-Fusion Cloning Primer Design Tool were used for finding proper primers as well as their Tm values for *CSF3* amplification. OligoAnalyzer and IDT DNA websites were used to determine other properties of primers such as hairpins, dimers, and mismatches. The properties of the designed primers, which have taken from NCBI/Primer-BLAST, are shown in Table 2.16. Primers are synthesized by PRZ Biotech, Ankara. The restriction enzymes, EcoRI-HF® (NEB, R3101S) and NdeI (NEB, R0111S), were added on 5' ends of both forward and reverse primer. The CSF3 sequence that have been used for

primer design were taken from NCBI and its sequence as well as properties have been shown in Appendix E.

Table 2.18: Sequences, length, Tm, and GC% of primers designed for human CSF3.

	Sequence (5'->3')	Length (bp)	Gene-specific Tm (°C)	GC%
Forward primer	AAGGAGATATACATATG ACCCCCCTGGGCCCTG	33	61.0	54.5
Reverse primer	GACGGAGCTCGAATTCT CAGGGCTGGGCAAGGTG	34	60.7	61.8

2.2.5 Restriction Enzyme Digestion

Restriction enzyme digestion of vector, insert product and final G-CSF plasmid was performed by using EcoRI-HF® (NEB, R3101S) and NdeI (NEB, R0111S) enzymes. Table 2.19 shows components of a mixture for 50 µl reaction. They are mixed in a tube and waited at 37°C for 3 and a half hours. To inactivate restriction enzymes, the tube was exposed to 65°C for 20 minutes.

Table 2.19: Restriction enzyme control of G-CSF plasmid for 50 µl reaction.

Component	Uncut	One Enzyme Digestion	Double Enzyme Digestion
G-CSF plasmid	1000 ng	1000 ng	1000 ng
10X CutSmart Buffer	5.0 µl	5.0 µl	5.0 µl
EcoRI	-	1.0 µl	1.0 µl
NdeI	-	-	1.0 µl
Nuclease-free H2O	to 50 µl	to 50 µl	to 50 µl

2.2.6 Polymerase chain reaction (PCR)

2.2.6.1 *CSF3* amplification

For the amplification of G-CSF from synthesized cDNA, Polymerase Chain Reaction (PCR) was performed. CloneAmp HiFi PCR Premix (Takara, 638500) was used for this purpose. The reaction mixture and the procedure were given in Table 2.20 and Table 2.21 below. The mix had been prepared, then given conditions were programmed on the thermal cycler respectively.

Table 2.20: Components of CloneAmp HiFi PCR Premix.

Components	Reaction Volume: 25 µl
Nuclease-free H_2O	up to 25 µl
Forward Primer	0.2–0.3 µM
Reverse Primer	0.2–0.3 µM
DNA Template	50 ng
2X CloneAmp HiFi PCR Premix	12.5 µl

Table 2.21: PCR conditions for CloneAmp HiFi PCR Premix.

Step	Temperature	Time	Number of Cycle
Initial Denaturation	98°C	10 sec	1
Denaturation	98°C	10 sec	
Annealing	61°C	15 sec	35
Extension	72°C	2 min	
Final Extension	72°C	5 min	1
Incubation	+4 °C	∞	

2.2.6.2 Colony PCR

The colonies selected from the transformed cell containing plates are subjected to Colony PCR by the following procedure. T7 Promoter Forward (5'-TAATACGACTCACTATAGGG -3') and T7 Terminator Reverse (5'-GCTAGTTATTGCTCAGCGG -3') used as primers and Taq polymerase SuperMix (QuantaBio, 95137) is used for DNA polymerase.

1. The selected colony from the agar plate with Kanamycin is dissolved within 20 µL nuclease-free water.
2. Components in Table 2.22 is prepared.
3. Components are exposed to several different temperatures for PCR according to Table 2.23.

Table 2.22: Components of Taq DNA Polymerase for Colony PCR.

Components	Reaction Volume: 25 µl
T7 Forward Primer	2.0 µl
T7 Terminator Reverse Primer	2.0 µl
DNA Template	1.5 µl
Polymerase SuperMix Buffer (2X)	12.5 µl
Nuclease-free H2O	to 25 µl

Table 2.23: PCR conditions for Taq DNA Polymerase.

Step	Temperature	Time	Number of Cycle
Initial Denaturation	94°C	15 min	1
Denaturation	94°C	30 sec	
Annealing	53°C	30 sec	35
Extension	72°C	1 min	
Final Extension	72°C	10 min	1
Incubation	+4 °C	∞	

2.2.7 Agarose gel electrophoresis

Agarose Gel Electrophoresis is a basic technique for separating and visualizing the varying size of nucleic acid fragments according to their length. Several different agarose gels were prepared according to the aim. For observing 528 bp PCR product of insert, 2.0% agarose gel; on the other hand, for observing 5 kbp plasmid, 1.0% agarose gel was prepared. To minimize the inhibition effect of agarose in nucleic acid extraction from agarose gel, 0.7% agarose gel was prepared. All of the agarose gels were prepared in 1X TAE Buffer.

The gel was run under 90V for 45 minutes only if it's for DNA extraction; otherwise, it was run under 110°C for 25 minutes. The gel is visualized by GelDoc™ XR+ System (Bio-Rad, 1708195) or UV.

2.2.8 DNA Extraction from Agarose Gel & DNA Purification

DNA Extraction from Agarose Gel, DNA Purification & Clean-up was performed by using PCR NucleoSpin® clean-up, gel extraction kit (MACHEREY-NAGEL, 740609.50S). The procedure followed for this method was taken from manufacturers' guidelines.

2.2.9 DNA Ligation

To ligate the linearized vector and PCR product of choice, DNA ligation was performed by using the 5X In-Fusion HD Enzyme Premix (Takara, 639648). The procedure was given below.

Table 2.24 reaction mixture was prepared in a tube. This tube was incubated in 15 min at 50 °C. After 15 minutes, it was placed on ice and proceeded to transformation procedure (mentioned in section1.2.1.2.2) or stored at -20°C.

Table 2.24: Components for DNA Ligation with 5X In-Fusion HD Enzyme Premix.

Component	Amount
Digested DNA fragment	150 ng
Linearized vector	100 ng
5X In-Fusion HD Enzyme Premix	2 µl
Nuclease-free H2O	to 10 µl

2.2.10 Sequencing

The constructed pET30+::hG-CSF plasmid was sequenced by Macrogen, Inc.. By using the NCBI/BLAST tool, sequenced results of the plasmid insert complementation with the targeted sequence were checked. The reference DNA and amino acid sequence were gathered from NCBI and the drugbank website was shown in Appendix A.

2.2.11 Growth Curve of *E. coli* BL21 (DE3)/pET30a+::hGCSF

In order to determine the when IPTG induction to be performed, growth curve of BL21 (DE3)/pET-30a(+) was plotted. For that, 10 ml overnight culture was diluted in a 1:50 ratio in 50 ml LB and it's OD at 600 nm was measured in every 15 to 30 minutes depending on the OD while it is incubated at 37°C for 12 hours.

2.2.12 Isolation of inclusion bodies and wash

The harvested pellet of BL21 (DE3) cells with pET30a+::hG-CSf plasmid was taken from -20 and dissolved with the lysis buffer in 1:10(v/v) ratio. Then, they were sonicated with appropriate probe by 8 seconds on 16 seconds off for 4 minutes. Sonication should make sonicated cells with buffer to be clearer. Then it was centrifuged at 17000xg for 25 minutes at +4°C. The soluble proteins that reside in the supernatant was discarded while inclusion bodies in the pellet was kept.

Pellet was washed with wash buffer (WB) and incubated with it for 30 minutes at shaker. It was centrifuged at 17000xg for 25 minutes at +4°C. The supernatant was discarded while pellet is kept. This washing step was repeated for one more time.

2.2.13 Solubilization and Refolding of hG-CSF

The washed pellet is then dissolved with solubilization buffer (SB) in 1:10 w/v ratio and incubated in it for at least 3 hours at shaker. It is centrifuged at 17000xg for 25 minutes at +4°C. The pellet is discarded.

The supernatant, that has solubilized inclusion bodies, was subjected dialysis from MWCO 3.5K (Thermo Scientific, 68035) for 16 hours against 25 mM sodium acetate pH 4.5.

2.2.14 Purification

The purification of recombinant hG-CSF from total protein was performed by cation exchange chromatography (CEX) using ÄKTA avant 25 (Cytiva, 28930842) system. For this purpose, 1 ml HiTrap SP-FF cation exchange column (Cytiva, 17505401) was used. Column is equilibrated with 25 mM sodium acetate pH 4.5 for 5 column volume (CV), washed with 25 mM sodium acetate pH 4.5 for 5 CV and eluted with linear gradient of 25 mM sodium acetate, 1 M NaCl pH 4.5 for 15 CV. In all steps, flow rate was maintained at 1 ml/min at room temperature. Collection of purified fractions are stored at +4°C. The purification steps are analyzed by 15% SDS-PAGE and immunoblotting assays.

2.2.15 Characterization

2.2.15.1 SDS-PAGE and immunoblotting

SDS-PAGE is a technique that helps to separate and identify proteins according to their molecular weight. Gels were prepared according to the preparation recipe in section 2.1.5. After preparing the gel solution, the rack was assembled for gel solidification for both the separation and stacking gel respectively. After placing the gel on the tank and placing the Running buffer, proteins are added on each well. Meanwhile, samples were prepared by mixing them with 5X Laemmli buffer and boiling them at 95°C for 5 minutes. Denatured protein samples were loaded on gels. Running is done under 90 V until marker passes stacking gel, after that, it is increased to 120 V for approximately 1.5 hours.

Electrotransfer, a procedure to transfer molecules on the gel into the nitrocellulose membrane under the electric field, performed by using Amersham WB Systems by

either 16 V for 16h or 100 V for 90 minutes. After the transfer has done, the membrane was blocked with 5% non-fat milk in TBS-T. Then, the membrane was incubated with a primary antibody in a 5% BSA solution overnight in +4°C with a rotation. Membranes were cleaned with TBS-T for 3 times for 5 -10 minutes each. Then, they were incubated with a secondary antibody, which was conjugated with HRP, at room temperature for an hour. With the help of the Clarity Western ECL Substrate (Bio-Rad, 1705060), the membrane was visualized via ChemiDoc™MP System (Bio-Rad, 1708280).

If the gel itself wanted to be observed, electrotransfer has not performed. The gel was incubated with Coomassie Brilliant Blue staining solution right after the gel run was completed. It is incubated overnight. Then, Coomassie Brilliant Blue staining was discarded and gel was incubated with a destaining solution until blue color on the gel is diminished and proteins are visible. Finally, the gel was visualized via Bio-Rad ChemiDoc™MP System (Bio-Rad, 1708280). Analysis were performed on ImageLab software (Bio-Rad).

2.2.15.2 SEC-HPLC

In order to analyze the oligamarizton as well as to determine the protein concentration of purified recombinant hG-CSF, size-exclusion chromatography (SEC) was performed by using UltiMate™ 3000 (Thermo Scientific, 5200.0355). This HPLC system is equipped with BioSuite 250, 10 µm SEC (Waters, 186002170) column (7.5 mm I.D. with a particle size of 10 µm). The detector was set at 280 nm. The areas of the peaks were integrated by using Chromeleon 7.2.9.11323 software system. The SEC-HPLC experiment was performed by using 1 X PBS, pH 7.4 as mobile phase at room temperature. Injection volume for each sample is 0.7 ml and flow rate were maintained at 0.6 ml/min for each run.

2.2.15.3 Circular dichroism spectrometry

Secondary structure evaluation of recombinant hG-CSF and reference has been measured by Jasco-J 1500 Circular Dichroism (CD) spectrophotometer (Jasco, Easton, MD). The measurements of spectra were taken from 195 to 260 nm with 1 nm bandwidth, 50 nm/min speed and 2 s response time using 1 mm path length quartz cell at 25°C. The protein was in the formulation buffer (FB) and spectra was smoothened.

α-helix structures usually have positive band at 190 nm and two negative bands at 220 nm and 200 nm.

2.2.15.4 Peptide mapping

Determination of the primary structure of purified hG-CSF and the reference product was confirmed by using peptide mapping analysis with Liquid Chromatograph-Mass Spectrometry/ Mass spectrometry (LC-MS/MS) analysis. For this purpose, proteins were separated in SDS-PAGE, then this bands are in-gel digested with Endoproteinase Glu-C from *Staphylococcus aureus* V8 (Sigma, P6181) 1:20 ratio for 16 hours. When digestion is performed in phosphate buffer at pH 7.8, it selectively cleaves the C terminus of the glutamic acid and aspartic acid. Peptides were subjected to reverse-phased chromatography on ACQUITY UPLC® Peptide BEH C18, 300Å, 1.7 µm, 2.1 mm x 100 mm column (Waters, 186003686).

The peptides were analyzed by using the integrated protein characterization system UNIFI of Waters proteomics systems.

2.2.15.5 Intact mass analysis

Intact mass analysis is performed in order to measure and compare the molecular weight of the recombinant G-CSF and reference. For this analysis samples were analyzed on SYNAPT G2-Si High Definition Mass Spectrometry. BEH C4 300Å, 1.7 µm, 2.1 mm x 50 mm column (Waters, 186004495) is used for this analysis. Flow rate is maintained at 0.3 ml/min. 5-10 µl of sample was used for analysis.

2.2.15.6 Capillary electrophoresis

SDS-MW Capillary electrophoresis analysis was performed on PA800 PLUS system (Beckman Coulter) with 50 µg sample. Samples were concentrated until their concentration reaches to 50 µg in 50 µl. Then, 45 µl SDS-MW sample buffer, 5 µl 250 mM Iodoacetamide (IAM) and 2 µl internal 10 kDa was added on the sample respectively. The mixture was heated on the heat block at 70°C for 10 minutes. The samples are placed on the sample tray and the buffers needed for the run is prepared and place on the inlet and outlet tray according to the Beckman Coulters' manual.

2.2.15.7 Host cell protein

Since recombinant proteins are manufactured in a *E. coli,* its remaining proteins in the product was estimated with *E. coli* host cell protein ELISA kit (Cygnus Technologies, F410). For that, 3 sets of 25 µl purified hG-CSF is assayed in duplicate. Assay is performed according to its manual guideline.

2.2.16 Biological activity analysis

In order to observe whether the purified recombinant human G-CSF is biologically active or not, target protein and reference product was given to human monocyte THP-1 cells, which cells also expresses G-CSF receptor highly. Cells were seeded into 12-well plate as $1x10^6$ cells/ml. 50 ng/ml either G-CSF or reference product is given within 1X PBS. Cells are harvested at after incubating them at 37°C with 5% CO_2 for various time.

THP1 suspension cells are centrifuged at 300xg for 6 minutes at +4°C. The cells are washed with 1X PBS and then solubilized with appropriate mammalian cell lysing buffer and incubated on ice for 25 minutes. Then, it is centrifuged for 20 minutes at 17000xg at +4°C. Pellet is discarded while supernatant is kept at -20°C until further use.

3. RESULTS

3.1 Construction, Amplification, and Cloning of hG-CSF

3.1.1 RNA isolation

We aimed to clone human G-CSF protein from a human malignant glioblastoma cell line, U87 MG. For this purpose, firstly, U87 MG cells were grown in cell culture and their RNA was isolated. Since the cDNA library aimed to be constructed from the isolated RNA, their integrity was checked by an agarose gel electrophoresis run. Its results are given in Figure 3.1.

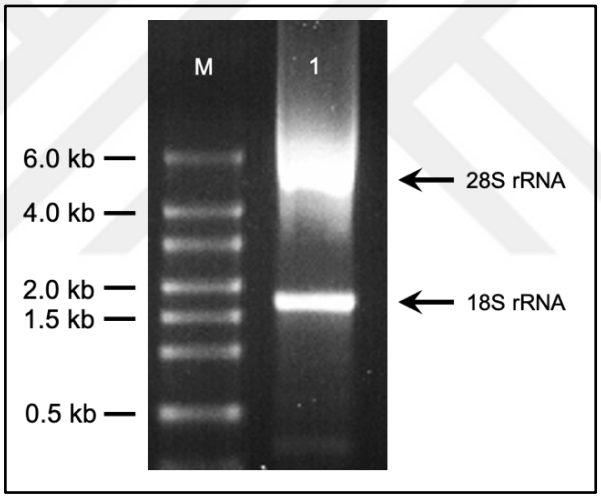

Figure 3.1: The integrity check result of isolated RNA's from U87-MG cells on the denaturing agarose gel. Lane M. RiboRuler High Range RNA Ladder, Thermo Scientific. Lane 1. 6 µl isolated total RNA. 1.5 % Agarose / TAE Gel.

In a successful total RNA isolation, both human 18S and 28S ribosomal RNAs (rRNAs) are expected to be detected around approximately 1900 bp and 5000 bp respectively. According to the results in Figure 3.1, both human rRNAs were spotted sharply in agarose gel electrophoresis results. Thus, this total RNA isolation was successful to translate them into cDNA.

3.1.2 G-CSF PCR

Having been RNA isolated, cDNAs were reverse transcribed into cDNA. The specific *CSF3* gene was targeted and amplified from those cDNAs using PCR with primers specific to the G-CSF sequence. The PCR results were run on an agarose gel electrophoresis system and is shown in Figure 3.2.

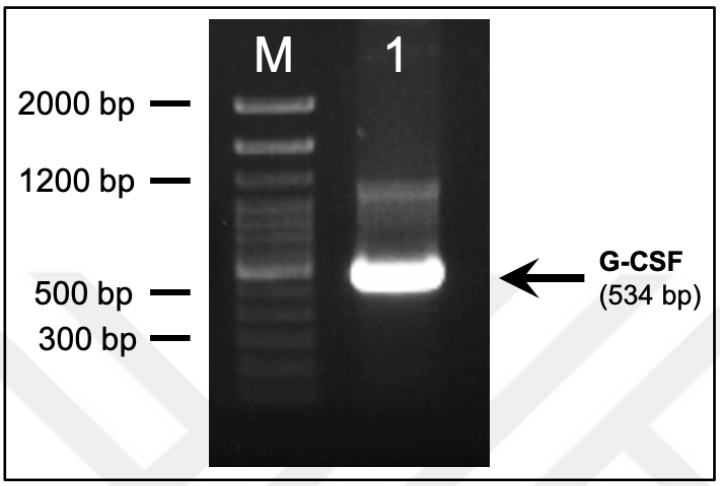

Figure 3.2: The G-CSF PCR results. Lane M. TrackIt! 100 bp marker, Thermo Scientific. Lane 1. 25 µl PCR product from cDNA with 1 µl 6X DNA Loading Dye. 1.0 % Agarose / TAE.

3.1.3 Enzyme digestion, ligation, and verification

The amplified PCR product was isolated from the gel; thereafter, digested with appropriate digestion enzymes, EcoRI and NdeI, to prepare them for ligation with bacterial vector. Meanwhile, the bacterial vector was linearized by digesting it with similar restriction enzymes. The digested, linearized vector and the digested insert product were ligated by using an in-Fusion kit. The ligated plasmid construct has been transformed into Stellar chemical competent cells. Later, several colonies have been selected from the LB agar growth to check colonies with successfully ligated constructions. Therein, colony PCR has been conducted and its result of the agarose gel electrophoresis run is shown in Figure 3.3.

Colony PCR primers have selected on both down and upstream of the ligated gene on the constructed plasmid. In other words, those sites lay upon the vector itself: therefore, if the ligation would be successful, the length of the colony PCR product should be around 745 bp.

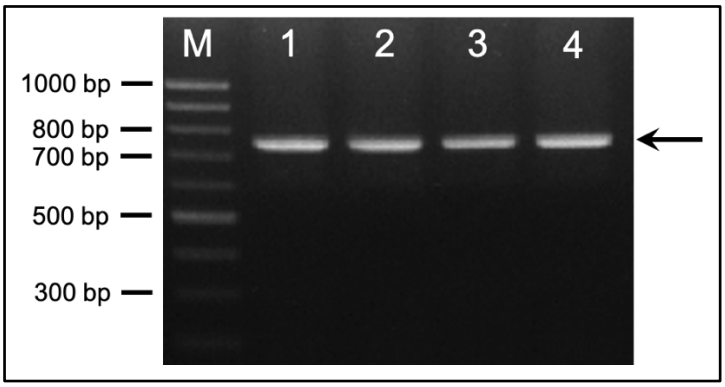

Figure 3.3: The colony PCR results of ligated pET30::hG-CSF plasmid. Lane M, 1kb DNA ladder, GeneON. Lane 1, 20 µl PCR product from colony 1. Lane 2, 20 µl PCR product from colony 2. Lane 3, 20 µl PCR product from colony 3. Lane 4, 20 µl PCR product from colony 4. 1.5 % Agarose / TAE Gel.

Among the positive and false-positive colonies, a colony has been selected for plasmid isolation and restriction enzyme digestion. Restriction enzyme digestion is the second verification for ligation success. The constructed plasmid was digested with both one and double restriction enzymes, EcoRI and NdeI. The expectation was the detection of the insert and ligated plasmid around 528 and 5260, respectively. Its agarose gel electrophoresis result is shown in Figure 3.4. Subsequently, they have sent to sequencing for verifying the accuracy of both plasmids and insert. This sequencing result has G-CSF Expression.

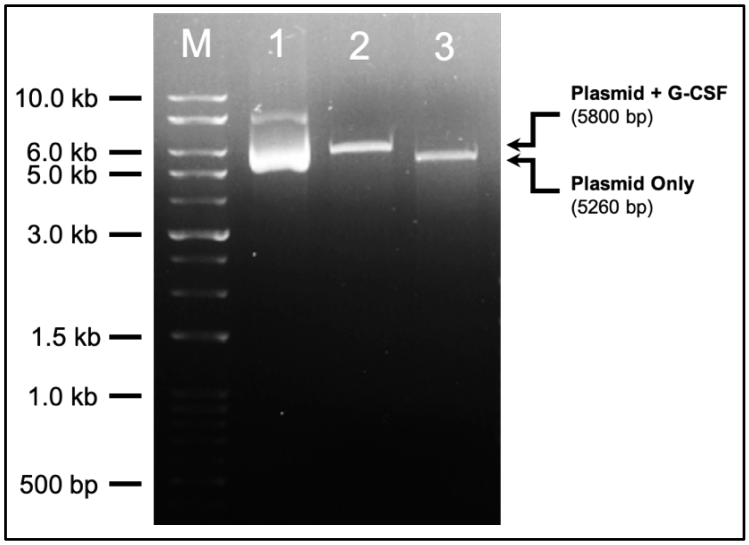

Figure 3.4: The restriction enzyme digestion results. Lane M, 1kb DNA ladder, GeneON. Lane 1, negative control. Lane 2, pET-30a(+) ::hG-CSF with a restriction enzyme digestion. Lane 3, pET-30a(+) ::hG-CSF with double enzyme digestion. 1.5 % Agarose / TAE Gel.

3.2 Production of hG-CSF

3.2.1 Growth curve of G-CSF plasmid contained bacteria

In order to determine the induction time of the *Escherichia coli* growth, the graph of OD_{600} versus time, or growth curve shown in Figure 3.5. According to that graph, induction time is selected when the OD is between 0.60-0.70.

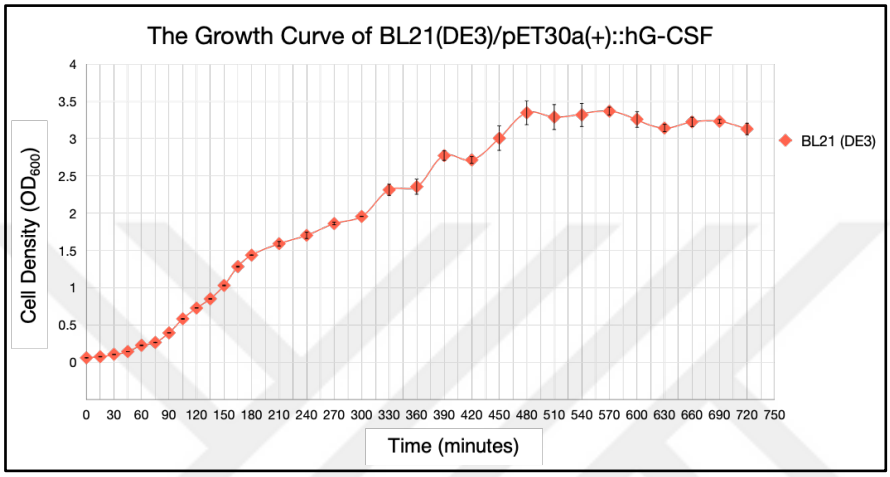

Figure 3.5: The growth curve of *E. coli* BL21(DE3) cells containing pET-30a(+) ::hG-CSF plasmid in LB for 12 hours at 37°C and 200 rpm.

3.2.2 The optimum protein expression

To obtain the optimum G-CSF production condition while minimizing the inclusion body formation, several strategies were followed. These strategies were on deciding the optimum bacterial strain, IPTG concentration for induction, time duration of IPTG induction, and temperature to perform IPTG induction.

Since pET-30a(+) ::hG-CSF has been artificially given to bacteria, whether those cell are producing it or not under IPTG condition was determined by growing them at 37°C for 4 hours. Its SDS-PAGE and the immunoblotting results are shown in Figure 3.6.

The optimum IPTG induction concentration for pET-30a(+) ::hG-CSF containing *E. coli* BL21(DE3) cells was decided after looking at the production amount of G-CSF at 4 different IPTG concentration, 0.25 mM, 0.5 mM, 0.75 mM and 1.0 mM. Its SDS-PAGE and the immunoblotting results are given in Figure 3.7 and Appendix C, respectively. Accordingly, the best IPTG concentration for induction was 0.50 mM.

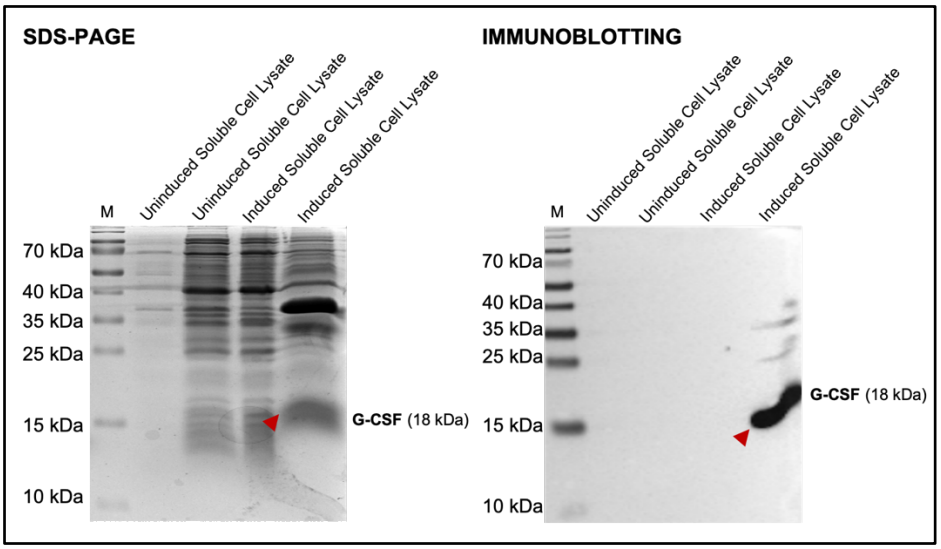

Figure 3.6: The IPTG induction results of the optimization of hG-CSF protein production in *E. coli* BL21 (DE3) cells. Left shows the SDS-PAGE results while right shows the Immunoblotting. M is PageRuler marker, Thermo Scientific.

The optimal incubation temperature after IPTG induction for pET-30a(+) ::hG-CSF containing *E. coli* BL21(DE3) cells were decided after looking at the production amount of G-CSF at 3 different temperatures: 18 °C overnight, 30°C for 6 hours and 37°C for 4 hours. Its SDS-PAGE and the immunoblotting results are given in Figure 3.7 and Appendix C, respectively. Consequently, the incubation temperature of these cells was 37°C.

The ideal incubation duration after IPTG induction for pET-30a(+) ::hG-CSF containing *E. coli* BL21(DE3) cells were decided after looking at the production amount of G-CSF at 4 different duration, 2 hours, 4 hours and 6 hours. Its SDS-PAGE and the immunoblotting results are given in Figure 3.7 and Appendix C, respectively. Herein, the best incubation duration of these was 3 hours.

3.2.3 Solubilization of inclusion bodies

In order to start purifying the human recombinant G-CSF from the pellet, hrG-CSF that in the inclusion bodies must already be solubilized in a solubilization buffer (SB). Before solubilization buffers, inclusion bodies that reside in the pellet are washed twice with the Wash Buffer (WB). Its SDS-PAGE and immunoblotting results are shown in Figure 3.8 below. Accordingly, G-CSF is successfully solubilized in the solubilization buffer and pellet is also cleared from other cell proteins.

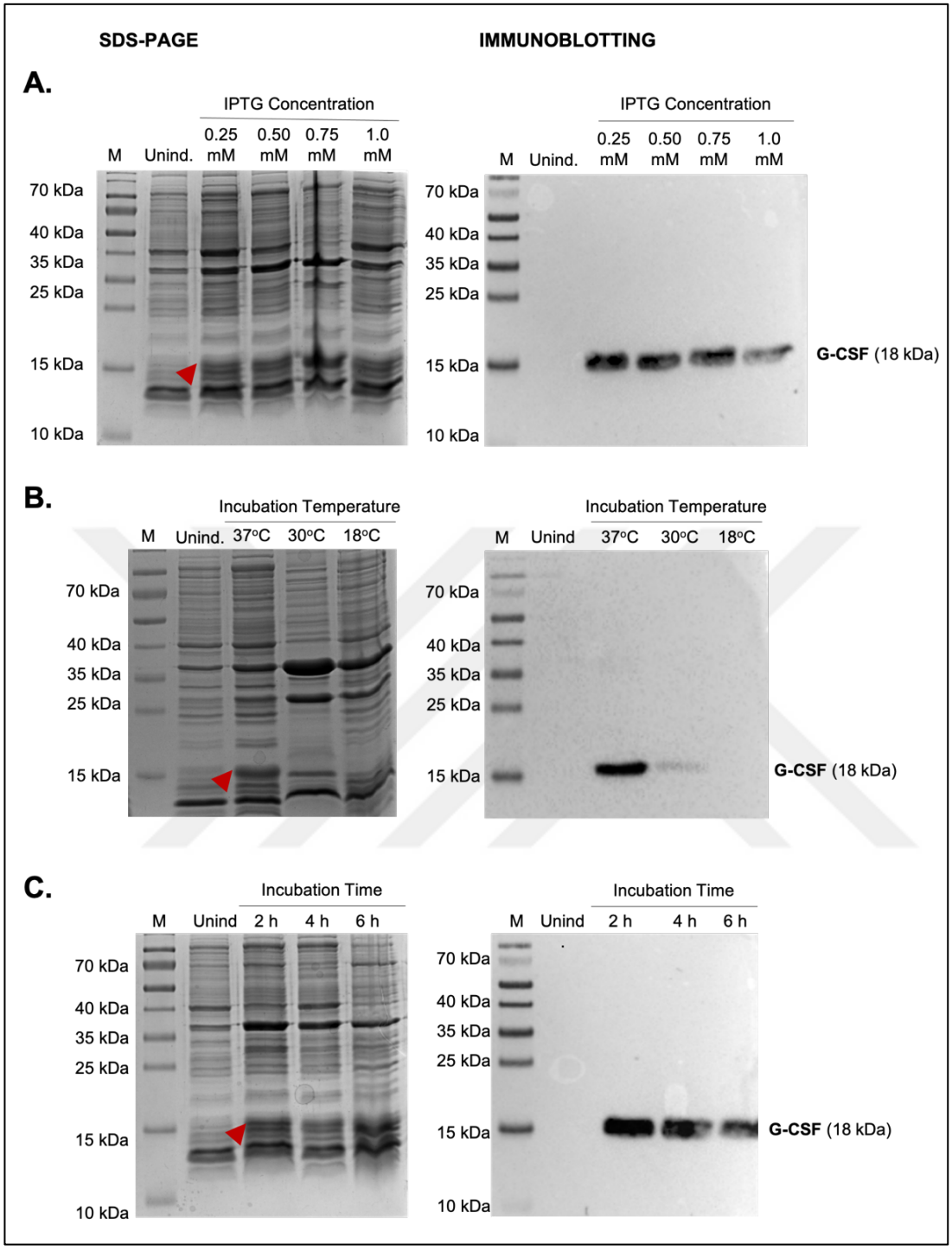

Figure 3.7: The SDS PAGE (on the left) and immunoblotting (on the right) results of the optimization of hG-CSF protein production from *E. coli* BL21 (DE3) cells pellet. (A.) shows different (0.25-1 mM) IPTG concentration result (B.) shows different temperature growth (37°C, 30°C, and 18°C) after IPTG induction result and (C.) shows different time duration (2, 4, 6 hours) after IPTG induction result. M is PageRuler marker, Thermo Scientific, Unind. is uninduced-control.

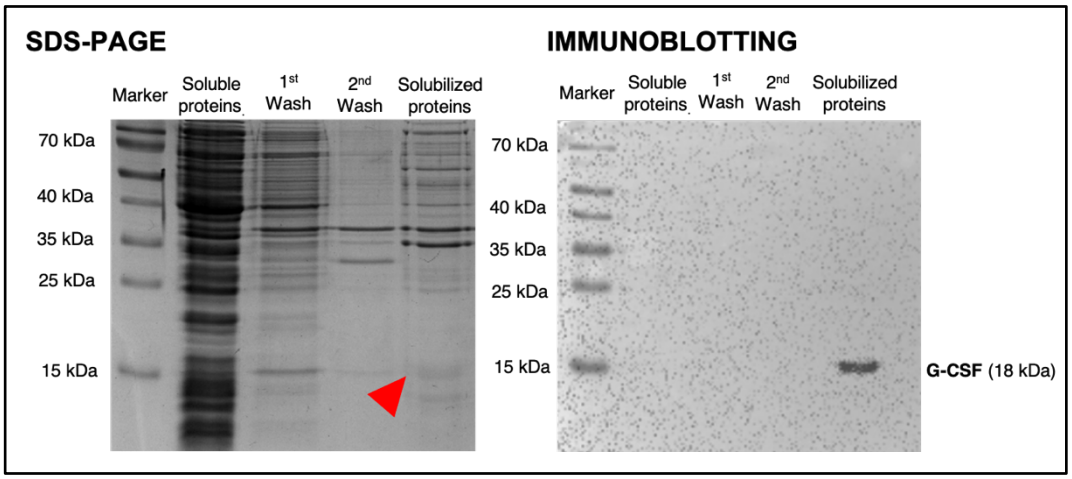

Figure 3.8: The (A). SDS-PAGE and (B). immunoblotting result of solubilization and denaturation of G-CSF. M is PageRuler marker, Thermo Scientific.

3.3 Cation exchange chromatography purification of target protein

When this protein is solubilized in 8 M Urea, its buffer is exchanged to binding buffer for purification via dialysis.

Then, this product is loaded on to HiTrap SP-FF 1 ml CEX column. Binding buffer is 25 mM Sodium Acetate, pH 4.5 and Elution Buffer is 25 mM Sodium Acetate, 1 M NaCl pH 4.5. Elution peaks numbered on the Figure 3.9 is loaded on to the both SDS-PAGE and Immunoblotting to detection the G-CSF (Figure 3.10).

According to Figure 3.10, recombinant hG-CSF has been eluted between fraction 3 and 4 mostly. The fractions in which elution of G-CSF is the highest (3-4) are collected. Then, the G-CSF contained solution has concentrated and buffer exchanged with 3K membranes against formulation buffer (FB). End product human G-CSF, which is in FB buffer, is characterized. Other replicates are given in Appendix C.

3.4 Characterization

3.4.1 Intact mass

Intact mass analysis was performed in order to assess and compare the molecular weight of recombinant human G-CSF and the reference product, Neupogen from Amgen by using mass spectrometry (MS). For this purpose, 10 µl of 0.1 mg/ml protein is used. Its chromatogram with the deconvolute mass spectrometry data is shown in Figure 3.11. The estimated molecular weight of recombinant hG-CSF and the reference is 18800 Da and 18798 Da, respectively.

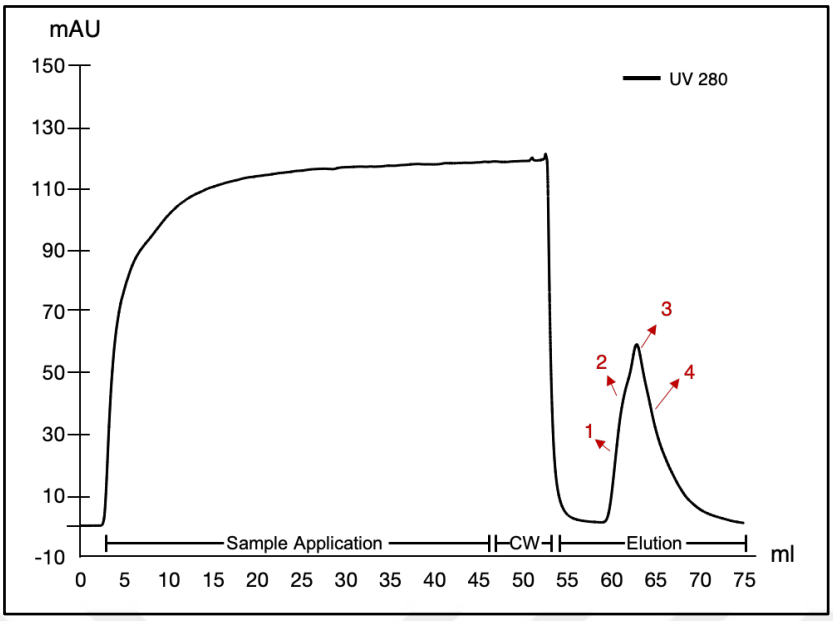

Figure 3.9: Cation Exchange chromatography results. Human recombinant G-CSF is isolated from BL21(DE3) cells that carries pET30(a)+::hG-CSF plasmid upon induction with IPTG. Cation exchange binding buffer is 25 mM sodium acetate pH 4.5 and elution buffer is 25 mM sodium acetate, 1 M NaCl pH 4.5. The collected elution fractions are showed in number.

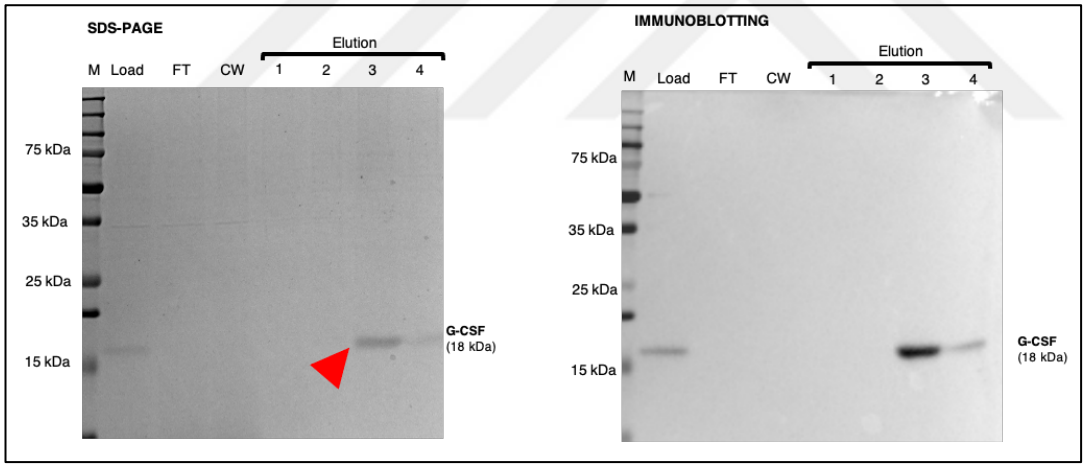

Figure 3.10: SDS-PAGE (15%) and immunoblotting results of purification of recombinant human G-CSF. M is Bio-Rad's PageRuler Precision Plus Protein™ Dual Color Standards, FT is Flow-through, CW is Column Wash. The peaks of the numbers in the elution are showed on chromatogram in Figure 3.9.

3.4.2 Peptide mapping

In order to determine the primary structure of the protein bands seen on SDS-PAGE gel of reference and purified product, peptide mapping method is performed on LC-MS/MS. Its results are shown in the Figure 3.12. Accordingly, the main band around 18 kDa of reference and the product belongs to G-CSF and their amino acid sequence is alike.

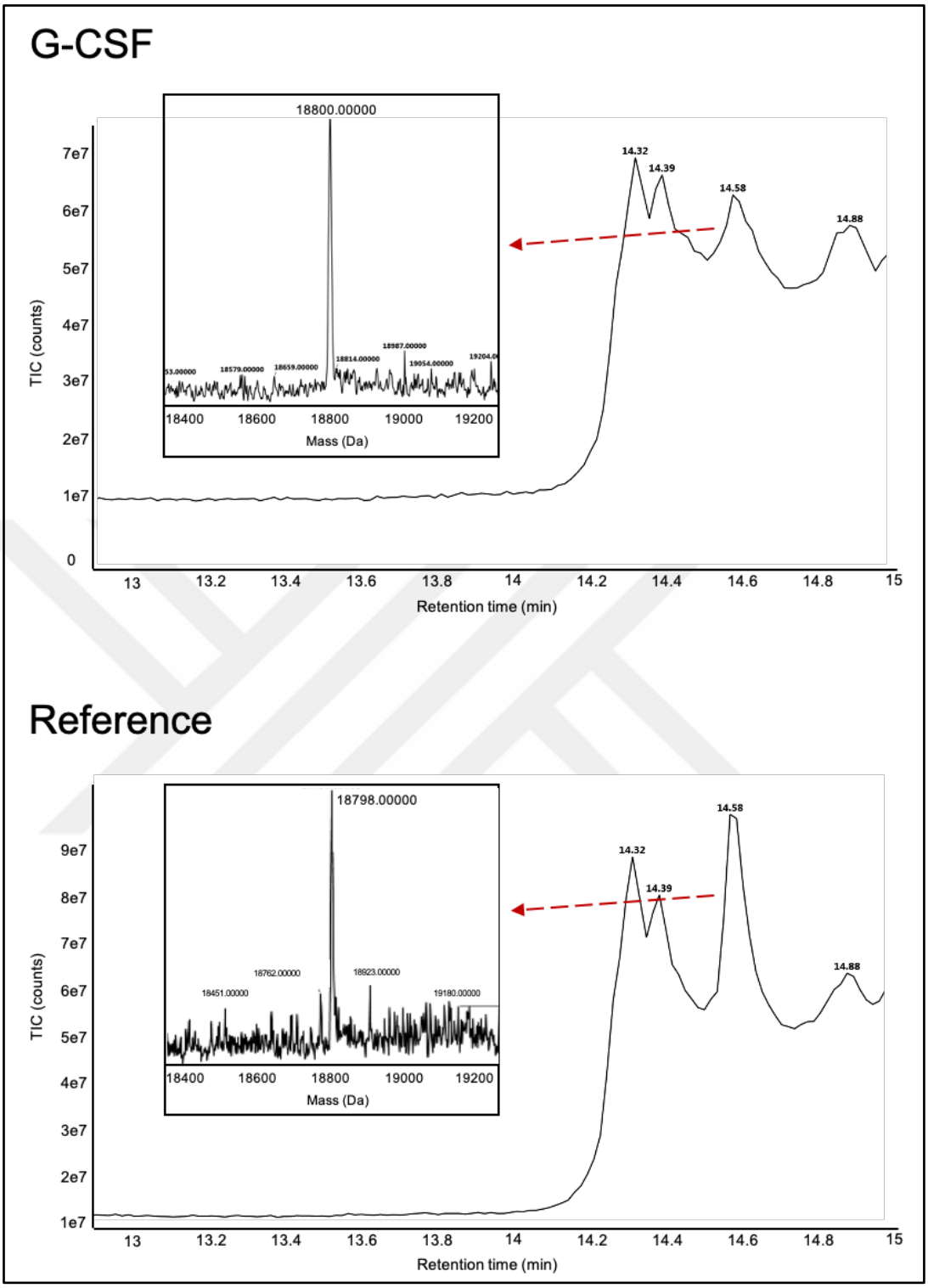

Figure 3.11: LC/MS chromatography of recombinant human G-CSF (above) and the reference product, Amgen (below). Inset: Deconvoluted mass spectra of peaks corresponds to G-CSF peak.

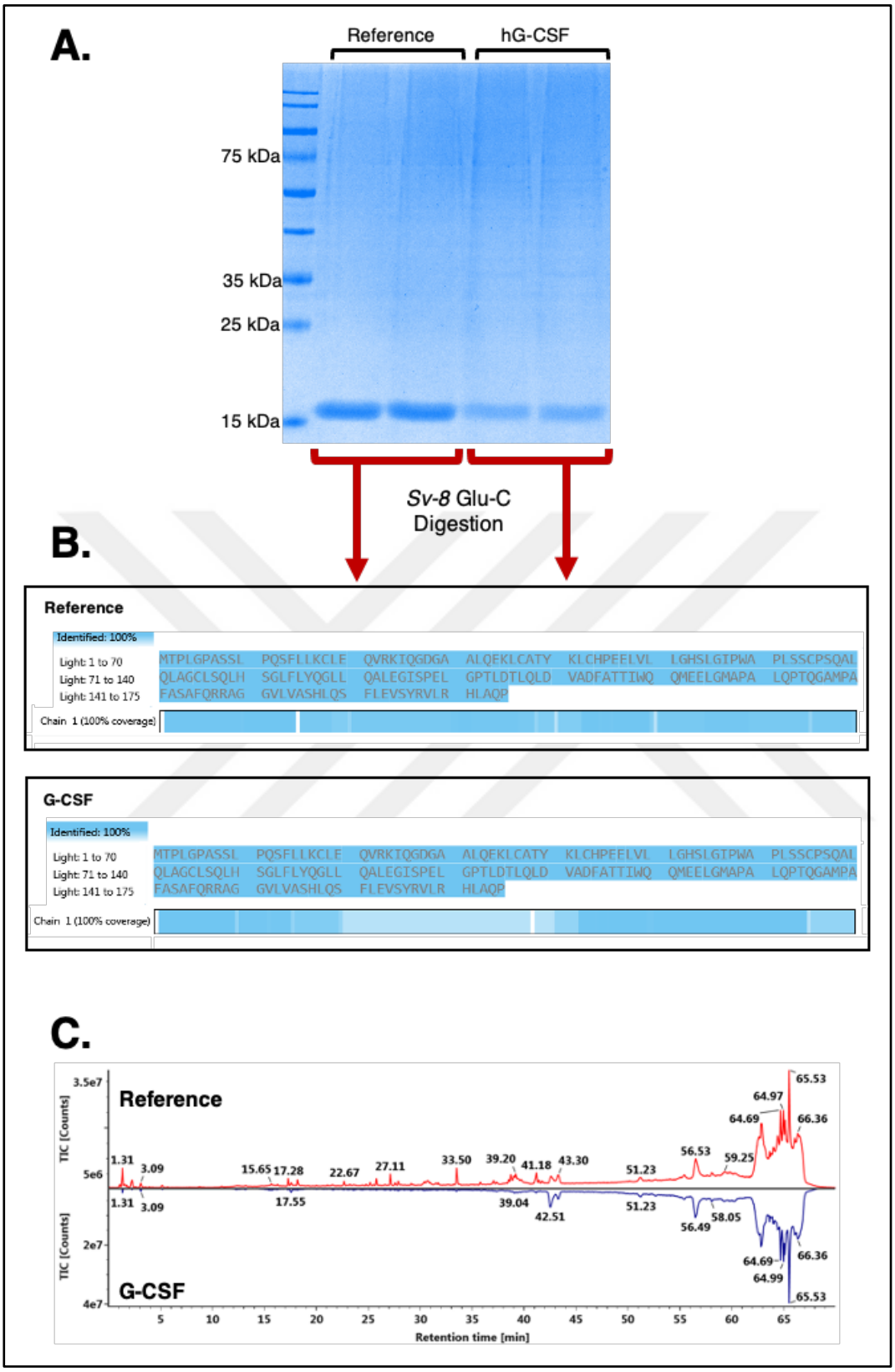

Figure 3.12: Peptide mapping results of G-CSF proteins. They are digested with *Sv*-8 Glu-C enzyme from SDS-PAGE on the A. The coverage of the reference (above) and hG-CSF (below) shown on the B. and C. shows the chromatography of reference (above) and hG-CSF (below).

3.4.3 Secondary structure analysis with Circular Dichriosm

To have a biologically active human G-CSF, it needed to be folded correctly. In other words, be in its native form. Therefore, after its denaturation with 8 M urea, it is refolded again to form its native form via step-wise dialysis from MWCO 3.5K membrane.

One of the methods to check the this refolding process' success is circular dichroism, CD analysis, whose data is collected at far UV, from ~195 to 260 nm. The result shown in Figure 3.13 presents the double negative extrema at 208 and 222 nm for both reference and our product. Thus, our product and reference have similar secondary structure.

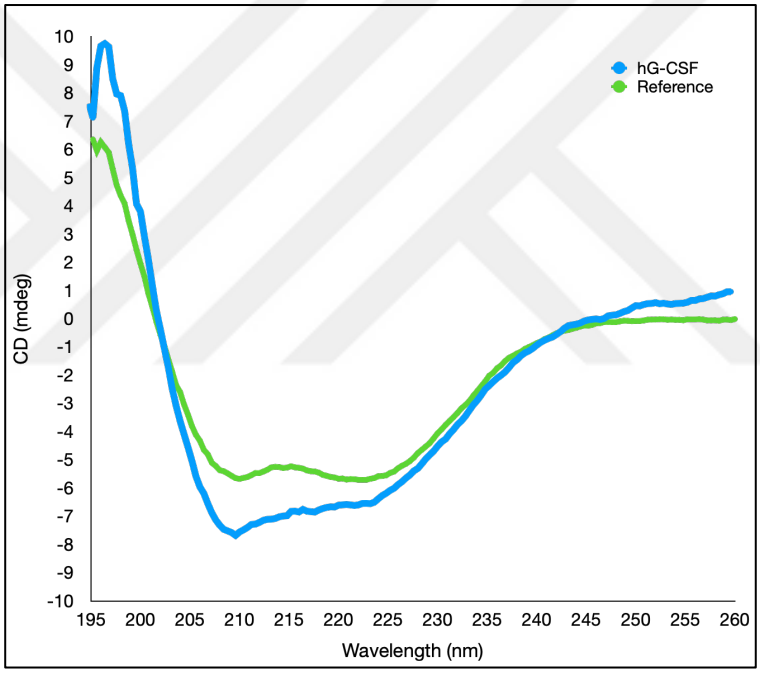

Figure 3.13: The Circular dichroism spectra of G-CSF product (blue) and reference (green) in formulation buffer (FB).

3.4.4 SEC-HPLC analysis

Human G-CSF in the formulation buffer is subjected to SEC-HPLC in order to determine the oligomeric state of the purified hG-CSF. Its chromatogram is presented in Figure 3.14 and migration time of three replicate is given in Table 3.1. According to the result and comparison with the standard SEC proteins given in Appendix E; human recombinant G-CSF that is eluted at 17.843 minutes, and it is purified as monomer successfully.

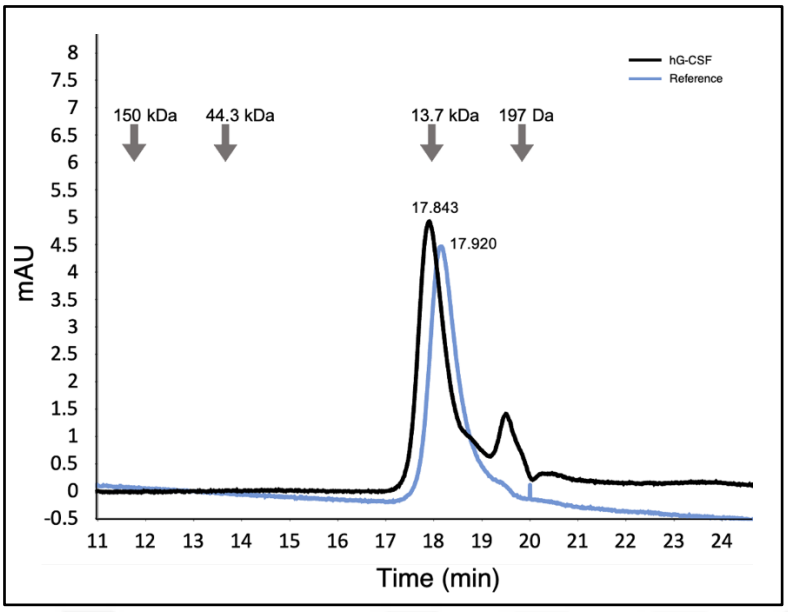

Figure 3.14: The SEC-HPLC chromatogram of the SEC analysis of 0.05 mg/ml purified hG-CSF and the reference. The black line shows purified hG-CSF; blue line shows reference Neupogen, Amgen. Size Standards are presented in Appendix D.

Table 3.1: Retention time of purified human G-CSF main peak.

Name	Retention Time (min)
G-CSF-1	17.843
G-CSF-2	17.904
G-CSF-3	17.984

Expect the determination of the oligomerization state, SEC-HPLC is used for estimating the concentration of the purified protein. Titer of the final hG-CSF product from 1 L growth is given in the Table 3.2.

Table 3.2: Titer of the final hG-CSF product per liter growth.

Name	Titer (mg/ml per liter bacterial culture)
G-CSF-1	0.109
G-CSF-2	0.087
G-CSF-3	0.081

3.4.5 Capillary Electrophoresis

The determination and the comparison of impurity as well as the purity assessment of both human G-CSF and the reference is achieved by subjecting them to the capillary electrophoresis system. The Figure 3.15 shows the G-CSF and the reference product has >95% purity.

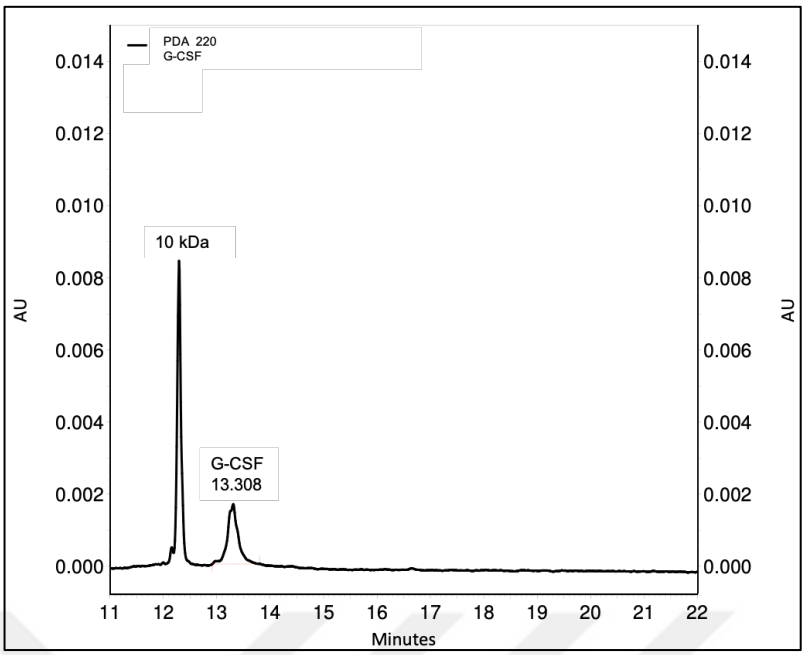

Figure 3.15: Electropherogram of the 50 µg non-reduced G-CSF (red) and reference (black) product.

3.5 Host Cell Protein

The recombinant protein production in *E. coli* may cause the contamination of host cell (*E. coli* BL21 (DE3)) protein. In order to determine whether this contamination is in an acceptable level, enzyme-linked immunosorbent assay (ELISA), which uses antibodies specific to host cell, is performed. Each sample is assayed in duplicate. According to the guidelines and literature, the acceptable HCP amount must be less than 100 ng/ml (100 ppm)(Bracewell, 2015). HCP amounts of produced G-CSF lower than the 100 ppm, thus it is in acceptable level.

Table 3.3: Host cell protein ELISA results of hG-CSF.

Name	HCP amount (ng/ml)
G-CSF-1	0.089
G-CSF-2	1.940
G-CSF-3	2.213

3.6 Biological Activity

In addition to purification and characterization, biological activity of hG-CSF product is tested in human monocytic THP-1 cells. Total protein of this monocytes is used for determination of biological activity of G-CSF product and reference. For this

experiment, the level of p-ERK is investigated upon stimulation of 50 ng/ml hG-CSF and reference product. The results are shown at Figure 3.16.

Upon stimulation of G-CSF either produced by us or the reference, the phosphorylated ERK 1/2 levels are started to increase after 30 minutes ($p < 0.001$ and $p < 0.05$ respectively) (Figure 3.16B and Figure 3.16D). Thus, MAPK pathway stimulation has reached its peak around 30 minutes, then started to decrease.

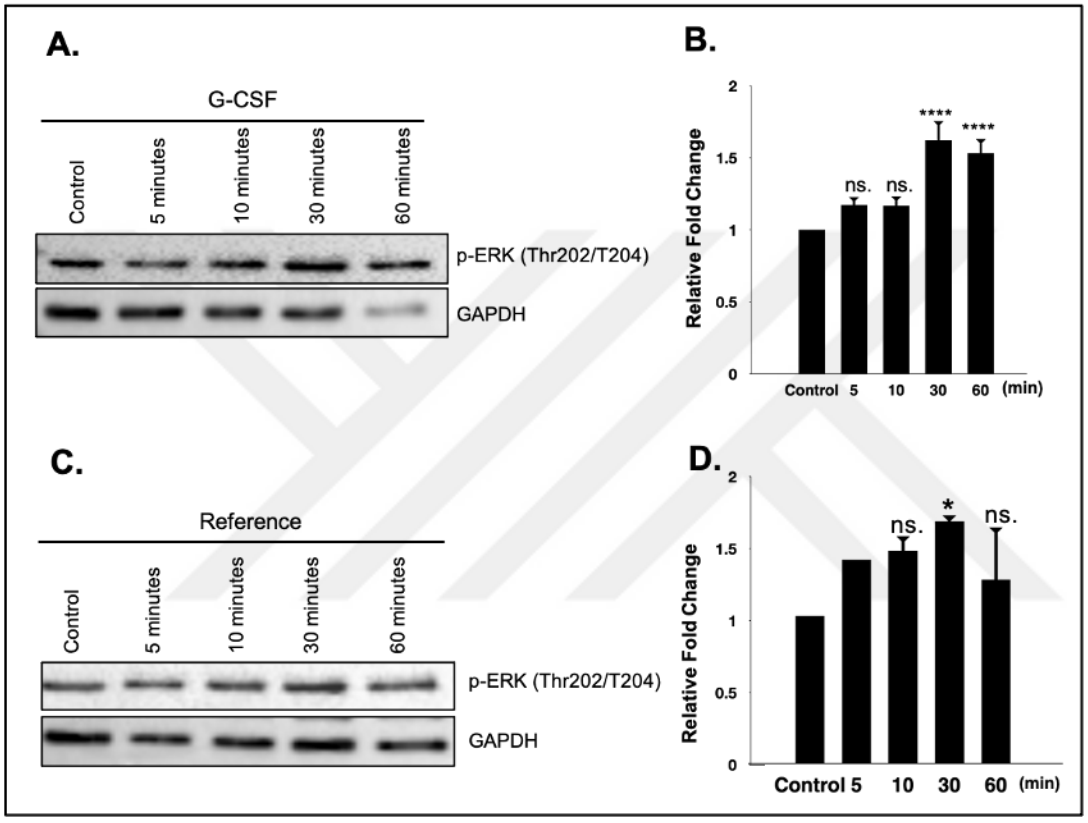

Figure 3.16: The p-ERK levels upon stimulation of (A and B.) G-CSF and (C. and D.) reference product.

4. DISCUSSION

Biosimilars, biologic medicines, are being developed from cells or living organisms; thus, these complex structures are not precisely the same product with reference. Consequently, FDA, EMA, and World Health Organization (WHO) have published a strict regulation of biosimilars in case of quality, efficacy, or safety (Reviewed in Araújo, Gonçalves, & Fonseca, 2016). Neupogen's biosimilar *Zarzio*, which only differs in its buffer system, has been mentioned to be on the European and the US markets since 2009 and 2015, respectively, and has allowed to save millions of euros in Europe (Gascón et al., 2013). Therefore, this small glycoprotein's cloning, production, and purification have been crucial for the pharmaceutical industry.

The major production hosts of recombinant human G-CSF are mainly bacteria *E. coli* and mammalian Chinese Hamster Ovary, CHO, cells. In lenograstim, latter cells have been used as production hosts because G-CSF has one O-linked glycosylation at the Thr^{133} position and these cells are able to present glycosylation pattern, while prokaryotic bacteria cell cannot (Boubevaa et al., 2012; Hill et al., 1993). Thus, some biosimilars of G-CSF have been produced in cells since these cells are able to present glycosylation. Yet, a report by Hill et al. mentioned that O-linked glycosylation is not important for biological activity (Hill et al., 1993). Additionally, they have been reported to have no significant differences between glycosylated and non-glycosylated G-CSF in case of pharmacokinetic and pharmacodynamic profiles *in vivo* (Tanaka et al., 1997). On the other hand, *E. coli* who cannot perform glycosylation has low nutrient requirements with a high growth rate and well-known genetics as well as physiology (Babaeipour, et. al, 2010). Therefore, this has been used as a major production host for recombinant human G-CSF at both a small and large scale. (Babaeipour, et. al, 2010). Consequently, bacteria systems have been used to optimize the production of hG-CSF in this thesis.

The cloning process of any gene starts with creating a DNA library which is a pool of DNA fragments of a particular organism. Typically, two kinds of libraries are used for cloning; they are either genomic DNA, gDNA library, or complementary DNA, cDNA library (Thieman & Palladino, 2004). The human genome contains nonprotein coding

sequences called introns as well as protein-coding sequences exons. Using the gDNA library for cloning would clone exons and introns as well. Since most of the eukaryotic genome consists of introns, these libraries would consist of majorly noncoding fragments (Thieman & Palladino, 2004). However, cDNA libraries are created from reverse transcription of isolated mRNA to its complementary DNA. Therefore, this library only contains expressed genes in the cells (Thieman & Palladino, 2004).

In order to capture the G-CSF protein-coding sequence, one of the most GCSF expressed cells, human malignant glioblastoma U87 MG cells' cDNA is used. Glioblastoma, U87-MG cells are presented to be one of the highly G-CSF expressed cells that do not require any induction for G-CSF production (Wang et al., 2012). For cloning purposes, U87 MG cells' RNA were isolated and this isolation was successful as most of the RNA in human cells are ribosomal 28S and 18S RNA – those of which were presented in Figure 3.1.

After cloning processes have been performed, the formed pET-30a(+)::hG-CSF plasmid has transformed with the protein expression *E. coli* strain: BL21 (DE3). hG-CSF gene is under the control of the T7 promoter, which is a strong bacteriophage promoter that is under the control of IPTG – a structural analog of lactose who does not become a part of the metabolic pathway. Therefore, an IPTG addition to the growth media would cause production G-CSF and, consequently, leads to the production of protein aggregates (inclusion bodies). In order to obtain active proteins, these insoluble inclusion bodies should be solubilized and refolded. (Hoffmann et.al., 2018). The washing of inclusion bodies and solubilization of them were successfully performed as shown in Figure 3.8. After this solubilization, G-CSF is refolded meanwhile buffer is exchanged by subjecting it in urea contained buffer.

The solubilized and refolded G-CSF is then purified by cation exchange chromatography and its chromatogram result is presented in Figure.3.9. The fractions of purification are collected and are analyzed via several characterization techniques. The purity and approximate molecular weight of the target protein in fractions are shown with SDS-PAGE and immunoblotting in Figure 3.10. Accordingly, the other cell lysate proteins are successfully separated from G-CSF. In order to preserve the native form of G-CSF, it is buffer exchange into FB.

The other way to estimate the molecular weight is intact mass analysis with mass spectrometry. G-CSF and reference is subjected to LC/MS and shown in Figure 3.11.

This result showed that, the molecular weight of both G-CSF contained our product and reference is similar and has 18800 and 18797 Da molecular weight respectively. Different concentrations of samples might have caused the noises seen on the deconvoluted mass spectra shown on the inset on the LC chromatography.

Amino acid sequence of our product and reference also their similarities have disclosed via peptide mapping by subjecting them to *SV-8* Endoproteinase Glu-C digestion followed by LC-MS/MS analysis. Our hG-CSF product overlaps with the reference product are shown.

Another important characterization technique, circular dichroism has revealed the secondary structure of 4 α-helix contained our G-CSF product and the reference. The characteristics of α-helix in literature referred to as having two negative peaks at 208 nm and 222 nm and one positive peak at 190 nm (Greenfield, 2007). The results in Figure 3.13 shows that, product and the reference had clear α-helix structure with mentioned peaks and dips, thus, it matches with reference.

G-CSF has been mentioned to be an oligomerization-prone protein (Krishnan et al., 2002). Thus, the oligomeric state of the protein is wanted to be analyzed by HPLC-Size exclusion chromatography (SEC) analysis, in Figure 3.14. The human G-CSF and reference main peak elution time is 17.84 minutes and 17.92 minutes respectively. This presents the solubilized, refolded and then purified G-CSF product and the reference G-CSF is monomer and similar. The oligomerization of G-CSF is mentioned to be seen at phycological conditions (pH 7.0 and room temperature) and eliminated when it is stored below 8°C at pH 5 (Krishnan et al., 2002). Therefore, we stored our purified hG-CSF product in pH 4.5 at +4° C.

Another method to determine the purity of target protein in formulation buffer is subjecting it to non-reducing capillary electrophoresis. Purity of many pharmaceutical product is assessed by capillary gel electrophoresis systems because this method is highly stable, robust and sensitive (Sastre Toraño, 2019). Even-though our G-CSF had low concentration, it can be seen that, G-CSF in FB buffer has high purity (>95).

The previous experiments have shown that, our G-CSF product have similar DNA sequence, primary and secondary structure, molecular mass and purity with the reference product. Yet, this does not prove our product is biologically active. Therefore, its biological activity is tested by inducing human monocyte THP-1 cells,

which cells expresses G-CSFR highly, with our G-CSF product and the reference G-CSF.

Pankaj et. al. (2017) has mentioned that upon binding to G-CSFR, G-CSF induces several pathways including the STAT, PI-3K and MAPK cellular signal pathways. Extracellular signal-regulated kinase (ERK) family is one of the major kinases in this pathway and ERK 1/2 module is activated upon a stimulation via growth factors for differentiation and cell growth (Morrison, 2012). A study by Saito et al. (2002) revealed that, G-CSF stimulation on monocytes causes upregulation of phosphorylated ERK 1/2. Similarly, when human monocytic THP-1 cells are stimulated with both our and reference G-CSF, phosphorylated ERK 1/2 levels are upregulated significantly in 30 and 60 minutes (Figure 3.17) This upregulation of p-ERK 1/2 indicates the functional activity of our refolded G-CSF.

Consequently, in this thesis, we efficiently constructed G-CSF gene into pET-30a (+) ::hG-CSF plasmid, expressed in *E. coli* BL21(DE3) host cell and purified using cation exchange chromatography.

REFERENCES

Adusumilli, A., & Krothapalli, S. R. (2012). The clinical applications of Hematopoietic growth factor - GCSF. *Asian Journal of Biomedical and Pharmaceutical Sciences*, *2*(13), 1–10.

Amgen. (2020). *2020 Biosimilar Trends Report*. *Amgen Biosimilars*.

Araújo, F. C., Gonçalves, J., & Fonseca, J. E. (2016). Pharmacoeconomics of Biosimilars: What Is There to Gain from Them? *Current Rheumatology Reports*, *18*(8). https://doi.org/10.1007/s11926-016-0601-0

Aritomi, M., Kunishima, N., Okamoto, T., Kuroki, R., Ota, Y., & Morikawa, K. (1999). Atomic structure of the GCSF-receptor complex showing a new cytokine- receptor recognition scheme. *Nature*, *401*(6754), 713–717. https://doi.org/10.1038/44394

Arvedson, T., O'Kelly, J., & Yang, B. B. (2015). Design rationale and development approach for pegfilgrastim as a long-acting granulocyte colony-stimulating factor. *BioDrugs*, *29*(3), 185–198. https://doi.org/10.1007/s40259-015-0127-4

Babaeipour, V., Abbas, M. P. H., Sahebnazar, Z., & Alizadeh, R. (2010). Enhancement of human granulocyte-colony stimulating factor production in recombinant E. coli using batch cultivation. *Bioprocess and Biosystems Engineering*, *33*(5), 591–598. https://doi.org/10.1007/s00449-009-0380-3

Bagnyukova, T., Serebriiskii, I. G., Zhou, Y., Hopper-Borge, E. A., Golemis, E. A., & Astsaturov, I. (2010). Chemotherapy and signaling: How can targeted therapies supercharge cytotoxic agents? *Cancer Biology and Therapy*, *10*(9), 839–853. https://doi.org/10.4161/cbt.10.9.13738

Birbrair, A. (Ed.). (2020). *Tumor Microenvironment Hematopoietic Cells*. *Advances in Experimental Medicine and Biology* (Vol. 1224). Springer. https://doi.org/10.1007/978-3-030-35723-8_5

Birbrair, A., & Frenette, P. S. (2017). Niche heterogeneity in the bone marrow Alexander. *Annals of the New York Academy of Sciences*, *1370*(1), 82–96. https://doi.org/10.1111/nyas.13016.Niche

Borregaard, N. (2010). Neutrophils, from Marrow to Microbes. *Immunity*, *33*(5), 657–670. https://doi.org/10.1016/j.immuni.2010.11.011

Boubevaa, R., Reicherta, C., Handrick, R., Müllera, C., Hannemannb, J., & Borchard, G. (2012). New expression method and characterization of recombinant human granulocyte colony stimulating factor in a stable protein formulation. *Chimia*, *66*(5), 281–285. https://doi.org/10.2533/chimia.2012.281

Boxer, L. A. (2012). How to approach neutropenia. *Hematology / the Education*

Program of the American Society of Hematology. American Society of Hematology. Education Program, *2012*, 174–182. https://doi.org/10.1182/asheducation.v2012.1.174.3798251

Bracewell, D. G., Francis, R., & Smales, C. M. (2015). The future of host cell protein (HCP) identification during process development and manufacturing linked to a risk-based management for their control. *Biotechnology and Bioengineering*, *112*(9), 1727–1737. https://doi.org/10.1002/bit.25628

Bratton, D., & Henson, P. (2011). Neutrophil clearance: when the party is over, clean-up begins. *Trends in Immunology*, *32*(8), 350–357. https://doi.org/10.1016/j.it.2011.04.009.Neutrophil

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., … Zychlinsky, A. (2004). Neutrophil Extracellular Traps Kill Bacteria. *Science*, *303*(5663), 1532–1535. https://doi.org/10.1126/science.1092385

Cieutat, A. M., Lobel, P., August, J. T., Kjeldsen, L., Sengeløv, H., Borregaard, N., & Bainton, D. F. (1998). Azurophilic granules of human neutrophilic leukocytes are deficient in lysosome-associated membrane proteins but retain the mannose 6-phosphate recognition marker. *Blood*, *91*(3), 1044–1058. https://doi.org/10.1182/blood.v91.3.1044.1044_1044_1058

Croker, B. A., Metcalf, D., Robb, L., Wei, W., Mifsud, S., DiRago, L., … Roberts, A. W. (2004). SOCS3 Is a Critical Physiological Negative Regulator of G-CSF Signaling and Emergency Granulopoiesis. *Immunity*, *20*(2), 153–165. https://doi.org/10.1016/S1074-7613(04)00022-6

Dale, D. C., Bolyard, A., Marrero, T., Makaryan, V., Bonilla, M., Link, D. C., … Spiekerman, C. (2018). Long-Term Effects of G-CSF Therapy in Cyclic Neutropenia, 377 N Engl J Med 2290–2292. https://doi.org/10.1056/NEJMc1709258

Dawn, B., Sanganalmath, S. K., & Bolli, R. (2012). G-CSF-Induced Mobilization of Bone Marrow Stem Cells and Cardiac Repair. *Milestones in Drug Therapy*, 435–463. https://doi.org/10.1007/978-3-0348-0218-5

Demetri, G., & Griffin, J. D. (1991). Granulocyte Colony-Stimulating Factor and Its Receptor. *The Jornal of the American Society of Hematology*, *78*(11).

Doeing, D. C., Borowicz, J. L., & Crockett, E. T. (2003). Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clinical Pathology*, *3*, 1–6. https://doi.org/10.1186/1472-6890-3-1

Eyles, J. L., Roberts, A. W., Metcalf, D., & Wicks, I. P. (2006). Granulocyte colony-stimulating factor and neutrophils - Forgotten mediators of inflammatory disease. *Nature Clinical Practice Rheumatology*, *2*(9), 500–510. https://doi.org/10.1038/ncprheum0291

Feldmann, M. (1998). Cytokines. In *Encyclopedia of Immunology* (pp. 719–722). https://doi.org/10.1016/0378-1127(91)90194-Z

Frampton, J. E., Lee, C. R., & Faulds, D. (1994). Filgrastim: A Review of its Pharmacological Properties and Therapeutic Efficacy in Neutropenia.

Drugs, *48*(5), 731–760. https://doi.org/10.2165/00003495-199448050-00007

Gascon, P. (2012). Presently available biosimilars in hematology-oncology: G-CSF. *Targeted Oncology*, *7*(SUPPL.1), 29–34. https://doi.org/10.1007/s11523-011-0190-9

Gascón, P., Tesch, H., Verpoort, K., Rosati, M. S., Salesi, N., Agrawal, S., … Turner, M. (2013). Clinical experience with Zarzio® in Europe: What have we learned? *Supportive Care in Cancer*, *21*(10), 2925–2932. https://doi.org/10.1007/s00520-013-1911-7

Greenfield, N. J. (2007). Using circular dichroism spectra to estimate protein secondary structure. *Nature Protocols*, *1*(6), 2876–2890. https://doi.org/10.1038/nprot.2006.202

Haddy, T. B., Rana, S. R., & Castro, O. (1999). Benign ethnic neutropenia: What is a normal absolute neutrophil count? *Journal of Laboratory and Clinical Medicine*, *133*(1), 15–22.

Häger, M., Cowland, J. B., & Borregaard, N. (2010). Neutrophil granules in health and disease. *Journal of Internal Medicine*, *268*(1), 25–34. https://doi.org/10.1111/j.1365-2796.2010.02237.x

Hauke, R. J., & Tarantolo, S. R. (2000). Hematopoietic Growth Factors. *Laboratory Medicine*, *31*(11), 613–615. https://doi.org/10.1309/HNTM-ELUV-AV9G-MA1P

Hill, C. P., Osslund, T. D., & Eisenberg, D. (1993). The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. *Proceedings of the National Academy of Sciences of the United States of America*, *90*(11), 5167–5171. https://doi.org/10.1073/pnas.90.11.5167

Hoffmann, D., Ebrahimi, M., Gerlach, D., Salzig, D., & Czermak, P. (2018). Reassessment of inclusion body-based production as a versatile opportunity for difficult-to-express recombinant proteins. *Critical Reviews in Biotechnology*, *38*(5), 729–744. https://doi.org/10.1080/07388551.2017.1398134

Horwitz, M. S., Corey, S. J., Grimes, H. L., & Tidwell, T. (2013). ELANE Mutations in Cyclic and Severe Congenital Neutropenia. Genetics and Pathophysiology. *Hematology/Oncology Clinics of North America*, *27*(1), 19–41. https://doi.org/10.1016/j.hoc.2012.10.004

Hsieh, M. M., Everhart, J. E., Byrd-Holt, D. D., Tisdale, J. F., & Rodgers, G. P. (2007). Prevalence of Neutropenia in the U.S. Population: Age, Sex, Smoking Status, and Ethnic Differences. *Annals of Internal Medicine*, *146*(7), 486–492.

Jarrett, S., & Dingermann, T. (2015). A hospital pharmacist's guide to educating health care professionals on biosimilars. *Hospital Pharmacy*, *50*(10), 884–893. https://doi.org/10.1310/hpj5010-884

Jung, K. H., Chu, K., Lee, S. T., Kim, S. J., Sinn, D. I., Kim, S. U., … Roh, J. K. (2006). Granulocyte colony-stimulating factor stimulates neurogenesis via vascular endothelial growth factor with STAT activation. *Brain*

Research, *1073–1074*(1), 190–201. https://doi.org/10.1016/j.brainres.2005.12.037

Kato, T. (2016). *Granulocyte Colony-Stimulating Factor*. *Handbook of Hormones*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-801028-0.00387-1

Kenne, E., Rasmuson, J., Renné, T., Vieira, M. L., Müller-Esterl, W., Herwald, H., & Lindbom, L. (2019). Neutrophils engage the kallikrein-kinin system to open up the endothelial barrier in acute inflammation. *FASEB Journal*, *33*(2), 2599–2609. https://doi.org/10.1096/fj.201801329R

Ketley, N. J., & Newland, A. C. (1997). Haemopoietic growth factors. *Postgraduate Medical Journal*, *73*, 215–221.

Koeffler, H. P., Gasson, J., & Tobler, A. (1988). Transcriptional and posttranscriptional modulation of myeloid colony-stimulating factor expression by tumor necrosis factor and other agents. *Molecular and Cellular Biology*, *8*(8), 3432–3438. https://doi.org/10.1128/mcb.8.8.3432

Köhler, A., De Filippo, K., Hasenberg, M., Van Den Brandt, C., Nye, E., Hosking, M. P., … Gunzer, M. (2011). G-CSF-mediated thrombopoietin release triggers neutrophil motility and mobilization from bone marrow via induction of Cxcr2 ligands. *Blood*, *117*(16), 4349–4357. https://doi.org/10.1182/blood-2010-09-308387

Kolaczkowska, E., & Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nature Reviews Immunology*, *13*(3), 159–175. https://doi.org/10.1038/nri3399

Krishnan, S., Chi, E. Y., Webb, J. N., Chang, B. S., Shan, D., Goldenberg, M., … Carpenter, J. F. (2002). Aggregation of granulocyte colony stimulating factor under physiological conditions: Characterization and thermodynamic inhibition. *Biochemistry*, *41*(20), 6422–6431. https://doi.org/10.1021/bi012006m

Lacy, P. (2006). Mechanisms of degranulation in neutrophils. *Allergy, Asthma and Clinical Immunology*, *2*(3), 98–108. https://doi.org/10.2310/7480.2006.00012

Layton, J. E., & Hall, N. E. (2006). The interaction of G-CSF with its receptor. *Frontiers in Bioscience*, *11*, 3181–3189.

Lieschke, G. J., Grail, D., Hodgson, G., Metcalf, D., Stanley, E., Cheers, C., … Dunn, A. R. (1994). Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood*, *84*(6), 1737–1746. https://doi.org/10.1182/blood.v84.6.1737.bloodjournal8461737

Martins, A., Han, J., & Kim, S. O. (2010). The Multifaceted Effects of Granulocyte Colony-Stimulating Factor in Immunomodulation and Potential Roles in Intestinal Immune Homeostasis Andrew. *Bone*, *62*(8), 611–617. https://doi.org/10.1002/iub.361.

Metcalf, D. (2010). The colony-stimulating factors and cancer. *Nature Reviews Cancer*, *10*(6), 425–434. https://doi.org/10.1038/nrc2843

Molineux, G., Foote, M., & Arvedson, T. (Eds.). (2012).*Twenty Years of G-CSF* (1st ed., Vol. 67). Basel: Springer Basel. https://doi.org/10.1023/A:1016123027290

Morrison, D. K. (2012). MAP Kinase Pathways. *Cold Spring Harbor Laboratory Press;* https://doi.org/10.1101/cshperspect.a011254

Nagata, S., Tsuchiya, M., Asano, S., Yamamoto, O., Hirata, Y., Kubota, N., … Yamazaki, T. (1986). The chromosomal gene structure and two mRNAs for human granulocyte colony-stimulating factor. *The EMBO Journal*, *5*(3), 575–581. https://doi.org/10.1002/j.1460-2075.1986.tb04249.x

N**agata, Shigekazu, Masayuki, T., Shigetaka, A., & Yoshito, K**. (1986). Molecular cloning and expression of cDNA for human granulocyte clony-stimulating factor. *Nature*, *319*, 415–418.

Nauseef, W. M., & Borregaard, N. (2014). Neutrophils at work. *Nature Immunology*, *15*(7), 602–611. https://doi.org/10.1038/ni.2921

N**ewburger, P. E., & Dale, D. C.** (2013). Evaluation and management of patients with isolated neutropenia. *Seminars in Hematology*, *50*(3), 198–206. https://doi.org/10.1053/j.seminhematol.2013.06.010

Niemiec, M. J., De Samber, B., Garrevoet, J., Vergucht, E., Vekemans, B., De Rycke, R., … Urban, C. F. (2015). Trace element landscape of resting and activated human neutrophils on the sub-micrometer level. *Metallomics*, *7*(6), 996–1010. https://doi.org/10.1039/c4mt00346b

Pankaj, D., & Greis, K. D. (2017). Granulocyte Colony Stimulating Factor Receptor (G-CSFR) signaling in severe congenital neutropenia, chronic neutrophilic leukemia and related malignancies. *Exp Hematology*, *176*(46), 9–20. https://doi.org/10.1016/j.physbeh.2017.03.040

Papayannopoulos, V., & Zychlinsky, A. (2009). NETs: a new strategy for using old weapons. *Trends in Immunology*, *30*(11), 513–521. https://doi.org/10.1016/j.it.2009.07.011

Pillay, J., Den Braber, I., Vrisekoop, N., Kwast, L. M., De Boer, R. J., Borghans, J. A. M., … Koenderman, L. (2010). In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood*, *116*(4), 625–627. https://doi.org/10.1182/blood-2010-01-259028

Rankin, E. B., & Sakamoto, K. M. (2018). Bone Marrow Failure. In G. Kupfer, G. H. Reaman, & F. O. Smith (Eds.), *Pediatric Oncology* (pp. 1–23). Springer, Cham. https://doi.org/10.1007/978-3-319-61421-2_1

Rankin, S. M. (2010). The bone marrow: a site of neutrophil clearance. *Journal of Leukocyte Biology*, *88*(2), 241–251. https://doi.org/10.1189/jlb.0210112

R**emijsen, Q., Berghe, T. Vanden, Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., … Vandenabeele, P.** (2011). Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Research*, *21*(2), 290–304. https://doi.org/10.1038/cr.2010.150

Renwick, W., Pettengel, L. R., & Green, M. (2009). Use of filgrastim and

pegfilgrastim to support delivery of chemotherapy: Twenty years of clinical experience. *BioDrugs*, *23*(3), 175–186. https://doi.org/10.2165/00063030-200923030-00004

Root, R. K., & Dale, D. C. (1999). Granulocyte Colony-Stimulating Factor and Granulocyte-Macrophage Colony-Stimulating Factor: Comparisons and Potential for Use in the Treatment of Infections in Nonneutropenic Patients. *The Journal of Infectious Diseases*, *179*(s2), S342–S352. https://doi.org/10.1086/513857

Rosales, C. (2018). Neutrophil: A cell with many roles in inflammation or several cell types? *Frontiers in Physiology*, *9*(FEB), 1–17. https://doi.org/10.3389/fphys.2018.00113

Saito, M., Kiyokawa, N., Taguchi, T., Suzuki, K., Sekino, T., Mimori, K., … Fujimoto, J. (2002). Granulocyte colony-stimulating factor directly affects human monocytes and modulates cytokine secretion. *Experimental Hematology*, *30*(10), 1115–1123. https://doi.org/10.1016/S0301-472X(02)00889-5

Sastre Toraño, J., Ramautar, R., & de Jong, G. (2019). Advances in capillary electrophoresis for the life sciences. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *1118–1119*(November 2018), 116–136. https://doi.org/10.1016/j.jchromb.2019.04.020

Schneider, A., Krüger, C., Steigleder, T., Weber, D., Pitzer, C., Laage, R., … Schäbitz, W. R. (2005). The hematopoietic factor G-CSF is a neuronal ligand that counteracts programmed cell death and drives neurogenesis. *Journal of Clinical Investigation*, *115*(8), 2083–2098. https://doi.org/10.1172/JCI23559

Schneider, A., Kuhn, H. G., & Schäbitz, W. R. (2005). A role for G-CSF (Granulocyte-colony stimulating factor) in the central nervous system. *Cell Cycle*, *4*(12), 1753–1757. https://doi.org/10.4161/cc.4.12.2213

Schrader, J. W. (1998). *Clony Stimulating Factor*. *Encyclopedia of Immunology*.

Schwartzberg, L. S. (2006). Neutropenia: Etiology and Pathogenesis. *Clinical Cornerstone*, *8*(SUPPL. 5). https://doi.org/10.1016/s1098-3597(06)80053-0

Skokowa, J., Dale, D. C., Touw, I. P., Zeidler, C., & Welte, K. (2018). Severe congenital neutropenias. *Nat Rev Dis Primers*, *3*. https://doi.org/10.1016/j.physbeh.2017.03.040

Skokowa, J., Klimiankou, M., Klimenkova, O., Lan, D., Gupta, K., Hussein, K., … Welte, K. (2012). Interactions among HCLS1, HAX1 and LEF-1 proteins are essential for G-CSF–triggered granulopoiesis. *Nature Medicine*, *18*(10), 1550–1559. https://doi.org/doi:10.1038/nm.2958. Interactions371

Summers, C., Rankin, S. M., Condliffe, A. M., Singh, N., Peters, A. M., & Chilvers, E. R. (2010). Neutrophil kinetics in health and disease. *Trends in Immunology*, *31*(8), 318–324. https://doi.org/10.1016/j.it.2010.05.006

Švajger, U., & Rožman, P. (2018). Induction of tolerogenic dendritic cells by endogenous biomolecules: An update. *Frontiers in Immunology*, *9*(OCT), 1–17. https://doi.org/10.3389/fimmu.2018.02482

Tanaka, H., Tanaka, Y., Shinagawa, K., Yamagishi, Y., Ohtaki, K., & Asano, K. (1997). Three types of recombinant human granulocyte colony-stimulating factor have equivalent biological activities in monkeys. *Cytokine*, *9*(5), 360–369. https://doi.org/10.1006/cyto.1996.0177

Thieman, W. J., & Palladino, M. A. (2004). *Introduction to Biotechnology*. Pearson/Benjamin Cummings, 2004.

Touw, I. P., & Van De Geijn, G. J. M. (2007). Granulocyte colony-stimulating factor and its receptor in normal myeloid cell development, leukemia and related blood cell disorders. *Frontiers in Bioscience*, *12*(3), 800–815. https://doi.org/10.2741/2103

U.S. Food and Drug Administration. (2017). Biosimilar Development, Review, and Approval.

Wang, J., Yao, L., Zhao, S., Zhang, X., Yin, J., Zhang, Y., … Li, G. (2012). Granulocyte-colony stimulating factor promotes proliferation, migration and invasion in glioma cells. *Cancer Biology and Therapy*, *13*(6), 389–400. https://doi.org/10.4161/cbt.19237

Watari, K., Asano, S., Shirafuji, N., Kodo, H., Ozawa, K., Takaku, F., & Kamachi, S. (1989). Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. *Blood*, *73*(1), 117–122. https://doi.org/10.1182/blood.v73.1.117.bloodjournal731117

Welte, K., Gabrilove, J., Bronchud, M., Platzer, E., & Morstyn, G. (1996). Filgrastim (r-metHuG-CSF): The First 10 Years By. *British Medical Journal*, *88*(1907–1929), 161. https://doi.org/10.1136/bmj.2.32.161-a

Weycker, D., Li, X., Barron, R., Li, Y., Reiner, M., Kartashov, A., … Garcia, J. (2016). Risk of chemotherapy-induced febrile neutropenia with early discontinuation of pegfilgrastim prophylaxis in US clinical practice. *Supportive Care in Cancer*, *24*(6), 2481–2490. https://doi.org/10.1007/s00520-015-3039-4

Witko-Sarsat, V., Pederzoli-Ribeil, M., Hirsh, E., Sozzani, S., & Cassatella, M. A. (2011). Regulating neutrophil apoptosis: New players enter the game. *Trends in Immunology*, *32*(3), 117–124. https://doi.org/10.1016/j.it.2011.01.001

Witko-Sarsat, V., Rieu, P., Descamps-Latscha, B., Lesavre, P., & Halbwachs-mecarelli, L. (2000). Neutrophils : Molecules , Functions and Pathophysiological Aspects. *Biology of Disease*, *80*(5), 617–653.

Xiao, B. G., Lu, C. Z., & Link, H. (2007). Cell biology and clinical promise of G-CSF: Immunomodulation and neuroprotection: Cellular Medicine. *Journal of Cellular and Molecular Medicine*, *11*(6), 1272–1290. https://doi.org/10.1111/j.1582-4934.2007.00101.x

APPENDICES

APPENDIX A: Laboratory Equipments, Chemicals, enzymes and commertial kits

APPENDIX B: The reference and results of nucleic acid sequence of human G-CSF

APPENDIX C: The replicate of the results

APPENDIX D: Molecular weight standards of SEC-HPLC

APPENDIX A.

Equipment used in this study are shown in Table A.2.

Table A. 1: Equipment used in this study.

Equipment	Brand
Amersham WB System	GE Healthcare Lifescience
Autoclave	Tuttnauer Systec 3870
Centrifuges	Beckman Coulter Allegra 25R ScanSpeed
Chromatographic System	GE Healthcare Lifescience Äkta Avant
Gel Screening Systems	Bio-Rad ChemiDoc™ MP Bio-Rad GelDoc™ XR+
Hemocytometer	Fisher Lab Scientific
Incubator with CO2	Thermo Scientific
Laminar Air Flow Cabinets	Faster BH-EN 2003
Laminar Flow Faster	BH-EN 2003
Light Microscope	Olympus CH30 (USA)
Magnetic Stirrer	Dragon Lab MS-H-S
Micropipettes	Eppendorf Thermo Scientific
Microplate Spectrophotometer	Bio-Rad Benchmark Plus
Orbital Shaker	Lab Companion™ ISF-7200
pH meter	Mettler Toledo
Power Supply for SDS-PAGE	BIO-RAD Power Pac Basic
Pure Water System	Merck Direct-Q®
SDS-PAGE Gel Electrophoresis System	Bio-Rad Mini Protean
Shaker	Heidolph Duomax 1030
Thermal Cycler	Bio-Rad T100™ Thermal Cycler
Ultrapure Water System	Merck Direct-Q®-3UV
UV-Visible Spectrophotometer	Thermo Scientific Nanodrop 2000 UVstar Plus UV transilluminator
Vortex	Dragon Lab MX-F
Water Bath	Memmer WB110

Chemicals and enzymes used in this study are shown in Table A.2.

Table A. 2: Chemicals and enzymes used in this study

Chemical and Enzymes	Brand
1X CutSmart Buffer	NEB
40% Acrylamide Solution	Bio-Rad
6X DNA Loading Dye Solution	iNtRON
Acetic Acid	Sigma
Agar (Bacteriological Grade)	Multicell
Agarose	Multicell
APS	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	Sigma
Clarity Western ECL	Bio-Rad
Coomassie Brilliant Blue	Sigma
DMEM	Gibco
DMSO	Biomatik
DNA Ladder	GeneON Thermo Scientific
EDTA	Sigma
EcoRI-HF® Restriction Enzyme	NEB
Ethanol	Merck
FBS	Thermo Scientific
Glycerol	Merck
Glycine	Sigma
Hydrochloric Acid	Sigma
Isopropanol	Merck
Iodacetemate	Bio-Rad
Kanamycin	Sigma
Lysosome	Sigma
Methanol	Merck
Non-fat dry milk	Santa Cruz
Neupogen	Amgen
NdeI Restriction Enzyme	NEB
PageRuler Prestained Protein Ladder	Thermo Scientific
PBS	Invitrogen
PMSF	Fluka
Protease cocktail inhibitor	Roche
RedSafe™	iNtRON
RiboRuler High Range RNA Ladder	Thermo Scientific
RT MasterMix 5X All-In-One	abm®
SDS	Sigma
Sodium Chloride	Sigma
Sodium Hydroxide	Multicell
Taq polymerase SuperMix	QuantaBio
TEMED	Sigma
Trisma base	Multicell
Trypsin-EDTA	Gibco
Tween-20	Biomatik
Yeast Extract	Sigma
β-Mercaptoethanol	Sigma

Commercial kits used in this study are shown in Table A.3

Table A. 3: Commercial kits

Equipment	Brand
In-Fusion Cloning kit	Takara
NucleoBond Xtra Maxi kit for transfection-grade plasmid DNA	MACHEREY-NAGEL
NucleoSpin® PCR clean-up, gel extraction	MACHEREY-NAGEL
NucleoSpin® RNA Midi kit	MACHEREY-NAGEL
E. coli Host Cell Protein ELISA kit	Cygnus Technologies, F410

APPENDIX B.

G-CSF PDB Details

PDB ID: 1CD9 2:2 Complex of G-CSF with its receptor

Released: 2000-03-08

Deposition Author: Aritomi et al., 1999

DOI: 10.2210/pdb1CD9/pdb

1CD9:C|PDBID|CHAIN|SEQUENCE

MTPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLG
HSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLD
TLQLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVA
SHLQSFLEVSYRVLRHLAQP

DrugBank sequence

MTPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLG
HSLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLD
TLQLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVA
SHLQSFLEVSYRVLRHLAQP

AJC19277.1:1-174 granulocyte-colony stimulating factor, partial [Homo sapiens]

TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGH
SLGIPWAPLSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTL
QLDVADFATTIWQQMEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASH
LQSFLEVSYRVLRHLAQP-

Human *CSF3* sequence (NCBI, Homo sapiens colony stimulating factor 3 (CSF3), transcript variant 5, mRNA, Sequence ID: NR_033662.2)

5’ATGACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCA
AGTGCTTAGAGCAAGTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCA
GGAGAAGCTGTGTGCCACCTACAAGCTGTGCCACCCCGAGGAGCTGGTG

CTGCTCGGACACTCTCTGGGCATCCCCTGGGCTCCCCTGAGCAGCTGCCC
CAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCCAACTCCATAGCGGC
CTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGA
GTTGGGTCCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCA
CCACCATCTGGCAGCAGATGGAAGAACTGGGAATGGCCCCTGCCCTGCA
GCCCACCCAGGGTGCCATGCCGGCCTTCGCCTCTGCTTTCCAGCGCCGGG
CAGGAGGGGTCCTGGTTGCCTCCCATCTGCAGAGCTTCCTGGAGGTGTCG
TACCGCGTTCTACGCCACCTTGCCCAGCCCTGA'3

Homo sapiens colony stimulating factor 3 (CSF3), transcript variant 5, non-coding RNA

Sequence ID: NR_033662.2 **Length:** 1680 **Number of Matches:** 1

Range 1: 302 to 826 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
965 bits(522)	0.0	524/525(99%)	0/525(0%)	Plus/Plus

```
Query  1    ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGCAA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  ACCCCCCTGGGCCCTGCCAGCTCCCTGCCCCAGAGCTTCCTGCTCAAGTGCTTAGAGCAA  361

Query  61   GTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCTACAAG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  GTGAGGAAGATCCAGGGCGATGGCGCAGCGCTCCAGGAGAAGCTGTGTGCCACCTACAAG  421

Query  121  CTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCTGGGCTCCC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  CTGTGCCACCCCGAGGAGCTGGTGCTGCTCGGACACTCTCTGGGCATCCCCTGGGCTCCC  481

Query  181  CTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCCAACTCCATAGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CTGAGCAGCTGCCCCAGCCAGGCCCTGCAGCTGGCAGGCTGCTTGAGCCAACTCCATAGC  541

Query  241  GGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTGGGT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  GGCCTTTTCCTCTACCAGGGGCTCCTGCAGGCCCTGGAAGGGATCTCCCCCGAGTTGGGT  601

Query  301  CCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACCACCATCTGGCAGCAG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  CCCACCTTGGACACACTGCAGCTGGACGTCGCCGACTTTGCCACCACCATCTGGCAGCAG  661

Query  361  ATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  ATGGAAGAACTGGGAATGGCCCCTGCCCTGCAGCCCACCCAGGGTGCCATGCCGGCCTTC  721

Query  421  GCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTAGTTGCCTCCCATCTGCAGAGCTTC  480
            |||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  722  GCCTCTGCTTTCCAGCGCCGGGCAGGAGGGGTCCTGGTTGCCTCCCATCTGCAGAGCTTC  781

Query  481  CTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTGA  525
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782  CTGGAGGTGTCGTACCGCGTTCTACGCCACCTTGCCCAGCCCTGA  826
```

Chain A, Protein (granulocyte Colony-stimulating Factor) [Homo sapiens]

Sequence ID: 1CD9_A **Length:** 175 **Number of Matches:** 1

See 10 more title(s)

Range 1: 2 to 175 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
345 bits(884)	1e-119	Compositional matrix adjust.	174/174(100%)	174/174(100%)	0/174(0%)	+1

```
Query  1    TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP  180
            TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP
Sbjct  2    TPLGPASSLPQSFLLKCLEQVRKIQGDGAALQEKLCATYKLCHPEELVLLGHSLGIPWAP  61

Query  181  LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ  360
            LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ
Sbjct  62   LSSCPSQALQLAGCLSQLHSGLFLYQGLLQALEGISPELGPTLDTLQLDVADFATTIWQQ  121

Query  361  MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP  522
            MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP
Sbjct  122  MEELGMAPALQPTQGAMPAFASAFQRRAGGVLVASHLQSFLEVSYRVLRHLAQP  175
```

APPENDIX C

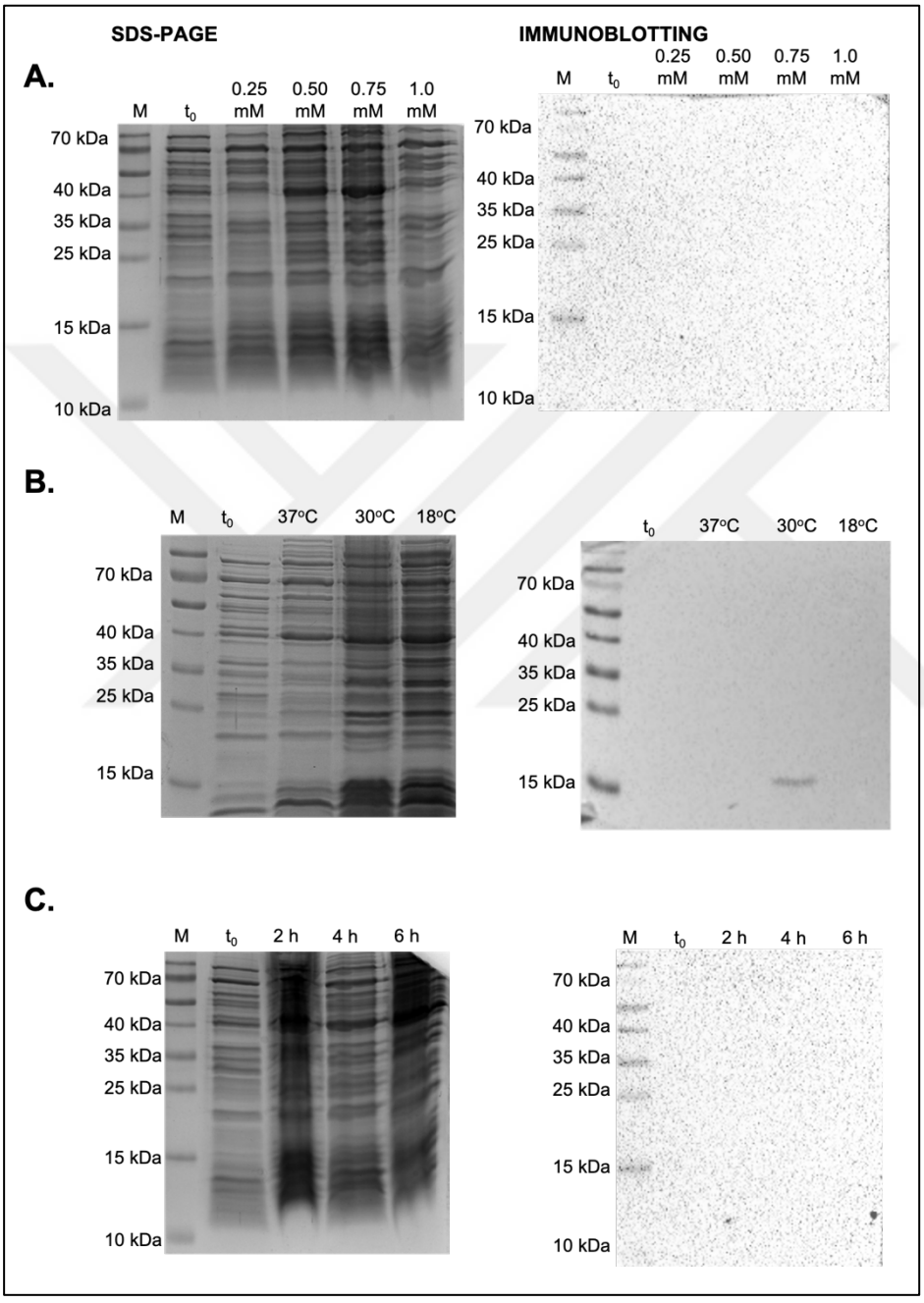

Figure C. 1: The SDS-PAGE (A) and immunoblotting (B) results of the optimization of hG-CSF protein production in *E. coli* BL21 (DE3) cells' supernatant. (A.) shows different (0.25-1 mM) IPTG concentration result (B.) shows different temperature growth (37°C, 30°C, and 18°C) after IPTG induction result and (C.) shows different time duration (2, 4, 6 hours) after IPTG induction result. M is PageRuler marker, Thermo Scientific. t0 is non-induced sample.

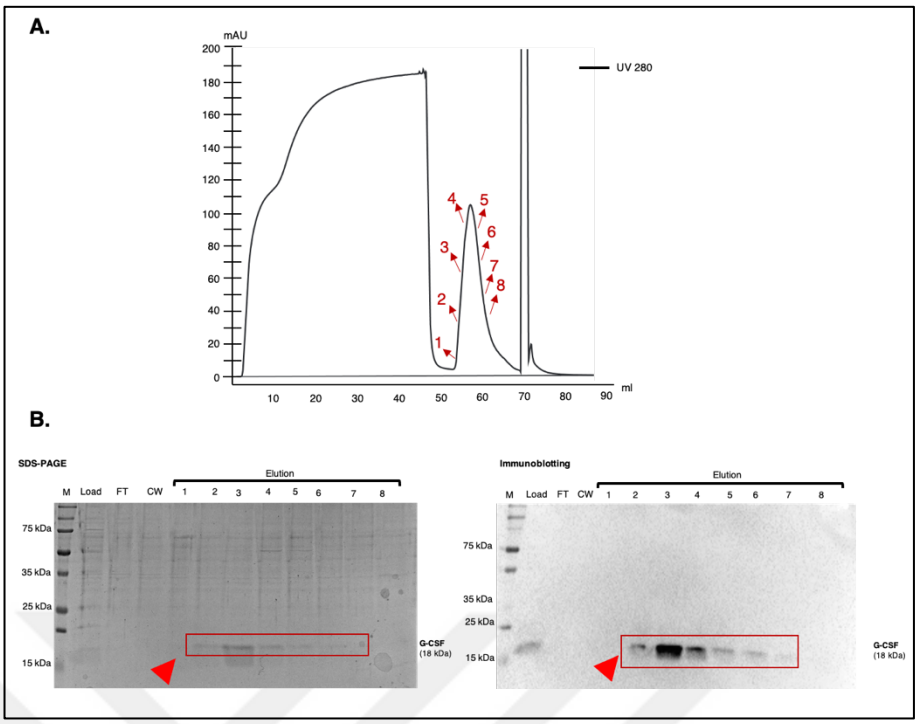

Figure C. 2: Replicate purification (A) chromatography, (B- Left)SDS-PAGE and (B-right) of human recombinant G-CSF isolated from BL21(DE3) cells that carries pET30(a)+::hG-CSF plasmid. Cation exchange binding buffer is 25 mM sodium acetate pH 4.5 and elution buffer is 25 mM sodium acetate, 1 M NaCl pH 4.5. The collected elution fractions are showed in number.

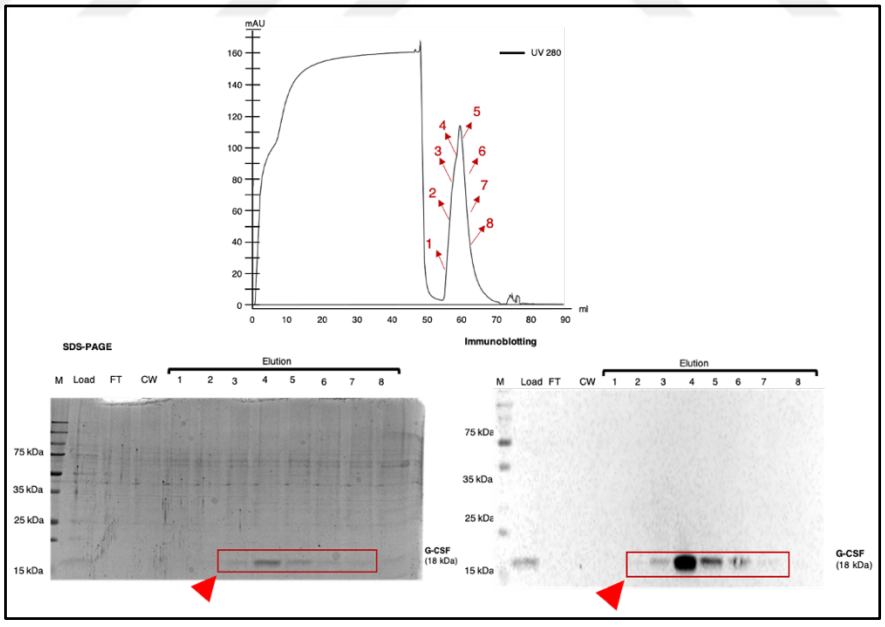

Figure C. 3: Replicate purification (A) chromatography, (B- Left)SDS-PAGE and (B-right) of human recombinant G-CSF isolated from BL21(DE3) cells that carries pET30(a)+::hG-CSF plasmid. Cation exchange binding buffer is 25 mM sodium acetate pH 4.5 and elution buffer is 25 mM sodium acetate, 1 M NaCl pH 4.5. The collected elution fractions are showed in number.

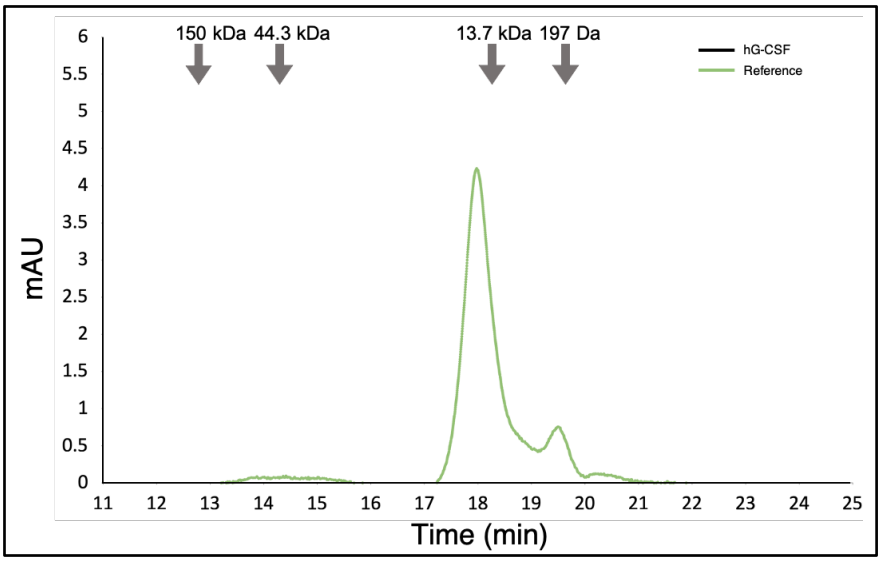

Figure C. 4: The SEC-HPLC chromatogram replicate of the SEC analysis of 0.05 mg/ml purified hG-CSF.

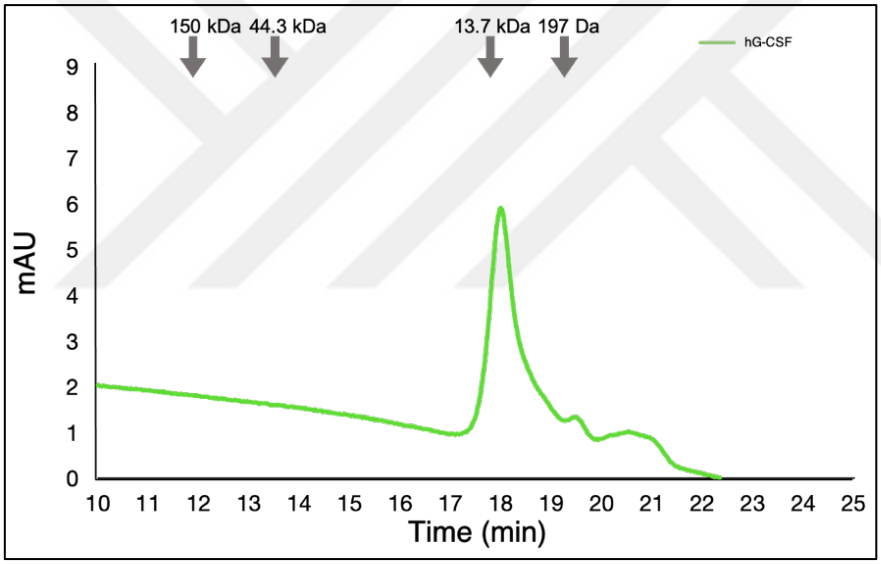

Figure C. 5: The SEC-HPLC chromatogram replicate of the SEC analysis of 0.05 mg/ml purified hG-CSF.

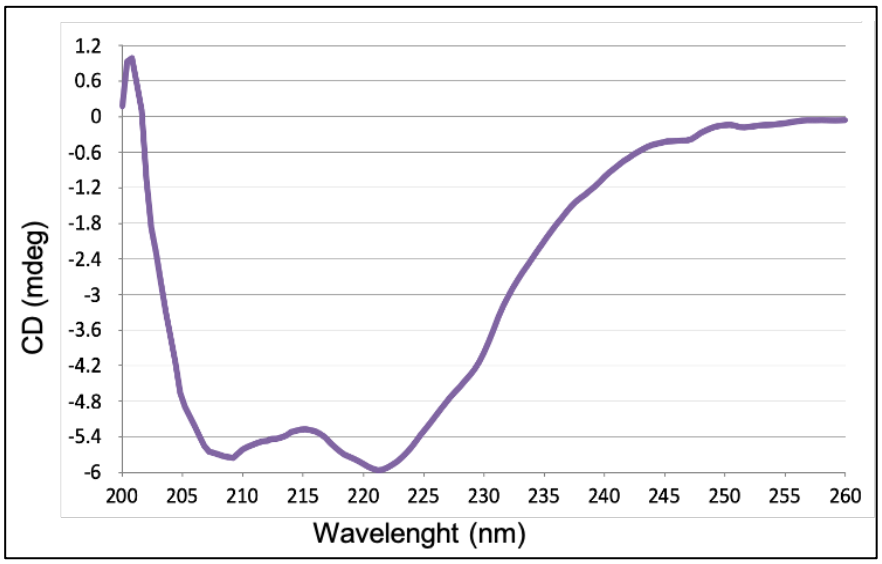

Figure C. 6: The circular dichroism spectra replicate of final G-CSF product.

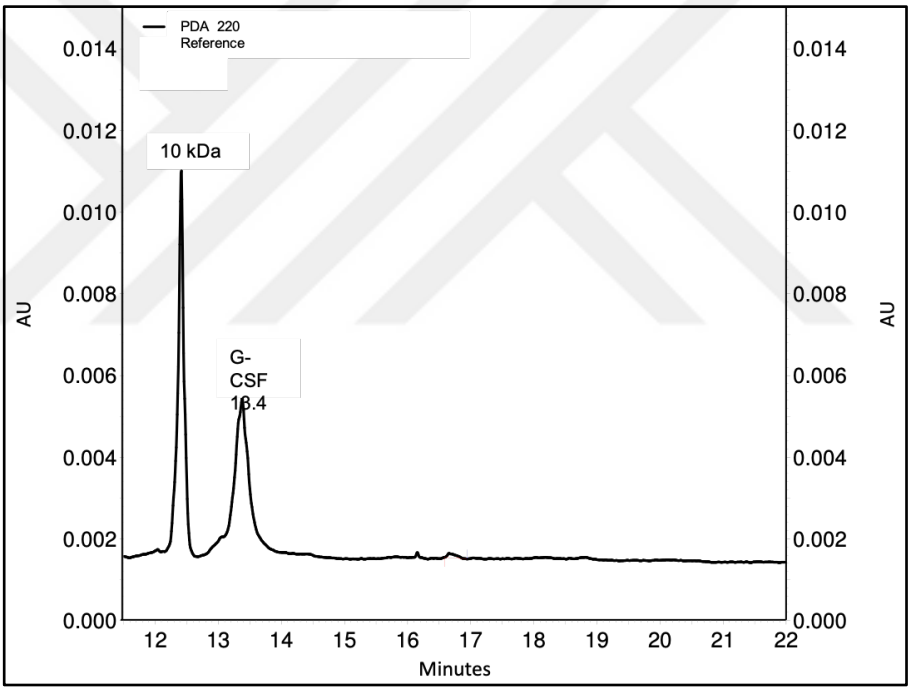

Figure C. 7: The capillary electrophoresis replicate of final G-CSF product.

APPENDIX D.

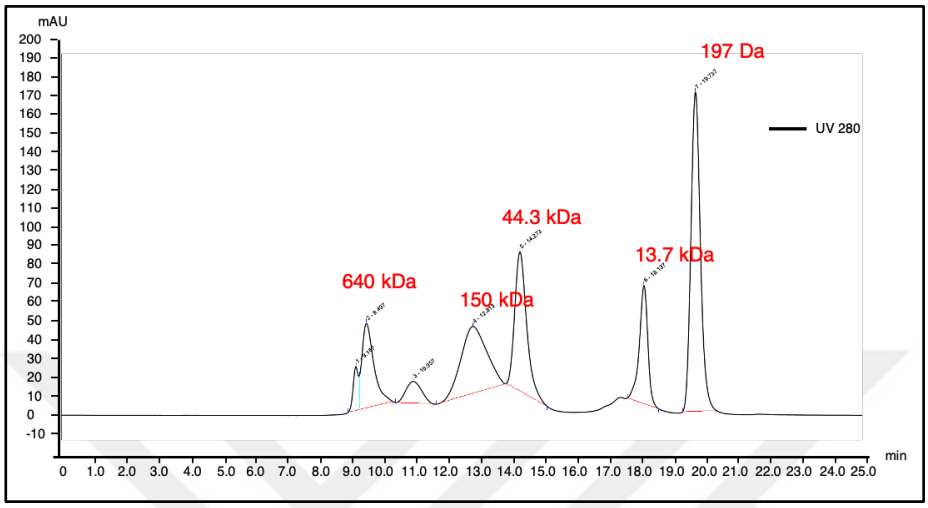

Figure D. 7: The SEC-HPLC analysis of protein standarts for reference.

CURRICULUM VITAE

Name Surname **:** Cansın KIRMAÇOĞLU

Place and Date of Birth **:** 28.08.1994, Çankaya

E-Mail **:**

EDUCATION **:**

- **B.Sc.** **:** 2017, Middle East Technical University, Art and Science Faculty, Biological Sciences Department

PROFESSIONAL EXPERIENCE AND REWARDS:

- 2018-2020 Istanbul Technical University, Istanbul - TUBITAK 1007 KAMAG Project: Biosimilar Drug Development, Scholar

OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

Türk, M., Alkurt G., Yıldırım N. G., Kırmaçoğlu C., & Dinler Doğanay, G.,(2018). Possible regulatory partner of Beclin 1 in apoptosis/autophagydecision: BAG 1, The FEBS Congress 2018. Prague, Czech Republic.

Türk, M., Alkurt G., Yıldırım N. G., Kırmaçoğlu C., & Dinler Doğanay, G.,(2018). Apoptoz/Otofaji Kararında Yeni Bir Düzenleyici: BAG-1, İmmünolojide Moleküller Sempozyumu