



ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

**PRODUCTION OF ANTI-CX3CR1 MONOCLONAL ANTIBODY FOR
USE IN THE PRECLINICAL TREATMENT OF PANCREATIC
CANCER AND CHRONIC PANCREATITIS**

DENİZ BAYBAĞ
M.Sc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
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ISTANBUL-2024



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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethical rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

31.05.2024

Deniz Baybağ

PREFACE AND ACKNOWLEDGEMENT

I would like to thank those who contributed and supported the writing of this thesis. But above all, I need to thank the thesis topic itself. I had lost my interest in the profession I was doing before starting this subject and the protocol. After I started my thesis experiments, I realized that I had not actually lost interest, I was just studying the wrong things. This work brought me back to myself, brought me back to life, and helped me become the best that I could be. I have learned so many techniques in so many areas in the past 3 years. For these reasons, I am grateful.

I have to thank my thesis advisor Prof. Dr. Gralp Onur Ceyhan. The day we first met, he said, "You are like coal now, but you have the potential to turn into a diamond." I feel like I'm starting to turn into that diamond in your hands. Thank you, sir, for never giving up on me and never stopping trusting me, even though I encountered many difficulties and setbacks during the thesis process. I am grateful to have the honor of working with you. I hope we will achieve much better things together.

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ABSTRACT

Development and Efficacy of a Novel Monoclonal Antibody Targeting CX3CR1 for Pain Relief in Pancreatic Cancer and Chronic Pancreatitis

Pancreatic cancer and chronic pancreatitis present daunting challenges due to the presence of severe pain and unfortunately the absence of effective treatments. We previously showed in both diseases, nervous system is activated within the tissue, and there is an increase in the expression and activity of the fractalkine chemokine and its specific receptor, CX3CR1, in parallel with the neuron-immune cell interaction. CX3CR1 is a special chemokine receptor that acts as both a chemoattractant and adhesion molecule and plays an important role in neural invasion process. Therefore, targeting this receptor holds promise for pain relief in afflicted patients. In this context, monoclonal antibody production specific to the 3rd terminal loop (binding location) of CX3CR1 receptor was carried out by hybridoma method. Female Balb/c mice were immunized, and the immunogenicity was checked through ELISA test from submandibular blood. In vitro dosage studies were conducted by SRB viability assay. Changes in abdominal pain were monitored during in vivo dosage studies in chronic pancreatitis-induced C57BL6 mice. Then, the histological and immunological progression of the disease were evaluated. Results indicated that our newly developed antibody exhibited higher efficacy on inhibiting pancreatic cancer cell proliferation compared to commercial antibody, without affecting healthy cell lines. Besides, in chronic pancreatitis mouse models, the antibodies effectively reduced abdominal pain. Due to the extremely high cost of existing inhibitory antibodies and the high renal toxicity of small molecule inhibitors, there is still a great need for effective antibodies to inhibit the CX3CR1 receptor. Our preliminary results show the potential of the CX3CR1 antibody produced by our group against chronic pancreatitis related pain; however, further research is needed to evaluate its potential on targeting pancreatic cancer.

Keywords: Antibody, CX3CR1, Fractalkine, Hybridoma, Chronic pancreatitis

ÖZET

Pankreas Kanseri ve Kronik Pankreatitte Ağrının Giderilmesinde CX3CR1'i Hedefleyen Yeni Bir Monoklonal Antikoru Geliştirilmesi ve Etkinliği

Pankreas kanseri ve kronik pankreatit, şiddetli ağrının varlığı ve ne yazık ki etkili tedavilerin bulunmaması nedeniyle göz korkutucu zorluklar sunmaktadır. Daha önce her iki hastalıkta da doku içerisinde sinir sisteminin aktive olduğunu ve nöron-immün hücre etkileşimine paralel olarak fraktalkin kemokin ve spesifik reseptörü CX3CR1'in ekspresyonunda ve aktivitesinde artış olduğunu göstermiştik. CX3CR1, hem kemoatraktan hem de adezyon molekülü olarak görev yapan ve nöral invazyon sürecinde önemli rol oynayan özel bir kemokin reseptörüdür. Bu nedenle, bu reseptörü hedeflemek, etkilenen hastalarda ağrının giderilmesi için umut vaat ediyor. Bu kapsamda hibridoma yöntemiyle CX3CR1 reseptörünün 3. terminal halkasına (bağlanma yeri) spesifik monoklonal antikoru üretimi gerçekleştirildi. Dişi Balb/c fareleri immünize edildi ve immünojenite submandibular kandan ELISA testi ile kontrol edildi. Farelerin dalağından ve lenf düğümlerinden izole edilen B hücreleri, F0 miyelom hücreleriyle birleştirildi ve uygun klon seçildi. Seçilen klonlardan toplanan süpernatantlar, afinite kromatografisiyle saflaştırıldı. İn vitro dozaj çalışmaları SRB canlılık tahlili ile gerçekleştirilmiştir. Kronik pankreatit kaynaklı C57BL6 farelerinde in vivo dozaj çalışmaları sırasında karın ağrısındaki değişiklikler izlendi. Daha sonra hastalığın histolojik ve immünolojik seyri değerlendirildi. Ayrıca kronik pankreatitli fare modellerinde antikoru ağrıyı etkili bir şekilde azalttı. Mevcut inhibitör antikoru son derece yüksek maliyeti ve küçük molekül inhibitörlerinin yüksek böbrek toksisitesi nedeniyle, CX3CR1 reseptörünü inhibe edecek etkili antikora hâlâ büyük bir ihtiyaç vardır. Ön sonuçlarımız, grubumuz tarafından üretilen CX3CR1 antikoru kronik pankreatite bağlı ağrıya karşı potansiyelini göstermektedir; ancak pankreas kanserini hedefleme potansiyelini değerlendirmek için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Sözcükler: Antikoru, CX3CR1, Fraktalkin, Hibridoma, Kronik pankreatit

1 INTRODUCTION

1.1 Pancreas

The main responsibility of the pancreas is to regulate the glucose and digestion metabolism. The endocrine and exocrine pancreas are the two main functional components (Miller, 1981). The main component is the exocrine part, which makes up a large part of this organ. Ductal and acinar cells are the primary cells in this area. During component metabolism; the exocrine component releases digestive enzymes and bicarbonate into the duodenum. In the endocrine part, the primary cells that can be found are Langerhans islets. From this component, different hormones are secreted, such as; glucagon ghrelin, somatostatin, pancreatic polypeptide, and insulin (Edlund, 2001; Navarro, 2014). In figure 1, four special cell types responsible for the secretion of these hormones from the islets can be seen. α - and β -cells, respectively, regulate glucose utilization by producing insulin and glucagon. PP and δ -cells, respectively, are the cell types responsible for the somatostatin and pancreatic polypeptide secretion. Both the endocrine and the exocrine components of the pancreas are innervated by the autonomic nervous system (Miller, 1981).

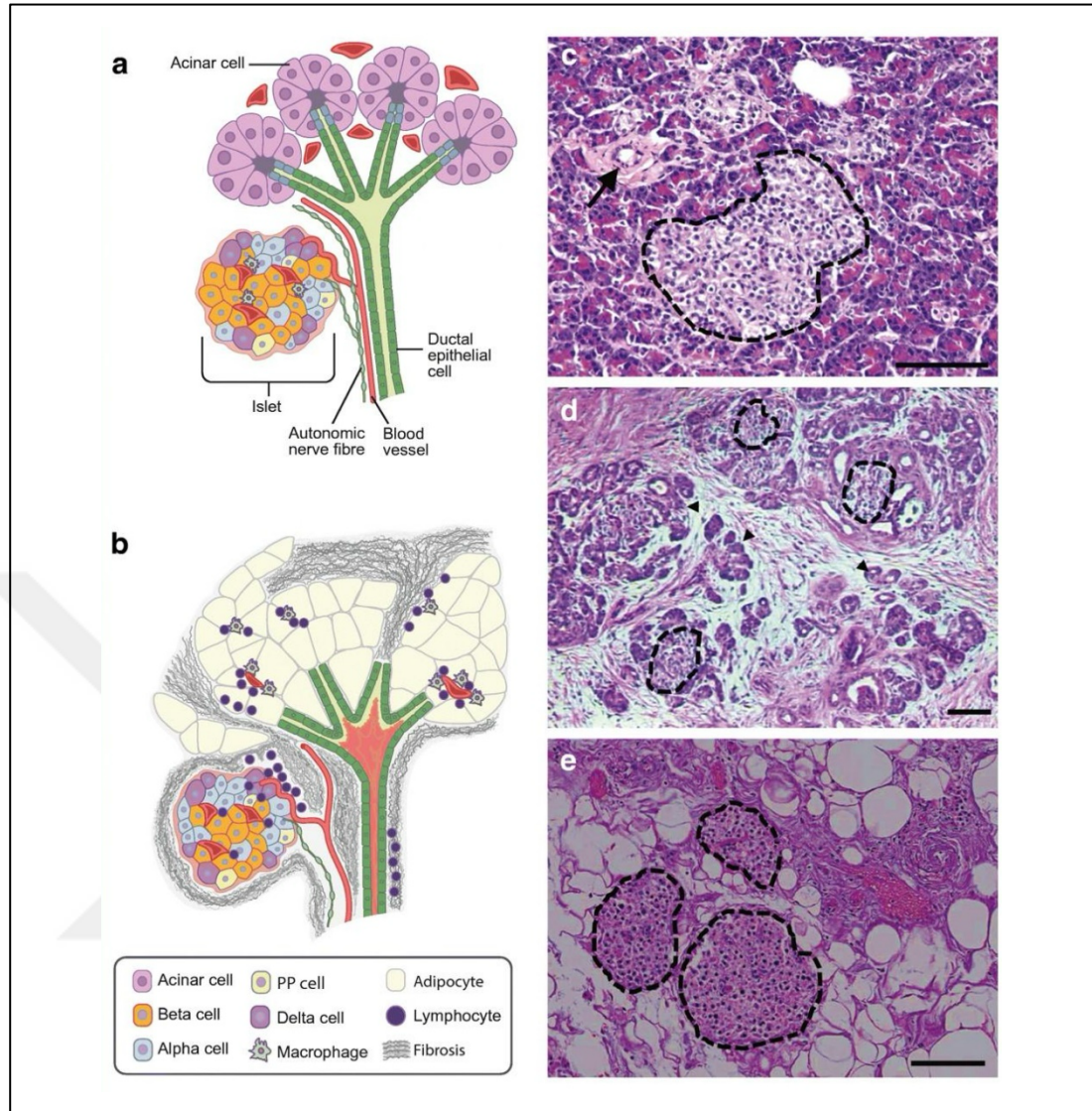


Figure 1. (a) Pancreas anatomy. (b) Exocrine component of the pancreas. (c) Single acinus. (d) Pancreatic islet (endocrine pancreas)

1.2 Pancreatic Cancer

Pancreatic cancer is one of the deadliest cancer types and ranked at seventh place in cancer-related deaths (Luo et al., 2023). Pancreatic ductal adenocarcinoma (PDAC), which arises from the exocrine cells, is the most common type of this disease, and 95% of the patients who are suffering from it cannot be cured. Pancreatic cancer is characterized by a poor prognosis, and it has the lowest five-year survival rate amongst all cancer types (Mizrahi et al., 2020). If the patient is untreated, the average survival time is accepted to be less than six months. In the United States, pancreatic cancer is

expected to be ranked in second place as the leading cause of cancer-related deaths (Yabar & Winter, 2016).

One of the main factors that causes poor pancreatic cancer prognosis is Neural invasion (NI) (Ceyhan et al., 2008). Neuropathic pain, local recurrence, and tumor progression are associated with NI. Usage of the term NI over the traditional term PNI (Perineural invasion) is a relatively more comprehensive and new term when compared with PNI (Demir et al., 2010). In some studies, the term NI has been used due to the fact that pancreatic cancer cells (PCa) are present all along the perineurium (Demir et al., 2015) but it was also demonstrated that it is possible to see PCa in the endoneural region which is located between the nerve fascicles (Demir et al., 2015).

NI is one of the most important problems which are related to nerves for clinical pancreatic cancer treatments (Demir et al., 2015). PNI can be explained as the neoplastic invasion around or into nerves by tumor cells. When compared with other types of gastrointestinal malignancies, the PNI prevalence is higher in PDAC. Also, it is worth mentioning that it is much more severe in PDAC (Ceyhan et al., 2008). It is still not clear why PNI incidence in PDAC has the highest rate.

Through PNI inhibition, it is possible to improve the prognosis of PDAC patients.

1.3 Chronic Pancreatitis

Chronic pancreatitis is another common disease of the pancreas with a progressive inflammation that might result in the loss of function of the organ and structural damage. Endocrine insufficiency resulting in diabetes mellitus, exocrine insufficiency, and recurrent abdominal pain are the characteristic symptoms. It significantly impacts the life quality of the patient and is associated with the risk of pancreatic cancer development. The stages of the condition can be listed as: initial injury, inflammation and fibrosis. In most cases, it results in high chronic pain and decreased life quality and expectancy (Braganza et al., 2011; Pham & Forsmark, 2018).

One of the driving factor leading to chronic pancreatitis is the activation of Pancreatic stellate cells (PSCs). PSCs play a big role in fibrosis development. Their activation leads to the production of ECM components and collagen, which results in pancreatic dysfunction and tissue scarring. Some of the pathways involved in inflammation are JAK/STAT and NF- κ B (Apte et al., 2011).

Management and early prognosis are important for progression slowing and improving the life quality of the patient. Some lifestyle modifications might be needed, like quitting alcohol and smoking, for the management. Some of the current approaches for treatment mainly focuses on the management of the pain. For the exocrine insufficiency, enzyme replacement therapy can be performed, or for diabetes, an insulin prescription is chosen. The fractalkine – CX3CR1 cross-talk holds promise for managing the inflammatory response, reducing fibrosis, and improving the life quality of the patient (Demir et al., 2011).

1.4 Fractalkine-CX3CR1

Fractalkine (CX3CL1) is one of the members of CX3C class chemokines, which is related to pain modulation, cancer progression, and inflammatory response with its receptor CX3CR1. The relationship of these two is associated with atherosclerosis, chronic pancreatitis, rheumatoid arthritis, and a wide range of cancer types. It is known that the crosstalk of this chemokine and its unique receptor is crucial in leukocyte recruitment to the inflammation side, which shows their importance in the immune response (Imai & Yasuda, 2016).

Fractalkine is highly expressed in many of the solid malignant tumors. Usually, chemokines possess 1 one or two properties. They either have adhesion or chemoattractant properties. What makes fractalkine special is that it can act as both. It can be found in 2 different forms as soluble form or membrane-bound. In particular, the endothelial cells, it is found in the membrane-bound form anchored to the cell surface. In this form, adhesion of the leukocytes is possible. Through the proteolytic cleavage soluble form is generated. In this form the chemokine acts as a

chemoattractant towards the cells that express CX3CR1. Both of them are involved in many different pathological and physiological processes like cell migration, immune response, and inflammation (D'Haese et al., 2010; Liu & Jiang, 2011).

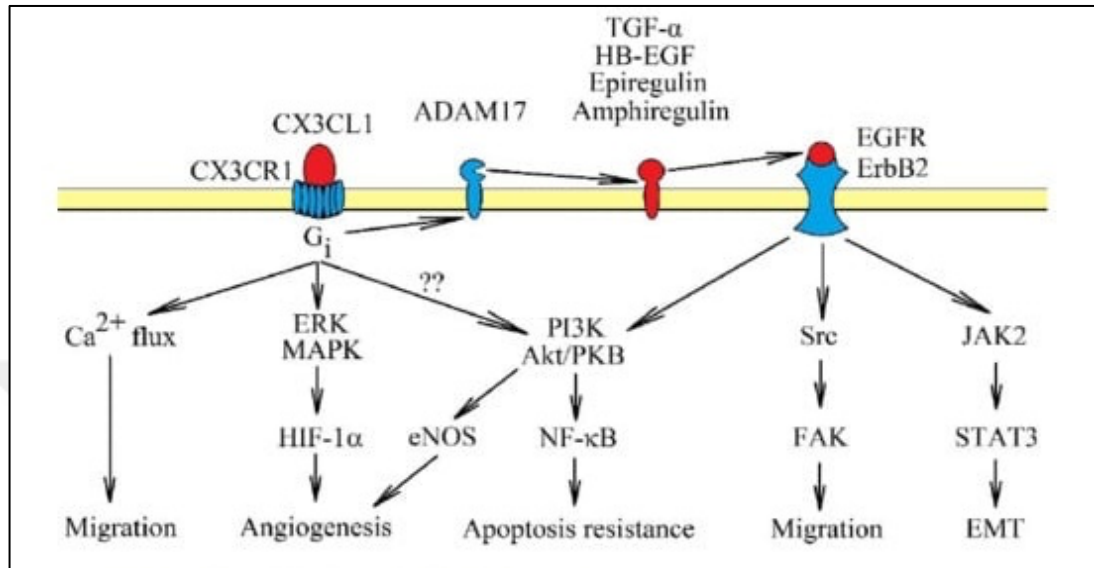


Figure 2. Signal transduction of CX3CL1-CX3CR1 (Korbecki et al., 2020)

1.5 Roles in Pancreatic Cancer

Tumor microenvironment: In the tumor microenvironment, the fractalkine is expressed and influences the interactions of immune cells and cancer cells. It has the ability to recruit T cells and macrophages to the site. Depending on the context, this interaction can play both an anti-tumorigenic or pro-tumorigenic role. The macrophages that were recruited to the area by fractalkine might get an M2-like phenotype and therefore result in immune evasion, angiogenesis, and tumor growth. It is also possible that the CX3CR1 can positively influence NK and T cells, resulting in cytotoxic effects against cancer cells (Huang et al., 2017; Wang et al., 2017).

Tumor progression and metastasis: In the studies performed on fractalkine and CX3CR1, it was seen that their expression is associated with increased invasion and migration, which indicates their role in metastasis. The binding of fractalkine to its receptor activates the PI3K/Akt pathway, which thrives of the promotion of proliferation, cell survival, and migration. It also activates the MAPK pathway, which

results in increased cell survival and proliferation. Fractalkine is also known to induce epithelial mesenchymal transition (EMT), which is a crucial process for metastasis. In this phenomenon, epithelial cells acquire the characteristics of mesenchymal cells which leads to invasive and migratory capability increase (Huang et al., 2017).

Therapeutic target: As fractalkine has the ability to influence tumor cell behavior and modulate the immune cells, it shows potential as a therapeutic target. The inhibition of this pathway may result in reduced metastasis and tumor growth through immune cell recruitment and altering the tumor microenvironment. The production of CX3CR1 antagonists can be used for the inhibition of this chemokine, which would result in reduced pro-tumorigenic immune cell recruitment, cell invasion, and migration inhibition. For this context, neutralizing antibodies can be used by altering the TME (Huang et al., 2017; Korbecki et al., 2020).

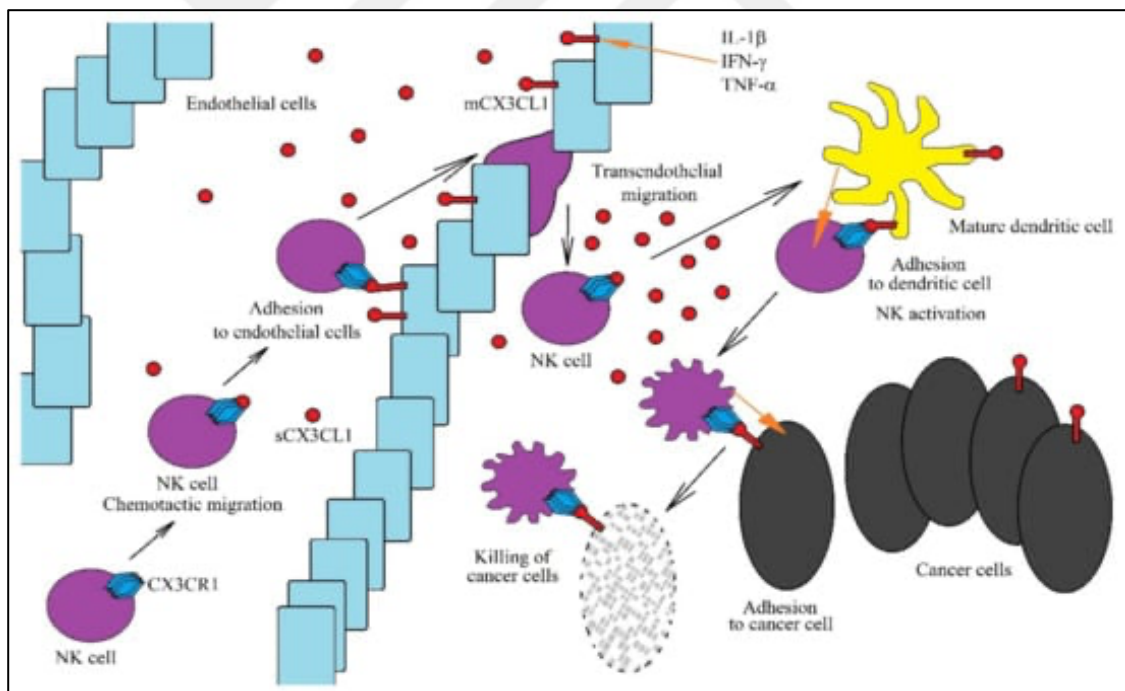


Figure 1. Role of fractalkine – CX3CR1 interaction on NK cell functions against cancer (Korbecki et al., 2020)

1.6 Roles in Chronic Pancreatitis

Inflammatory response: In the course of chronic pancreatitis, prolonged fibrosis and inflammation in the pancreas are observed. Under these conditions, the chemokine fractalkine recruits the immune cells to the area in order to sustain the inflammatory response and fibrosis development. The monocytes recruited by the fractalkine have the ability to differentiate into macrophages at the site of the inflammation. The differentiated macrophages can release cytokines like $\text{TNF-}\alpha$, $\text{IL-1}\beta$ which would result in the sustained response of inflammation, therefore acting as pro-tumorigenic. Also, T cells are recruited to the area by fractalkine. T cells contribute to the chronic inflammatory milieu. Fractalkine can influence the activation of the $\text{NF-}\kappa\text{B}$ pathway in immune cells, which leads to the perpetuation of inflammation and the production of inflammatory cytokines. Another pathway activated by this chemokine is JAK/STAT, which promotes fibrosis and inflammatory response (Ceyhan et al., 2009; Huang et al., 2012).

Fibrosis development: Pancreatic stellate cells (PSCs) are known to be the main actors in fibrosis development. The interaction of fractalkine in the cells that express its receptor can activate the stellate cells. Through the effect of fractalkine, PSCs transform into myofibroblast-like. These cells produce ECM components and collagen, which leads to organ dysfunction and tissue scarring. Activated PSCs also produce $\text{TGF-}\beta$ which is crucial for fibrogenesis. The activation of this pathway leads to fibrosis and ECM production stimulation (Ceyhan et al., 2009; Huang et al., 2012).

Potential as a biomarker and therapeutic target: Due to the discussed facts, fractalkine has the potential to be a biomarker for the progression and severity of chronic pancreatitis. Also, as it is involved in both of the main complications related to chronic pancreatitis, targeting this pathway can be effective for keeping the symptoms under control (Ito, 2007).

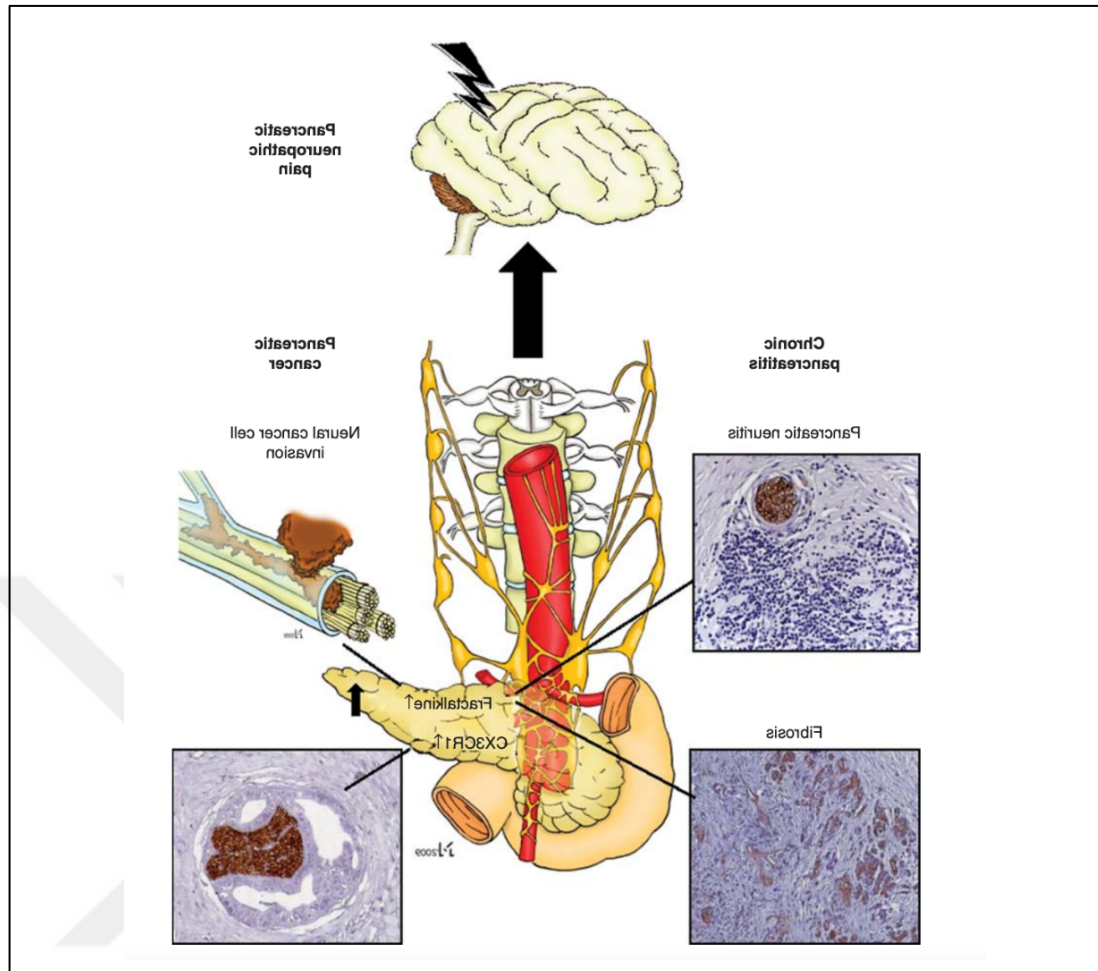


Figure 2. The role of CX3CR1/Fractalkine crosstalk in pancreatic cancer and chronic pancreatitis (Ceyhan et al., 2009).

This couple is known to be affected by various histopathological phenomena, and they are the crucial elements in the sensation of pain in pancreatic cancer and chronic pancreatitis. The upregulation of fractalkine in the chronic pancreatitis tissues is related to the degree of the neuritis neuropathic pain sensation and fibrosis. In pancreatic cancer, the fractalkine, which is expressed on the neural structures, binds to CX3CR1 on the pancreatic cancer cells, which results in neuropathic pain and neural invasion (Ceyhan et al., 2009).

In conclusion, fractalkine is a special chemokine with dual functions that can affect the immune environment and tumor progression in pancreatic cancer while

involving inflammation and fibrosis development in chronic pancreatitis. Therefore, it shows promise as a therapeutic target and a biomarker.

1.7 Fractalkine and Pain

In the studies performed, it was observed that the expression of CX3CR1 and fractalkine is performed by the nervous system, and they take a crucial role in neuromicroglial communication (Ceyhan et al., 2009; Zhuang et al., 2007). The neurons located in the spinal cord can cleave the fractalkine attached to the membrane through stimulation that results in the soluble chemokine release (Figure 2) (Ceyhan et al., 2009; Zhuang et al., 2007). In the experimental models on neuropathic pain, it was observed that the dorsal root ganglions (DRG) release endogenous fractalkine, and in response to that, CX3CR1 is upregulated in the spinal cord microglia, causing pain. It was also seen that the high immunoreactivity of CX3CR1 is related to macrophage migration. These findings show the role of the Fractalkine/CX3CR1 relationship on microglial cross-talk in nociceptive processes (Ceyhan et al., 2009).

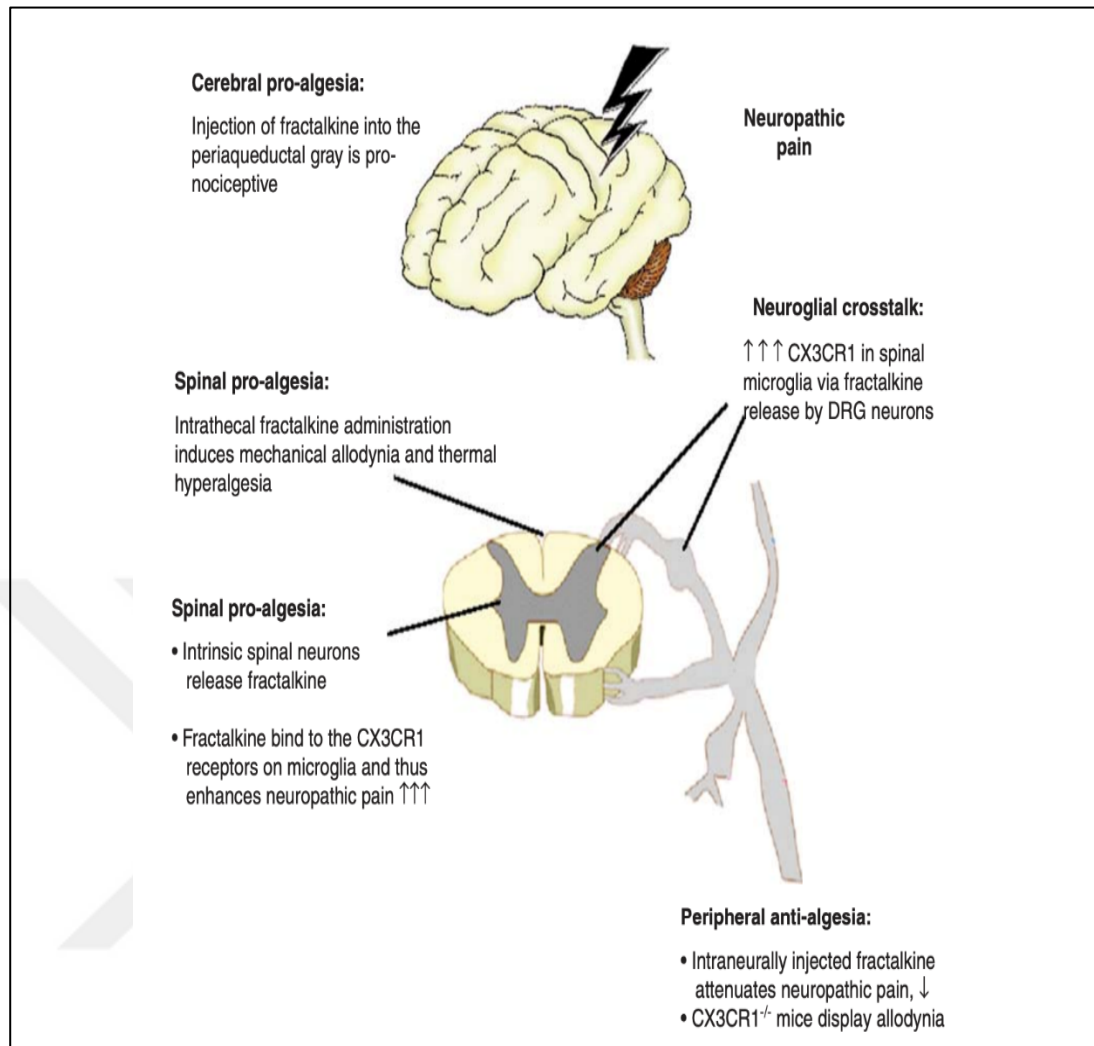


Figure 3. CX3CR1 and fractalkine involvement in peripheral and central mechanisms of neuropathic pain (Ceyhan et al., 2009)

1.8 CX3CR1 amino acid sequence

CX3CR1 - HUMAN

MDQFPESVTENFEYDDLAEACYIGDIVVFGTVFLSIFYSVIFAIGLVGNLL
VVFALTNSKKPKSVTDIYLLNLALSDLLFVATLPFWTHYLINEKGLHNAMCK
FTTAAFFFIGFFGSIFFITVISIDRYLAIVLAANSMNNRTVQHGVTISLGVWAAAI
LVAAPQFMFTKQKENECLGDYPEVLQEIWPVLRNVETNFLGFLPLLIMSYC
YFRIIQTFLFCKNHKKAKAIKLILLVVIVFFLFWTPYNVMI FLETCLKLYDFFPS

CDMRKDLRLALSVTETVAFSHCCLNPLIYAFAGEKFRRYLYHLYGKCLAVL
CGRSVHVDFFSSSESQSRHGSVLSSNFTYHTSDGDALLLL

The N terminus and the sequence resulting with FLET are the important parts for the binding process (Chen et al., 2006).

CX3CR1 - MOUSE

MSTSFPELDLENFEYDDSAEACYLGDIVAFGTIFLSVFYALVFTFGLVGN
LLVVLALTNSRKPKSITDIYLLNLALSDLLFVATLPFWTHYLISHEGLHNAMC
KLTTAFFFIGFFGGIFFITVISIDRYLAIVLAANSMNNRTVQHGV TISLG VWAA
AILVASPQFMFTKRKDNECLGDYPEVLQEMWPVLRNSEVNILGFALPLLIMS
FCYFRIIQTLSCKNRKKARAVRLILLVVF AFFLFWTPYNIMIFLET LKFYNFF
PSCDMKRDLRLALSVTETVAFSHCCLNPF IYAFAGEKFRRYLGHLYRKCLAV
LCGHPVHTGFSPESQSRQDSILSSFTHYTSEGDGSLLL

- Percent sequence similarity
- BLAST – seq similarity

CX3CR1 - HUMAN

MDQFPESVTENFEYDDLAEACYIGDIVVFGTVFLSIFYSVIFAIGLVGNLL
VVFALTNSKKPKSVTDIMSTFPELDLENFEYDDSAEACYLGDIVAFGTIFLSV
FYALVFTFGLVGNLLVVLALTNSRKPKSITD

YLLNLALSDLLFVATLPFWTHYLINEKGLHNAMCKFTT AFFFIGFFGSIFF
ITVISIDRYLAIVLAANSMNNRTVQHGV TISLG VWAAAILVAAPQFMFTKQK
ENECLGDYPEVLQEIWPVLRNVETNFLGFLPLLIMSYCYFRIIQTLSCKNH
KKAKAIKLILLVVIVFFLFWTPYNVMIFLET LKLYDFFPSCDMRKDLRLALSV
TETVAFSHCCLNPLIYAFAGEKFRRYLYHLYGKCLAVLCGRSVHVDFFSSSES
QSRHGSVLSSNFTYHTSDGDALLLL

CX3CR1 - MOUSE

IYLLNLALSDLLFVATLPFWTHYLISHEGLHNAMCKLTТАFFFIGFFGGIF
FITVISIDRYLAIVLAANSMNNRTVQHGVТISLGVWAAAILVASPQFMFTKРK
DNECLGDYPEVLQEMWPVLRNSEVNILGFALPLLIMSFСYFRІIQTLFSCKNR
KKARAVRLILLVVFAFFLFWTPYNIMIFLETЛKFYNFFPSCDMKRDRLALSV
TETVAFSHCCLNPFIYAFAGEKFRRYLGHLYRKCLAVLCGHPVHTGFSPESQ
RSRQDSILSSFTHYTSEGDGSLLL

Domain similarity Human vs Mouse

- Extracellular domain -I
- Human >sp|P49238|1-31
- MDQFPESVTENFEYDDLAEACYIGDIVVFGT
- Mouse
- >sp|Q9Z0D9|1-32
- MSTSFPELDLENFEYDDSAEACYLGDIVAFGT

Human

- sp|P49238|91-103
- LINEKGLHNAMCK

Mouse

- >sp|Q9Z0D9|92-104
- LISHEGLHNAMCK

Human

- >sp|P49238|168-195
- TKQKENECLGDYPEVLQEIWVLRNVET

Mouse

- >sp|Q9Z0D9|169-196
- TKRKDNECLGDYPEVLQEMWPVLRNSEV

Human

- >sp|P49238|257-273
- KLYDFFPSCDMRKDLRL

Mouse

- >sp|Q9Z0D9|258-274
- KFYNFFPSCDMRKDLRL

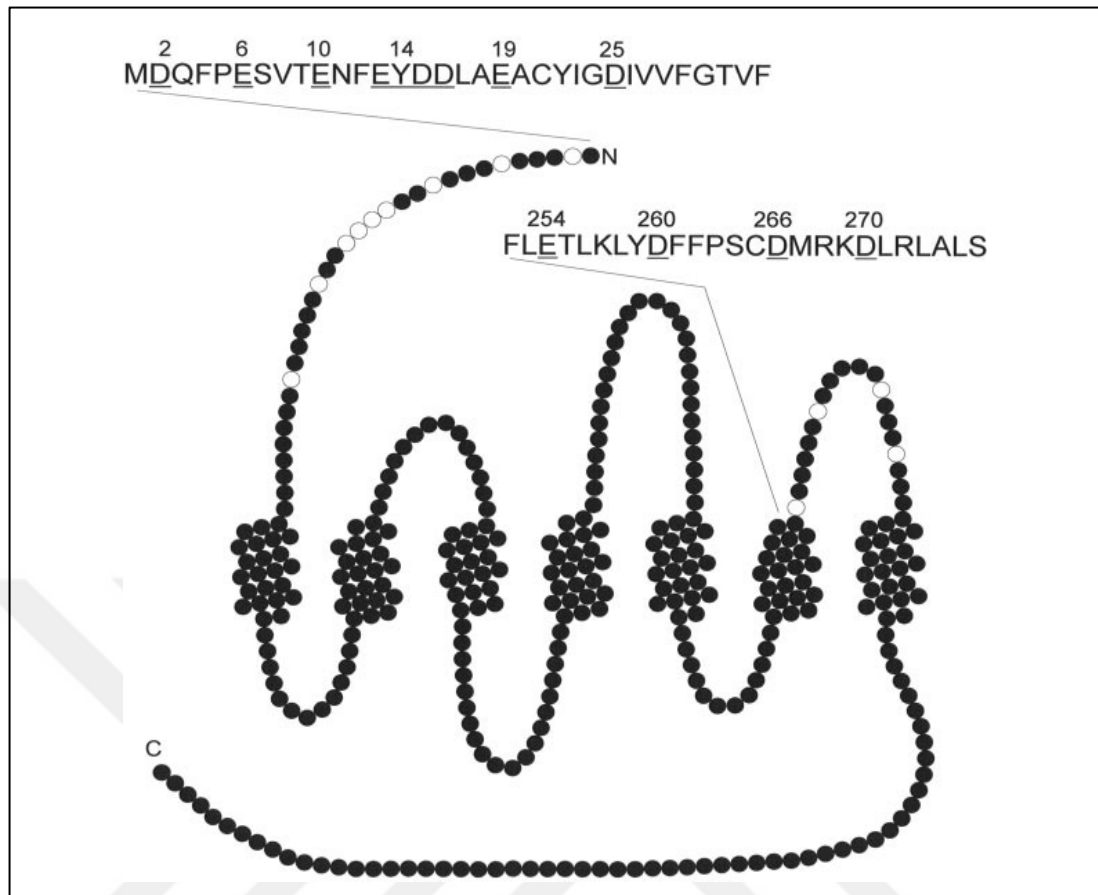


Figure 4. Partial amino acid sequence and structural diagram of CX3CR1. FLET sequence is the N-terminus third extracellular loop (binding position) (Chen et al., 2006).

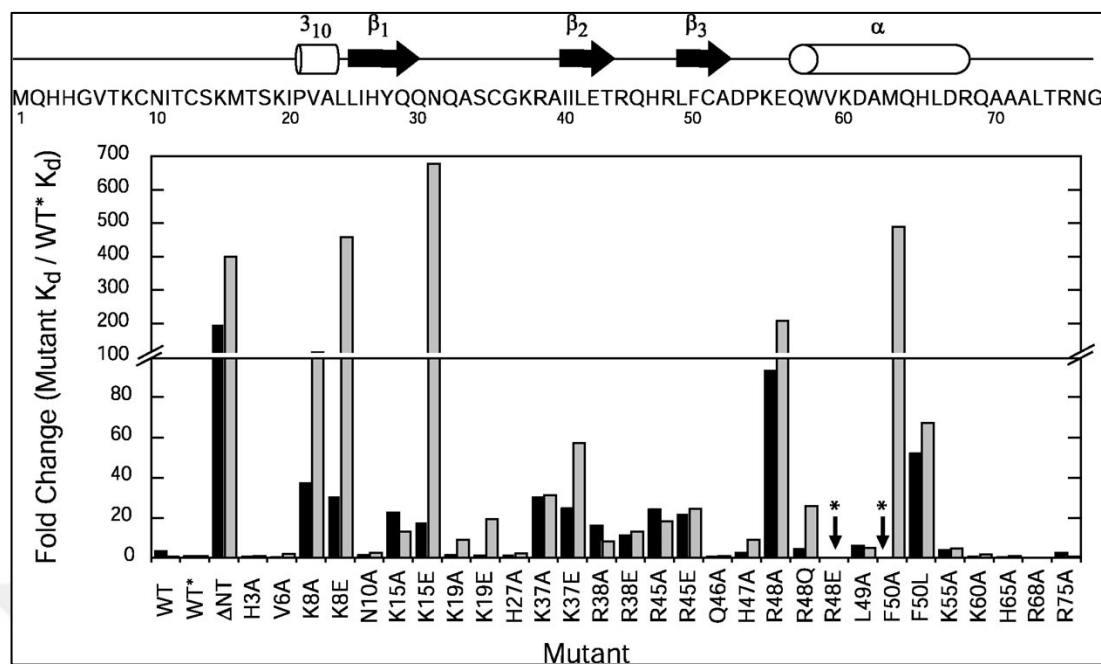


Figure 5. Binding affinity summary of the fractalkine mutants to the CX3CR1 and microglia transfectants. Gray columns Show the transfectant cell binding and black columns show microglia of rat

1.9 Monoclonal Antibody Production

Antibodies that play crucial roles in the body's immune system and are known to be glycoproteins, and immunoglobulins they are produced by B lymphocytes, which can be seen in Figure 7 (M. Tomita & K. Tsumoto, 2011). They are the main components of the humoral immune system and protect the body against pathogen invasions like viruses or bacteria (Epstein & Epstein, 1986; Zurawski & McLendon, 2020). They are composed of two main structural units which can be named as heavy and light chains (Vlasak & Ionescu, 2011). Each of the light chains contains one variable and one constant region, and each of the heavy chains is composed of one variable and three constant regions. The variable regions play a role in the recognition of the pathogens. The main recognition system can be resembled to a key and lock principle. The specific antigen to be recognized (key) and the specific paratope (lock) are recognized by only each other. Attached to the antigen-binding fragments, there are crystallizable fragments (Fc) (Vlasak & Ionescu, 2011). Based on Fc, there are five different heavy chain types, which can be given as IgE, IgD, IgM, IgG, and IgA (Spiegelberg, 1989).

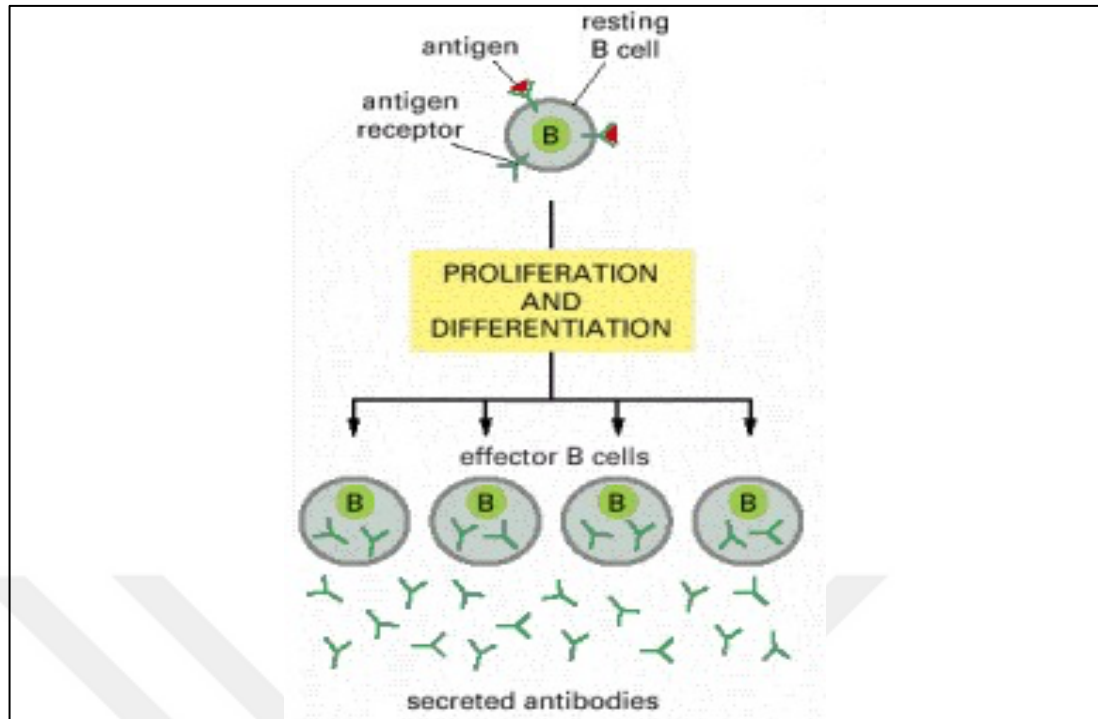


Figure 6. Antibody production and activation of the B cells (Alberts et al., 2002)

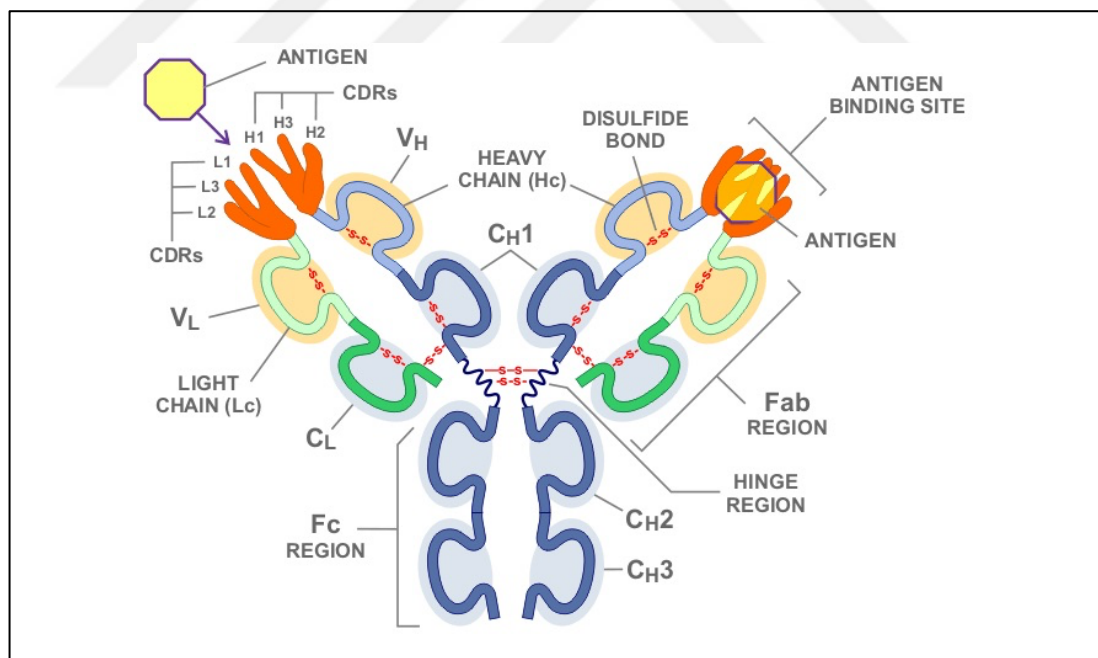


Figure 7. Structure of an IgG antibody (Negritto & Manthey, 2016).

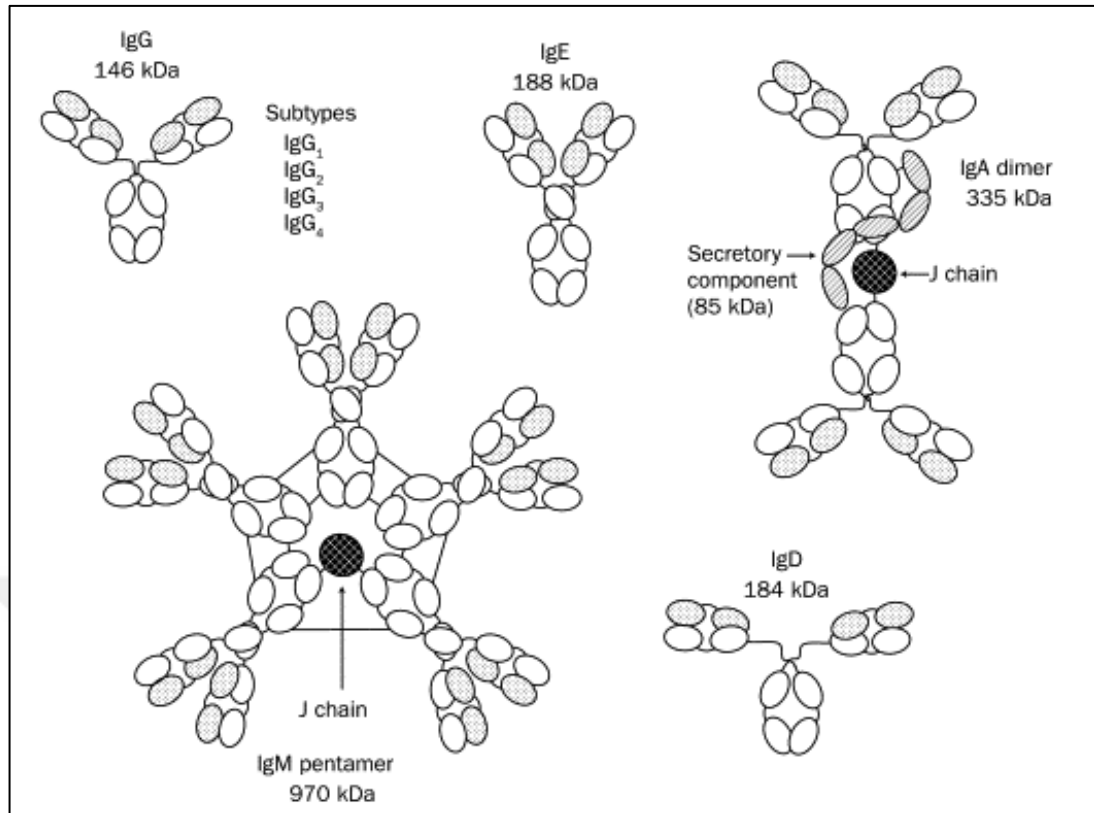


Figure 8. Immunoglobulin classes in humans (Zola & Roberts Thomson, 2001)

There are many different applications of antibodies in biomedical research area. The selective binding and high specificity of the antibodies made it possible for them to be used in applications like flow cytometry, magnetic cell sorting, ELISA, Western blot, etc. (Tetin & Stroupe, 2004; Zola & Roberts Thomson, 2001). Two main types of antibodies are present, which are monoclonal and polyclonal antibodies. This classification is originated through production from lymphocytes (Jelinek, 2000; Tetin & Stroupe, 2004). Both of them have their own disadvantages and advantages depending on the application which they will be used (Jelinek, 2000).

1.10 Polyclonal Antibodies

Polyclonal antibodies (pAbs) are produced by B cells and they react towards multiple regions of antigens (Lipman et al., 2005). They are able to recognize more than one epitope because they consist of a mixture of various antibodies. Due to their potential to recognize multiple epitopes, one of the main uses of this antibody type is

to be used in viral, bacterial disease toxin treatment (Chambers & Johnston, 2003). The pAbs production can be performed in order to generate a high antibody titer against an antigen and it is performed by a prime boost immunization strategy through the injection of an immunogen to animal (Stills, 2012). After the immunizations are performed and the production of the antibodies are validated, generated pAbs can be purified from other components or can be directly used.

The production can vary from batches as they are a mixture, and they also contain the possibility of transmission of blood-borne diseases. Another fact that limits the use of pAbs is the fact that they low specificity. Therefore, a higher dosage may need to be used in order to get the desired effect. The use of them in chronic diseases is not really possible. From the disadvantages that were mentioned, it can be seen that the need for monoclonal antibody (mAb) arises. Due to their high affinity, sensitivity, specificity, and homogenous nature, they are easily used in research (Rasmussen et al., 2007; Singh et al., 2018).

1.11 Monoclonal Antibodies

Different from pAbs, mAbs are specific to only one epitope, which makes them more sensitive and targeted. They are produced from a single B lymphocyte clone. One of the most common ways to produce mAbs is hybridoma technology, which was first used in 1975. The hybridoma method makes it possible to produce an infinite and continuous amount of mAbs with a minimum variation between batches (Köhler & Milstein, 1975; Vázquez-Gutiérrez & Langton, 2015).

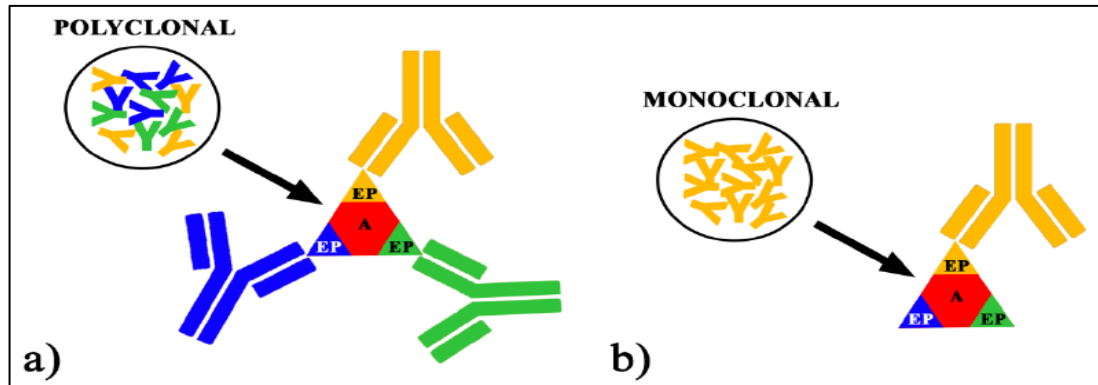


Figure 9. Monoclonal and Polyclonal antibody bindings a) polyclonal antibody; b) monoclonal antibody. A: antigen; EP: epitope (Vázquez-Gutiérrez & Langton, 2015)

1.12 Hybridoma Technology

Cesar Milstein and Georges Kohler invented the hybridoma technology in 1975 and received a Nobel prize in physiology and medicine for it (Köhler & Milstein, 1975). Hybridoma cells are produced by the fusion of immortal myeloma cells and antibody-producing B cells (Köhler & Milstein, 1975). The hybridoma cells can produce mAbs and can be cryopreserved.

In the early uses of this application, only murine antigens were used (Köhler & Milstein, 1975; Taggart & Samloff, 1983). Over time, the development of this method made it possible to produce mAbs from different species, such as humans, sheep, goats, chickens, cows, rabbits, mice, etc.. The main choice as the host is mice (Goldsby et al., 1987; Nishinaka et al., 1991; Weber et al., 2017).

It is possible to classify hybridomas into two types: hetero- and homohybridomas. In homohybridomas, fusion of B cells and myeloma cells is isolated from the same species, while in heterohybridomas, the isolation is performed from different species. In general, homohybridomas are stable genetically. This is caused by chromosomal recombinant loss, while clonal selection is performed in heterohybridomas (Page & Thorpe, 2002).

1.13 Monoclonal Antibody Production with Hybridoma Technology

Production of mAbs through hybridoma is mainly based on the immunization of a laboratory animal the isolation of the antibody-producing organ and lymph nodes and the fusion of the cells obtained from these organs with immortal myeloma cells, which can be seen in Figure 11. After the fusion process is performed, these cells are incubated in a hypoxanthine, aminopterin, thymidine) supplemented medium, and the clones are screened for the antibody of interest (Wood, 1984).

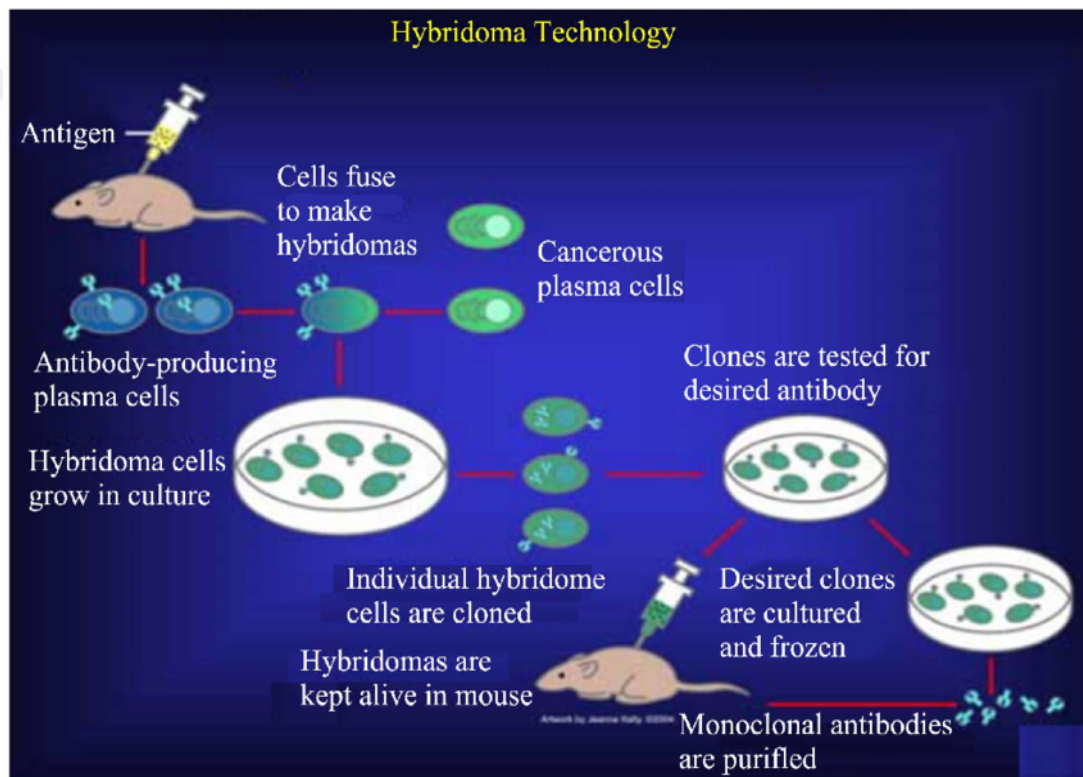


Figure 10. Hybridoma technology protocol (Alam et al., 2023)

1.13.1 Immunization

As mentioned, the first step is the immunization process. In this stage, the aim is to stimulate the B cell differentiation into memory and plasma B cells (48). The main choice of type for mouse for this procedure is Balb/C. The selected immunogen is mixed with an adjuvant like Freund's complete/incomplete adjuvant and injected

intraperitoneally. This injection can be performed at 7-10 day intervals. 3 days after the immunization, blood is collected from the submandibular vein of the mice and tested with appropriate testing methods like ELISA for blood antibody levels. It is expected to have a sufficient amount after 3-4 injections. If the amount is found to be enough, the mice are sacrificed for B cell and lymph node isolation (Wood, 1984).

1.13.2 B lymphocyte isolation

After the mice are scarified, the spleen and lymph nodes need to be isolated under aseptic conditions. Collected organs are stored in appropriate medium until further use. In this step, it is necessary to act fast, as B cells are fragile and cannot last long. The collected organs can be ground on the top of a cell strainer to isolate B cells (Carson & Freimark, 1986; Wood, 1984).

1.13.3 Preparation of myeloma cells

At least one week before the fusion process, myeloma cells need to be treated with 8-azaguanine to inhibit the work of the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) gene. This gene is related to DNA replication through the salvage pathway. The inhibition of this gene results in unfused myeloma cell death in the HAT supplement (Kontsekova et al., 1991).

1.13.4 Fusion

In this step, the isolated B cells and myeloma cells, which are HAT-sensitive, are fused together to make two cells into one with the usage of polyethylene glycol 4000(PEG-4000). The cells get combined in a 1/5 ratio (1 myeloma, 5 B cell). PEG-4000 creates pores on the cell membrane, and with the centrifugal force applied, the cells form a cell with more than one nucleus. Also, electro-fusion is another method to obtain hybridoma cells. In this method, an electrical field is applied on the cells, which results in pore formation (Jelinek, 2000; Masahiro Tomita & Kanta Tsumoto, 2011).

1.13.5 Selection in HAT medium

After the fusion process is performed, cells are incubated in HAT-supplemented media for 10 to 14 days. There can be three different cells found in the media. 1 out of 100 cells survive through this process. The first cell line that can be seen are unfused B cells, which die due to their limited lifespan. Unfused myeloma cells die when exposed to aminopterin in HAT. Fused hybridoma cells have the ability to perform DNA replication through a salvage pathway, so they are the only cell type expected to survive in the process. B cells have functional HGPRT genes, and myeloma cells are immortal, and as hybridoma cells can show the properties of both of the cells, they can grow indefinitely (Kontsekova et al., 1991; Masahiro Tomita & Kanta Tsumoto, 2011).

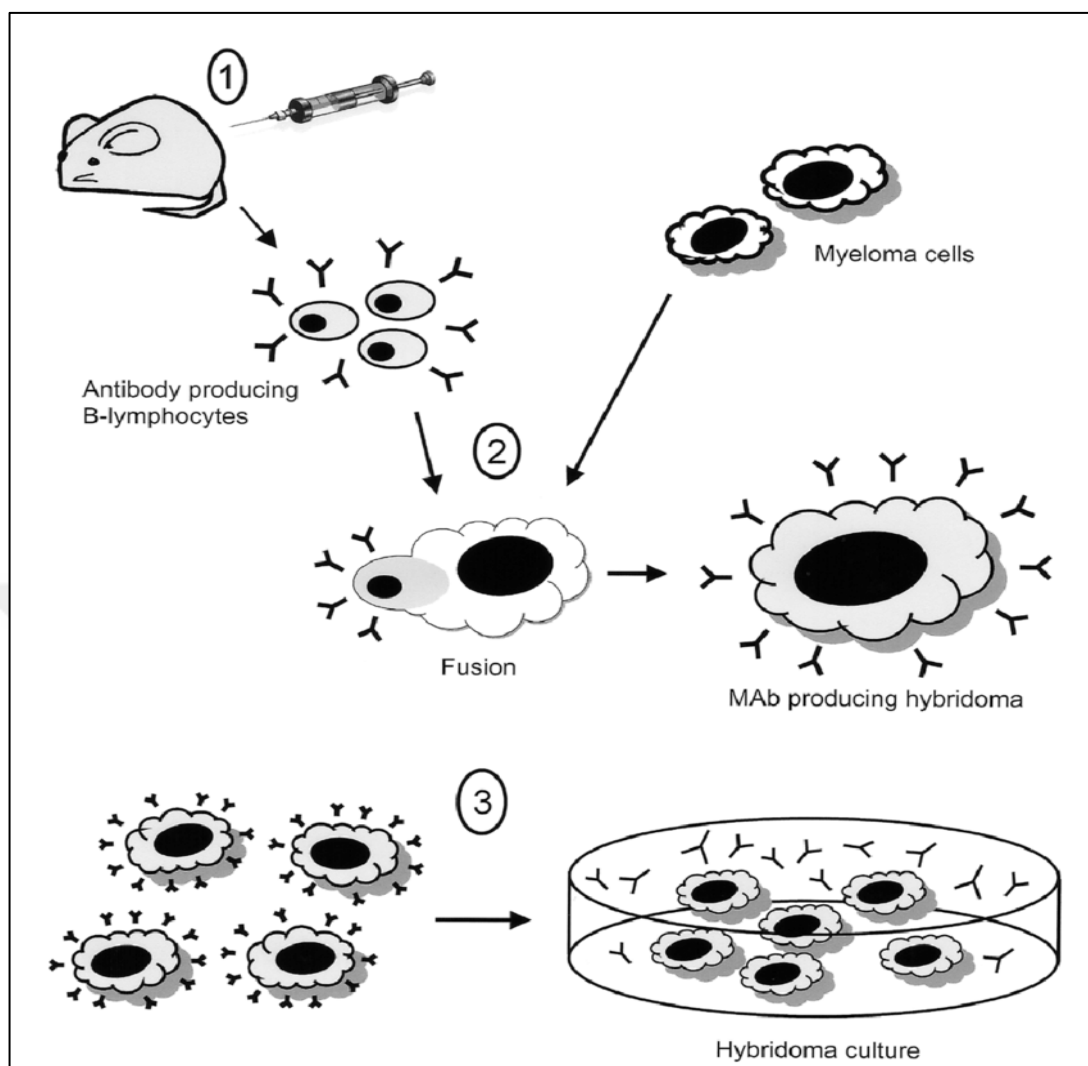


Figure 11. Hybridoma technology. (1) Immunization step and isolation of antibody-producing B lymphocytes from the spleen. (2) Fusion of B lymphocytes with myeloma cells and formation of hybridoma cells. (3) Propagation of hybridoma cells producing desired antibodies in culture (Koppe et al., 2006)

1.13.6 Screening

In the screening process, HAT supplement is removed from the media, and instead, HT supplement is added. Colonies are incubated for 4-5 days, and supernatants are collected to be tested with an appropriate test such as ELISA for desired antibody production. All clones don't have to produce the desired sample. Some cells maybe producing antibodies against the same antigen but not the epitope wanted. In this process, the cells that are producing the specific mAb are selected (Vaidya et al., 1985).

1.13.7 Expansion

Hybridoma cells that are chosen by ELISA are transferred to large culture flasks. In this part it is important to cryopreserve some of the cells and check the cells routinely as hybridoma cells are not really stable (Vaidya et al., 1985).

1.13.8 In vivo

Around 105 – 110 hybridoma cells can be injected to mouse intraperitoneally. After 2-3 weeks from the injection, acidic fluid that has been accumulated in the mice's abdomen is collected with a syringe to be purified (Vaidya et al., 1985).

1.13.9 In vitro

Hybridoma cells are grown in cell culture flasks. From these growing cell line, every 4-day supernatant is collected from the flask to be purified. This method provides antibodies that are more pure and don't have the risk of contamination from the mice (Jeong & Wang, 1995; Vaidya et al., 1985).

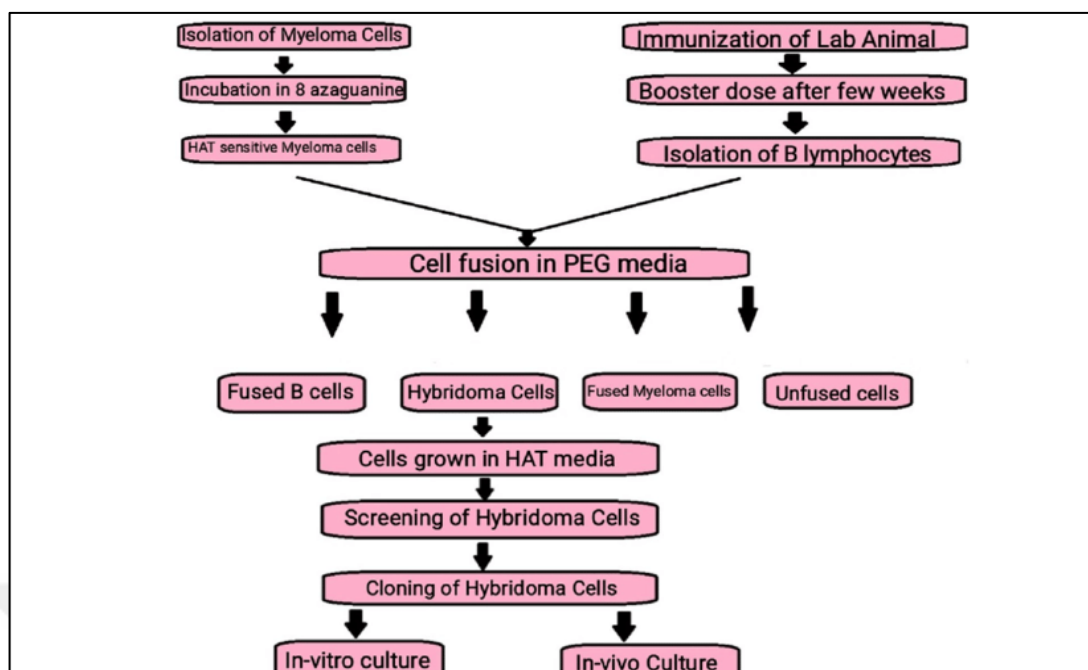


Figure 12. mAb production through Hybridoma technology (Mitra & Tomar, 2021)

The usage of the hybridoma method for mAb production has many advantages. An unlimited amount of mAb which is highly specific and pure can be obtained. The screening part allows to choose the particular antibody against different epitopes of the antigen. The antibodies produced by this method can be used directly in vaccine productions or chemotherapeutic regimens.

One of the challenges that this method presents is in the immunization process. The effectiveness of the protocol depends on the effectiveness of the immunization. This part depends on different factors like the number of boosts, dose of the antigen, adjuvant choice, and the protocol selected. Also, it takes time to identify the best suitable clone. This protocol is time-consuming and labor-intensive but effective (Corrigan, 1988; Moraes et al., 2021).

Using the semi solid media reduces the time needed for screening. In this media, only the viable hybridoma clones grow to be isolated. Semi solid media is acquired through methylcellulose addition. As only the viable hybridoma cells grow into distinct colonies it is not needed to check all of the wells in a 96 well plate and test them for positivity (Mitra & Tomar, 2021; Moraes et al., 2021).

The use of hybridoma technology can be said to revolutionize the immunology, biotechnology toxicology and many other fields. It provided an effective way of mAb production which can be widely used in many areas (Dharshanan & Hung, 2014).

1.14 Solid Phase Peptide Synthesis

The emergence of solid-phase peptide synthesis (SPPS) eliminated the need for repetitive purification steps from the solution-phase peptide synthesis method (Behrendt et al., 2016). For up to 50 amino acids, this method can be used. Introduction of the SPSS was done by Bruce Merrifield in 1963, and he received a Nobel prize for his invention in 1984. In this method, peptides are attached to resin (insoluble solid polymer support) and get chemically synthesized through repetitive additions of amino acids. It is based on the covalent bonding of the first amino acid from the C-terminus to a resin and the coupling of the amino acids. In the end, the synthesized peptide is removed from the resin. The excess byproducts and reagents are removed from the sample by washing and filtering (Chan & White, 1999).

1.15 Principles of Solid Phase Peptide Synthesis

1.15.1 Solid phase peptide synthesis

By using a solid resin bead, the assembling of amino acids from the C- to N terminus is performed through solid-phase peptide synthesis (Fields et al., 1992). In order to prevent more than one amino acid linking, the N^α-amino group is protected. Initially, the C-terminus carboxy group is attached to a linker group, resin. The protection on the α-amino group gets removed, and the next amino acid gets connected to the first one from the C- terminus, forming an amide bond. Washing and filtration steps are performed in order to get rid of impurities. The protecting groups located on the formed peptide's N-terminus are removed through the whole process. This protocol goes on until the final product (Figure 2).

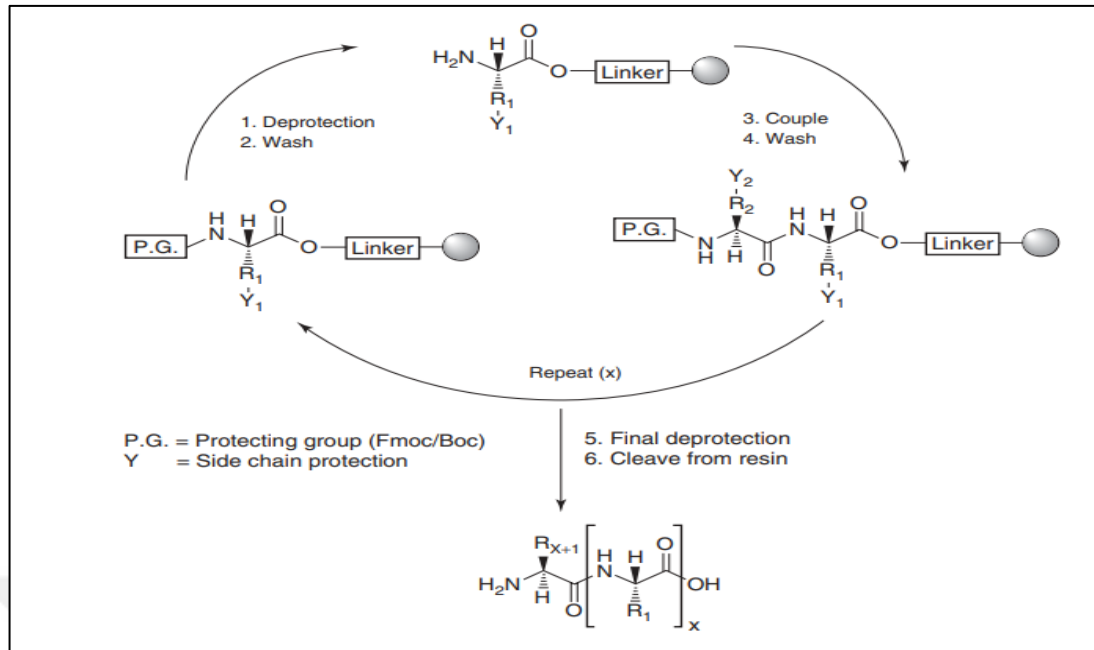


Figure 13. Solid-phase peptide synthesis (Rzeigui et al., 2021)

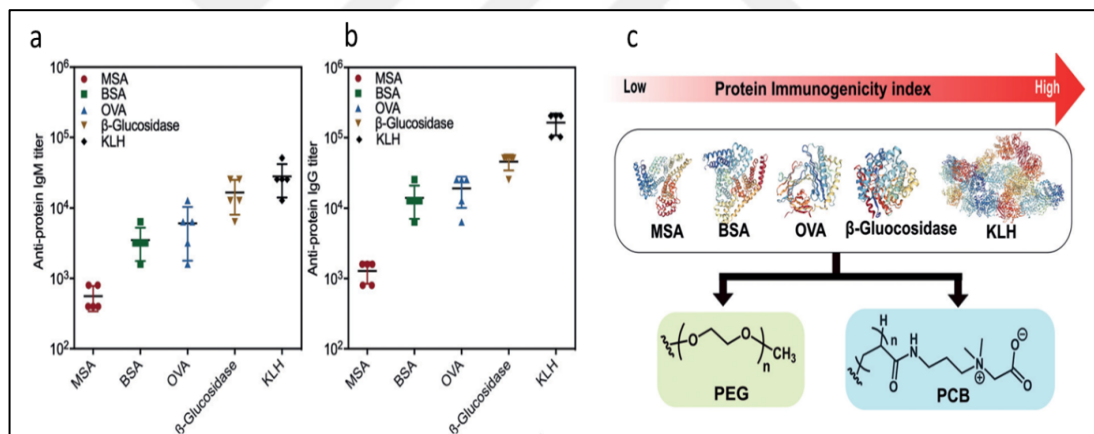


Figure 14. Immunogenicity comparison of different proteins.

IgM and IgG immunogenicity amounts are seen in A and B. IgM is the body's main defense mechanism and has 5 binding sites in total, so its avidity is higher, but the affinity is lower, meaning it is not specific. After an immunogen enters the body, the first thing it encounters is IgM. However, later, with the effect of the humoral response, the body begins to recognize this immunogen, and specifically, counter IgG begins to be produced. IgGs are more specific, and their half-life in the body is approximately twice as long (Morell et al., 1970). One of the main reasons for a total of 3 injections in the hybridoma procedure is that after the 3rd injection, the IgG rate

begins to exceed the IgM rate as the body recognizes the immunogen. As seen in the figure, since the immunization rate of KLH is higher than that of BSA, KLH was used in injections with peptide, and BSA-conjugated peptide was used as the coating material for the ELISA test. Then, the concentration measurement of peptides was carried out using the BCA method (Betting et al., 2008).

1.15.2 Isostrip

Essentially, the isostrip kit contains latex beads carrying anti-mouse kappa and anti-mouse lambda antibodies that react with any mouse monoclonal antibody, regardless of isotype. Each side of the strip carries a positive control band; this indicates that the antibody-coated beads are moving up the lane. Within approximately 5-10 minutes, the latex beads aggregate into blue bands in two sections corresponding to the isotype of the monoclonal antibody and the nature of its light chain. Stripe development is completed when the positive control band in the strip turns blue (Eftekharian et al., 2005).

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipments

Peptide Synthesizer (CEM), Peptide Razor (CEM), HPLC (Agilent), C18 Semi Preparative HPLC Column (Agilent), Xevo G2-XS QToF (MS) (Waters Corp), Vortex (VWR), Pure Water Device (Advantage Milli Q), Freeze Dryer (Lyophilizer) (Labconco), Biosafety Cabinets (Thermo Scientific), Microplate Reader (Thermo Scientific Varioskan), NanoDrop One (Thermo Scientific). Centrifuge (Thermo Scientific), CO₂ Incubator (NUVE), Laminar flow hood (NUVE), Microplate Reader (BioTek), Hemacytometer, Inverted microscope (Zeiss), 96-well plates (High binding) (Greiner Bio-One), 96-well Black (Thermo Scientific), 75 cm² cell culture flasks (Sarstedt), 5-10-25 mL serological pipettes (Isolab), Serum gel blood tube (500 µL) (Sarstedt), Cell scraper (Corning), 15-50 mL falcon tubes (Greiner Bio-One), Cryotubes (Corning), 96-well cell culture plates (Greiner Bio-One), 40 µm cell strainer (Corning), Petri dishes (Greiner Bio-One), Injectors (B Braun Omnican), 21G needle (B Braun Omnican), Scissors (Geuder), Forceps (Geuder).

2.1.2 Chemicals

Oxyma (Sigma-Aldrich), Rink Amide Resin (Sigma-Aldrich), Acetonitrile HPLC Grade (Merk), N,N-Dimethylformamide (DMF) (aber), N,N-Diisopropylcarbodiimide (DCM) (Merck), Trifluoroacetic acid (TFA) (Sigma), Triisopropylsilane (TIS) (Across), Diethyl ether (Carlo Erba), Acetic acid (Sigma-Aldrich), Tween 20 (Sigma-Aldrich), Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific). Freund's complete adjuvant (FCA) (Sigma-Aldrich), Incomplete Freund's adjuvant (IFA) (Sigma-Aldrich), Dulbecco's phosphate-buffered saline (DPBS) (Gibco), Skim milk (BioFrox), Anti-Mouse Polyvalent Immunoglobulins (G,A,M)–Alkaline Phosphatase antibody produced in goat (Sigma-Aldrich), p-NPP alkaline phosphatase substrate (Merck Millipore), Dulbecco's Modified Eagle's Medium

(DMEM) (Sigma-Aldrich), Fetal Bovine Serum (FBS) (Sigma-Aldrich), Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich), Gentamicin (Sigma-Aldrich), DMEM HG (Dulbecco's Modified Eagle's Medium with High Glucose) (Sigma-Aldrich), HEPES (Sigma-Aldrich), L-Glutamine (Sigma-Aldrich), Sodium Pyruvate (Gibco), Penicillin-Streptomycin (Gibco), Isoflurane (Dechra), Ethanol (Isolab), Polyethylene glycol (PEG) 4000 (Merck Millipore), Red Blood Lysis Buffer (ThermoFisher Scientific), Hypoxanthine-Aminopterin-Thymidine (HAT) supplement (50X) (Gibco), Hypoxanthine-Thymidine (HT) supplement (50X) (Gibco), Isostrip Mouse Monoclonal Antibody Isotyping Test Kit (Sigma-Aldrich), Fetal Calf Serum (FCS) (Sigma-Aldrich), 8-Azaguanine (Abcam), ClonaCell™-HY Hybridoma Kit (StemCell Technologies), Cerulein ammonium salt (Bachem).

2.2 Methods

2.2.1 Solid-phase synthesis and characterization

N-terminus antigen binding side of CX3CR1 synthetic molecule has been synthetically synthesized with Solid-Phase peptide synthesis method and characterized.

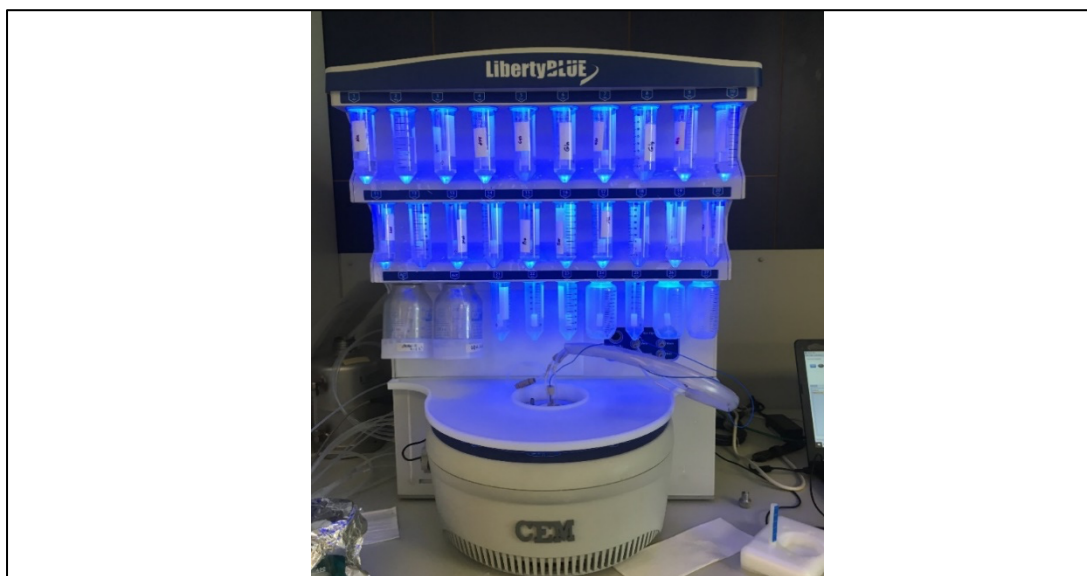


Figure 15. CEM, Liberty™ Blue Automated Microwave Peptide Synthesizer

Decoding studies on the 31 amino acid sequence of the CX3CR1 N-terminal region was performed. Purification studies of the synthesized sequence, the region between amino acids 252 and 277 belonging to the extracellular 3rd loop, continued in parallel with the experiments. Studies have been initiated to determine the binding affinity of CX3CR1, whose activity is controlled, with the fractalkine protein encoded by CX3CL1 and known as chemokine ligand. The binding site of the fractalkine protein was identified and the 17 amino acid sequence was synthesized. Sequence of the fractalkine binding region; HHGVTKCKITCSKMTSK

Peptide synthesis was carried out by the Solid Phase Peptide Synthesis method using the Liberty Blue™ unit and Discover™ unit, a microwave-powered peptide synthesis device. In Solid-Phase Peptide Synthesis, discovered and developed by Merrifield, peptides are synthesized on resin (Merrifield, 1985). The Liberty™ Blue unit has 20 positions to accommodate natural amino acids and 5 positions to accommodate unnatural amino acids. The Discover unit is the unit that provides microwave energy. The Solid Phase Peptide Synthesis method has become a frequently used method in research as it overcomes the purification steps required at each step during peptide synthesis in solution. With this method, peptides are synthesized extending from C-terminus to N-terminus.

Peptides were synthesized bearing a C-terminal amide group. Rink Amide (0.70 mmol/g loading capacity) resin was used in the synthesis of amide-terminated peptides. The resins were used by incubating them in DMF for at least 30 minutes and allowing them to swell before synthesis. Amino acids used during synthesis contain protective groups to prevent undesirable reactions. In the first step, the first amino acid is attached to the resin via the carboxy group at the C-terminus. Piperidine (20% in DMF) was used to remove the Fmoc protecting group on the N-terminus of the first amino acid bound to the resin when synthesis was initiated. The next amino acid to be bound was activated using Oxyma® and DIC. 0.2 M of the corresponding amino acid was used for each coupling step. After the reaction between two amino acids, an amide bond (peptide bond) is formed and the chemicals used in the reaction are removed by the washing process. Following these procedures, the protecting group of the last

added amino acid was removed, the N-terminus was released, and the synthesis on the device was completed. After the synthesis was completed, the peptide bound to the resin was taken out of the device. Separation of the peptide from the resin and simultaneous removal of the protecting groups on the side chains of the amino acids were carried out in a TFA/H₂O/TIS (95/2.5/2.5, v/v/v) mixture at 38°C for 30-45 minutes, depending on the amino acid content. Precipitation of peptides was carried out by centrifugation using cold ether (-20 °C). The precipitation process was repeated 3 times, the final precipitate was washed and dried.

HPLC (Agilent Technologies 1260 Infinity) analyzes of the synthesized peptides were carried out using a C-18 column (AdvanceBio Peptide Plus 2.1 × 150 mm 2.7-Micron) with the help of a UV detector at 214 nm and 280 nm wavelengths. Analyzes were performed with a linear gradient of 5-80% B (0.25% TFA in acetonitrile) in 30 minutes.

When the peptide synthesis was finished, the resin was transferred to the CEM, Razor (Rapid Peptide Cleavage System) device and washed three times with DMF before cleavage process. Peptide cleavage from the resin and deprotection was performed with a mixture of trifluoroacetic acid (TFA) /triisopropylsilane (TIS)/H₂O (95: 2.5: 2.5 v/v/v) for 30-45 minutes at 38°C. The peptides were precipitated with cold diethyl ether (-20 °C) and centrifuged at 4500 rpm for 3 min. The precipitation process was repeated 3 times and the final precipitate was washed and dried. Information about synthesized peptides is given in Table 1.

Table 1. Liquid chromatography (LC) experimental parameters

LC Parameters					
Mobile Phase A	%100 Ultra-pure water				
Mobile Phase B	%100 Acetonitrile				
Mobile Phase C	%1 Trifluoroacetic Acid				
Column Feature	ACQUITY UPLC Peptide CSH C18, 130 Å, 1.7 µm, 2.1 × 100 mm, (Part No:186006937)				
Injection Volume	10 µL				
Column Temperature	40 °C				
Sampler Temperature	10 °C				
UV Wavelength	214 nm				
Analysis Time	25 minutes				
Gradient Table and Flow Rates	Time (min)	%A	%B	%C	Flow rate (mL/min)
	0	85	5	10	0.200
	2	85	5	10	0.200
	9	70	20	10	0.200
	12	50	40	10	0.200
	16	20	70	10	0.200
	18	0	90	10	0.200
	20	0	90	10	0.200
	21	85	5	10	0.200
	25	85	5	10	0.200

High resolution mass measurements and amino acid sequence information analyzes of peptides were performed with an MS device. Experimental parameters of the mass spectrometer are listed in Table 2.

Table 2. Mass spectrometry experimental parameters used in analyzes

Mass Spectrometry parameters	
Mass Spectrometry	Xevo G2-XS QToF
Software	UNIFI v1.9.4
ESI Polarity	Positive
Analysis method	Sensitive
Source Parameters	Capillary Voltage (kV): 2.0
	Cone Voltage (V): 50
	Source Temperature (°C): 100
	Desolvation Temperature (°C): 300
	Cone Gas Flow Rate (L/h): 50.0
	Desolvation Gas Flow Rate (L/h): 600.0
Scan Time	0.500 sec
Low Collision Energy	4 eV
Collision Energy Range	25 eV – 30 eV
m/z Mass Range	50 – 2000 Da
Data Collection Time	0 – 25 minutes

FractalkineTK	
Identified: 49%	
1: 1 to 17	HHGVTKCKIT CSKMTSK
2: 1 to 16	HHGVTKCKIT CSKMTS
3: 1 to 15	HHGVTKCKIT CSKMT
4: 1 to 14	HHGVTKCKIT CSKM
5: 1 to 13	HHGVTKCKIT CSK
6: 1 to 12	HHGVTKCKIT CS
7: 1 to 16	HGVTKCKITC SKMTSK
8: 1 to 15	GVTKCKITCS KMTSK
9: 1 to 14	VTKCKITCSK MTSK
10: 1 to 13	TKCKITCSKM TSK
11: 1 to 12	KCKITCSKMT SK

Figure 16. Amino acid mapping result of the fractalkine sequence.

The impurities identified in HPLC analysis in the synthesized Fractalkine peptide were found by amino acid mapping and the sequences were extracted. According to the results, sequences marked in blue were detected. Single amino acid differences are not expected to affect binding assays.

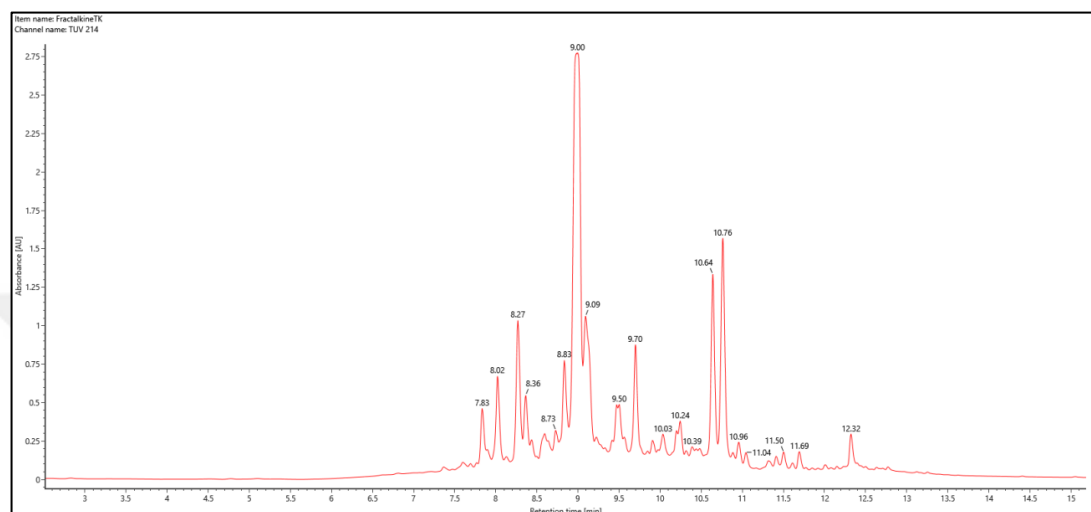


Figure 17. UV chromatogram of the fractalkine peptide sequence

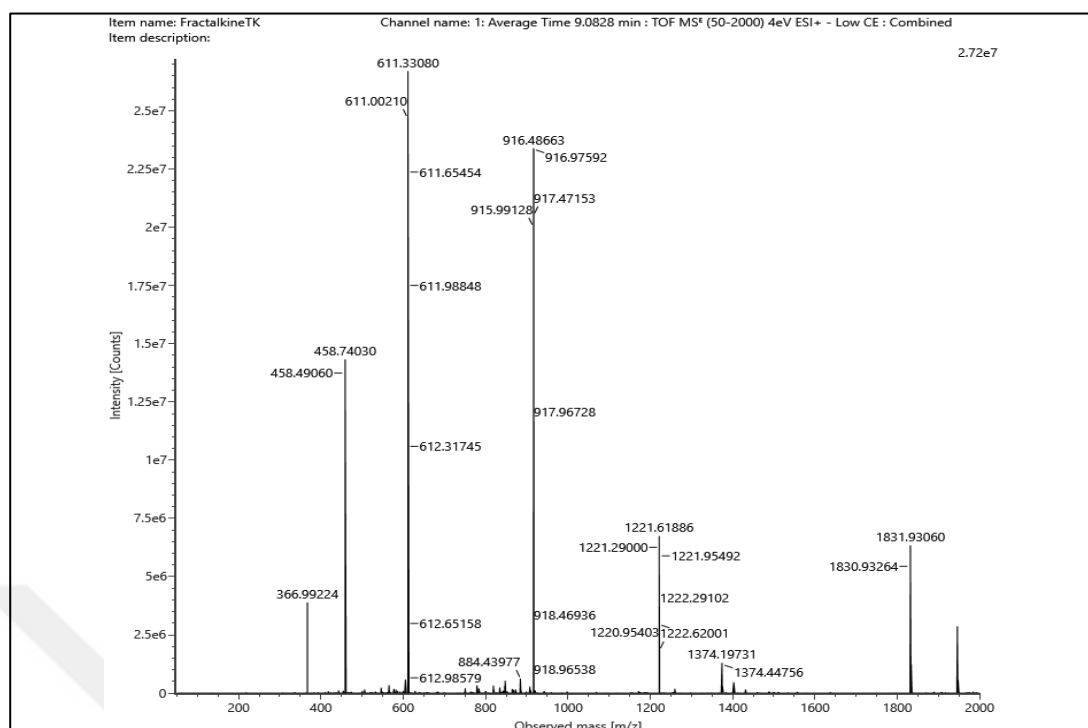


Figure 18. MS chromatogram of fractalkine sequence

Purification was carried out at 214 nm and 280 nm wavelengths with the help of a UV detector using Agilent Technologies VariTide RPC 250x10mm ID column, which is suitable for semi-prep system and peptide purification. Fraction Collector method; Peak based parameter, Slopes = 1, Threshold = 100 was used. Purified peptides were lyophilized with a lyophilizer and made ready for use in experiments. The gradient method used was determined according to the retention time and %B range determined in the analytical HPLC analysis of the peptide.

Binding affinity of fractalkine and CX3CR1 peptide will be performed with the Nanotemper Monolith device. Work has begun to determine the features and method to be used on the device. The experiments will be carried out in the 3rd reporting period.

2.2.2 Final peptide

The desired antibody of CX3CR1 consists of amino acids. The peptides synthesized in our thesis study is the 24 amino acid sequence and the amino acid composition of these sequences are given below:

Table 3. Final peptide sequence to be synthesized

Position	Amino Acid	Sequence Start	Sequence End	Peptide	Antigenicity Score
4	K	1	7	TKQKENE	0,895
5	E	2	8	KQKENEC	0,966
6	N	3	9	QKENECL	1,012
7	E	4	10	KENECLG	0,992
8	C	5	11	ENECLGD	0,983
9	L	6	12	NECLGDY	1,027
10	G	7	13	ECLGDYP	1,068
11	D	8	14	CLGDYPE	1,068
12	Y	9	15	LGDYPEV	1,064
13	P	10	16	GDYPEVL	1,064
14	E	11	17	DYPEVLQ	1,084
15	V	12	18	YPEVLQE	1,082
16	L	13	19	PEVLQEI	1,081
17	Q	14	20	EVLQEIW	1,056
18	E	15	21	VLQEIWP	1,087
19	I	16	22	LQEIWPV	1,087
20	W	17	23	QEIWPVL	1,087
21	P	18	24	EIWPVLR	1,067
22	V	19	25	IWPVLRN	1,056
23	L	20	26	WPVLRNV	1,089
24	R	21	27	PVLRNVE	1,083
25	N	22	28	VLRNVET	1,061
26	V	23	29	LRNVETN	0,974
27	E	24	30	RNVETNF	0,951

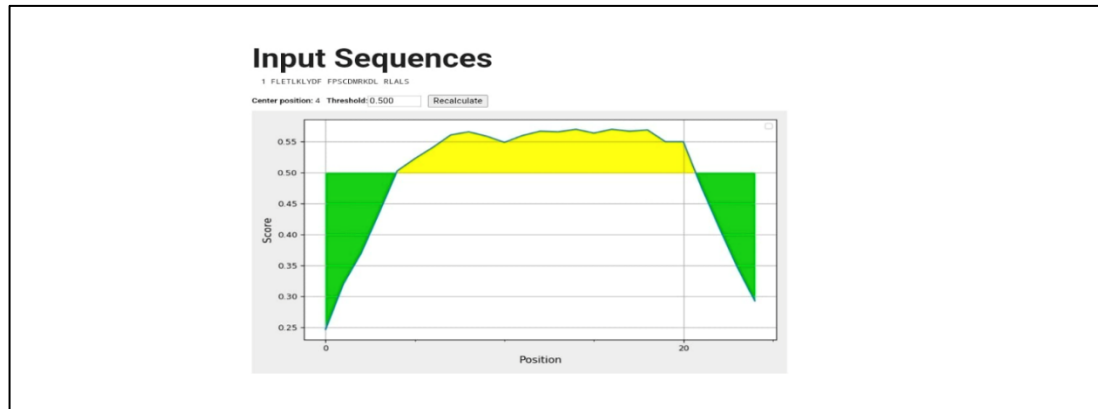


Figure 19. Affinity graph of the designed sequence (Average: 0.494, Min: 0.247, Max: =0.570)

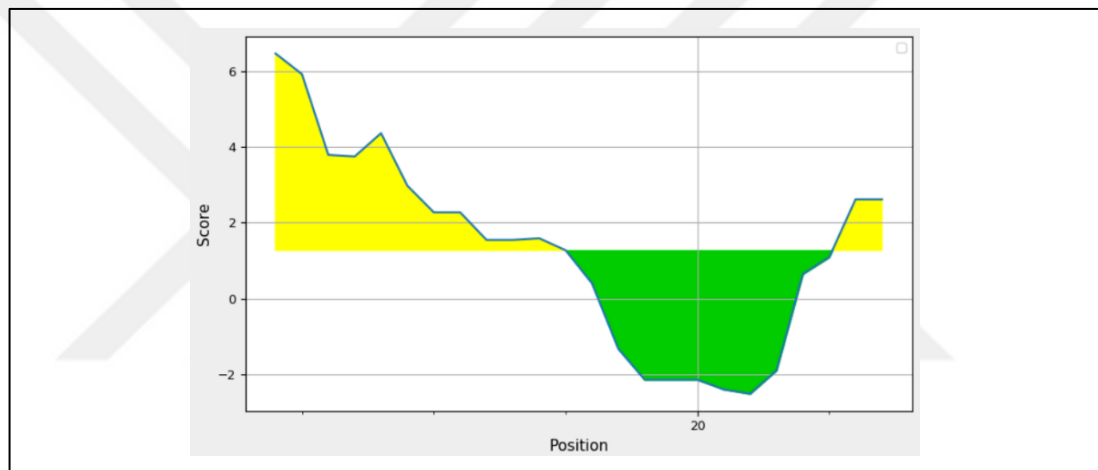


Figure 20. Hydropathy graph of the designed sequence (Average: 1.271, Min: -2.514 Max: =6.457)

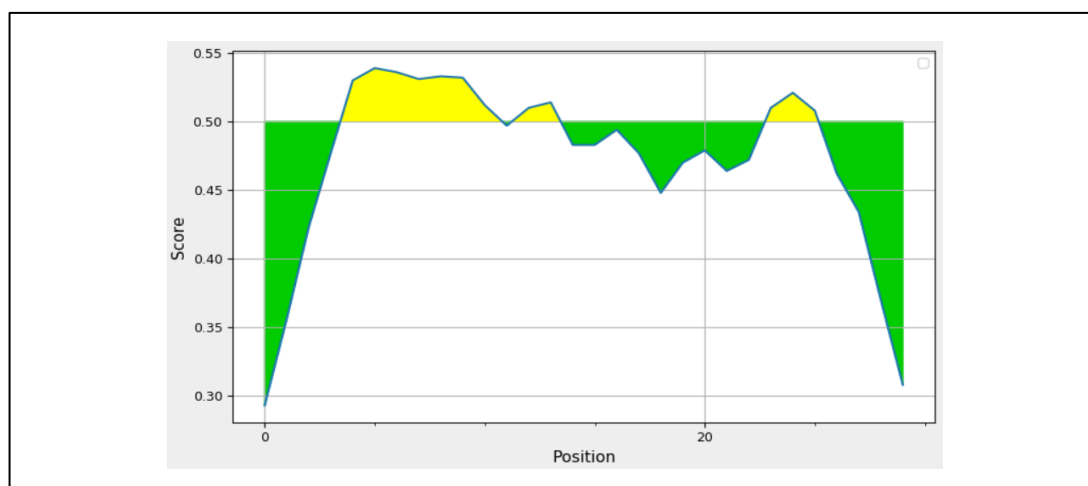


Figure 21. Antigenicity graph of the designed sequence (Average: 1.041 , Min: 0.895 Max: =1.089)

The peptide was synthesized carrying a C-terminal amide group. Rink Amide (0.70 mmol/g loading capacity) resin was used in the synthesis of amide-terminated peptides. The resins were used by incubating them in DMF for at least 30 minutes and allowing them to swell before synthesis. Amino acids used during synthesis contain protective groups to prevent undesirable reactions. In the first step, the first amino acid is attached to the resin via the carboxy group at the C-terminus. Piperidine (20% in DMF) was used to remove the Fmoc protecting group on the N-terminus of the first amino acid bound to the resin when synthesis was initiated. The next amino acid to be bound was activated using Oxyma® and DIC. 0.2 M of the corresponding amino acid was used for each coupling step. After the reaction between two amino acids, an amide bond (peptide bond) is formed and the chemicals used in the reaction are removed by the washing process. Following these procedures, the protecting group of the last added amino acid was removed, the N-terminus was released, and the synthesis on the device was completed. After the synthesis was completed, the peptide bound to the resin was taken out of the device. Separation of the peptide from the resin and simultaneous removal of the protecting groups on the side chains of amino acids were carried out in a TFA/H₂O/TIS (95/2.5/2.5, v/v/v) mixture at 38°C for 30-45 minutes, depending on the amino acid content. Precipitation of peptides was carried out by centrifugation using cold ether (-20 °C). The precipitation process was repeated 3 times, the final precipitate was washed and dried. As a result, 1mg of peptide was

produced with over 90% purity. Then, the produced peptide was conjugated with KLH and BSA.

2.3 Peptide Injection/Immunization

Table 4. Immunization schedule of mice with CX3CR1 antigen

Mice	Peptides used as an immunogen	Peptide Conc. (µg)	Time interval for immunization
F1-F5	CX3CR1 peptide	50	At 10-day intervals
F6-F10	CX3CR1 peptide	50	At 7-day intervals
F11-F15	CX3CR1 peptide	100	At 7-day intervals
F16-F20	CX3CR1 peptide	125	At 7-day intervals
F21-F25	CX3CR1 peptide (36)	125	At 7-day intervals

Until F21, the peptide synthesized by our group was used. For F21-25, the peptide sequence has been changed. After synthesized CX3CR1 peptide for the monoclonal antibody production and hybridoma process was obtained peptide has been injected intraperitoneally to female 5 week old Balb/C Mouse as shown in figure 16. Complete Freund's adjuvant was used for the first injection, in the following injections incomplete adjuvant was used. Adjuvant and the peptide mixed in 1/1 concentration.

2.3.1 Plasma collection

In order to detect the immunogenicity of the mouse, indirect sandwich ELISA method was used. Blood was collected from the submandibular vein of the mice. Collected blood was centrifuged for 10 minutes at 4C at 16,000 x G to separate the plasma and stored at -20 C. Collection of the blood was performed 3 days after each injection. The process was shown in Figure 2.5.

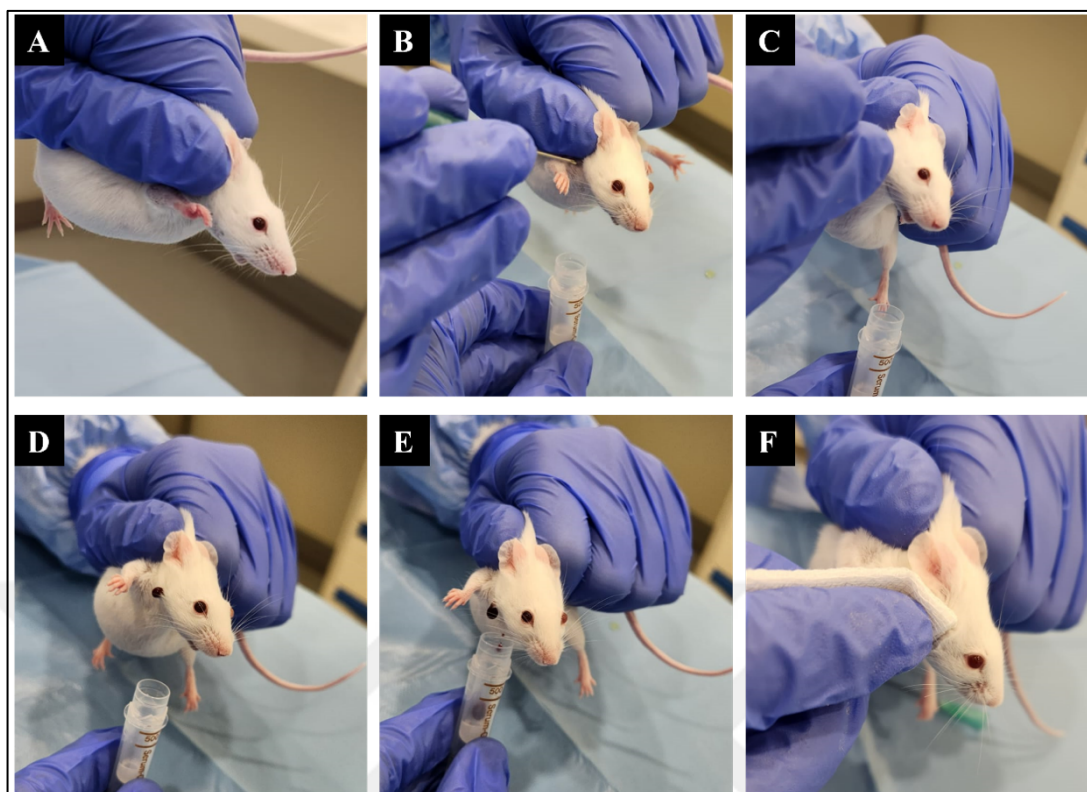


Figure 22. Collection of blood sample from BALB/c female mice (shown in order of all stages from A to F)

2.3.2 Detection of antibody response by ELISA method

40 ng BSA conjugated peptide was coated to 96 well plates and kept at 4 C overnight. After the coating process wells were washed with 250 ul 0.025% Tween-20 added PBS 3 times. Blocking solution of 100 ul 1% skim milk in PBS was added to wells and the 96 well plate was incubated at 37 C CO2 incubator for 1 hour. After the incubation process was completed, wells were washed and collected plasma was added to wells as 100 ul and incubated for 1 hour. After 1 hour, wells were washed and secondary antibody was added to wells as 100 ul and incubated for 1 hour. Following the incubation process wells were washed and alkaline phosphatase solution was added to wells as 100 ul and incubated for 1 hour. In the end, the absorbance was measured with a nano-spectrophotometer at 405 nm wavelength. The mice with the highest result were sacrificed and the hybridoma process was performed.

2.3.3 *In vitro* studies

All the necessary culture mediums were prepared in the cell culture cabin, laminar flow hood in order to prevent contaminations that might occur. The flow hood has been sterilized with 70% alcohol and UV light. The liquid materials that were used in the processes has been heated to 37 C with the use of a waterbath. The cell types used in this study are shown in Table 10.

Table 5. Cell types used in this study

Source	Name of Cell
F0, CRL-1646	Myeloma Cells
BALB/c mouse peritoneal cells (<i>fibroblasts</i>)	Feeder Cells
Cells from spleen of BALB/c mouse immunized with CX3CR1	B Lymphocytes

2.3.4 Cell thawing

Cells were taken from -80 C and thawed by hand. After they are thawed the contents of the cryovial was transferred into a 15 ml centrifuge tube and 8 ml of culture medium was added slowly. Centrifuge tube was placed in the centrifuge and cells were centrifuged for 5 minutes at 300 xG. Supernatant was removed and the pellet was dissolved in 1 ml culture medium. 11 ml of culture medium was added into a T75 cell culture flask and cells were transferred into the flask.

2.3.5 Cell counting

Cells were detached from the culture flask with the use of a cell scraper and centrifuged for 5 minutes at 300 xG. Supernatant was discarded and pellet was dissolved in 1 ml culture medium. 10 ul sample was taken from the pellet and mixed with 90 ul culture medium for the counting to be more viable. 10 ul of the sample was loaded onto the hemocytometer and counted.

2.3.6 Subculture of myeloma cells

Cells were detached from the culture flask with the use of a cell scraper and centrifuged for 5 minutes at 300 xG. Superantant was discarded and pellet was dissolved in 1 ml culture medium. After the counting was done, appropriate amount of cells were transferred into a cell culture flask.

2.3.7 Myeloma cell freezing

Cells were detached from the culture flask with the use of a cell scraper and centrifuged for 5 minutes at 300 xG. Superantant was discarded and pellet was dissolved in 1 ml culture medium. After the counting was done, appropriate amount of cells were taken and mixed with 90% FCS and 10% DMSO. Mixture was transferred into a labeled cryovial and the cryovial was put into a freezing container, then -80 C fridge.

2.3.8 Feeder cell isolation

For isolation, DMEM HG (Dulbecco's Modified Eagle's Medium High Glucose) was prepared in a biosafety cabinet under sterile conditions by adding 50 µg/ml Gentamicin antibiotic solution. For the feeder cell isolation process, 10 ml DMEM HG with Gentamicin medium was transferred to a new 50 ml falcon tube, labeled, sealed with paraffin wax, and placed in an ice-filled foam container. The mouse was sedated with Isoflurane and sacrificed using the cervical dislocation technique. The sacrificed mouse was fixed from its legs and arms with the help of a needle (attached to the dissection tub), and its skin was carefully peeled off without damaging the peritoneum. Stripping, injection, and isolation procedures were performed under laminar flow to avoid contamination. 5 ml DMEM HG-Gentamicin was injected into the peritoneal cavity with a 21G needle. The needle is carefully inserted just below the diaphragm, taking care not to enter another organ or puncture internal tissues during injection. Media injection was applied from the upper side of the mouse and the injected medium was kept in the visceral cavity for 1-2 minutes. The dissection cuvette is placed

inclined to accumulate feeder cells (fibroblasts and macrophages) in the lower abdomen. Using the same syringe, up to 3-5 ml of feeder cells were taken and transferred to a new 50 ml falcon tube placed on ice. For the fusion procedure, harvested cells were collected in 10 ml DMEM HG containing 20% FBS (or FCS), 1% HEPES (1 M), 1% L-Glutamine, 1% Sodium Pyruvate, 50 µg/ml Gentamicin or 1% Pen/Strep solution. Resuspended cells were planted in 100 µl wells. For each fusion experiment, 10x 96-well plates can be used, requiring approximately 100 ml of cell suspension.

2.3.9 Hybridoma fusion manual

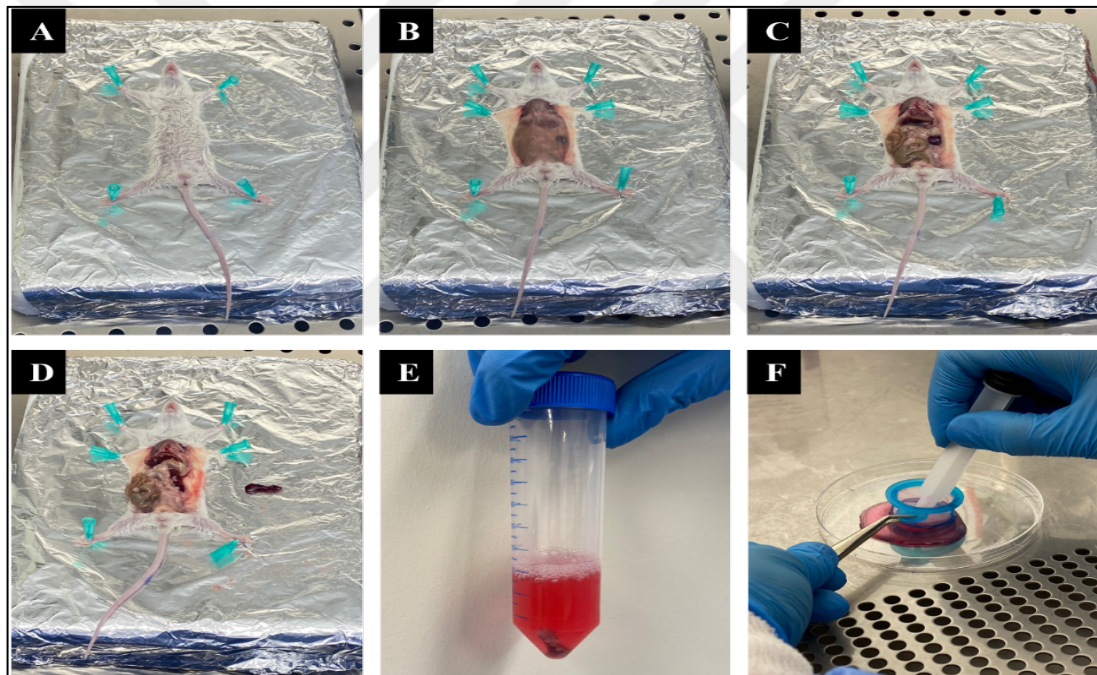


Figure 23. Steps of the spleen isolation process

2.3.10 Splenocyte isolation steps

Several materials must be prepared before the fusion process. PEG 4000 must be autoclaved and prepared for fusion (dissolve 1 g of PEG 4000 in 1 ml of PBS and 1 ml of dH₂O – Autoclaved PEG 4000 is incubated overnight). Feeder cells must be checked to prevent any contamination. HAT medium supplements should be prepared with appropriate DMEM aliquots (10% and 20% FBS/FCS). After the mouse was

sterilized with ethanol, it was fixed in the dissection cuvette for spleen extraction. The mouse skin was carefully peeled off through a small incision applied to the lower abdomen and cleaned with ethanol. Peripheral lymph nodes were taken from just under the rib cage, depending on the skin and fat tissue. The removed lymph nodes were transferred to a sterile falcon tube containing 10% FBS DMEM HG with Hepes, L-glu and Sodium Pyruvate. With the help of forceps, the tissues were transferred to a sterile petri dish and filtered by mashing on a 40 μ m strainer. 30 ml 10% FBS DMEM HG was used to wash cells from the strainer. The filtered cells were collected in a falcon tube and centrifuged at $300 \times g$ for 5 min (+4 °C). This washing step was repeated three times. Supernatants were discarded, 2.5 ml cold Red Blood Lysis Buffer (eBioscience™ 1X RBC Lysis Buffer) was used to resuspend the cells, incubated at room temperature for 2.5 mL. The resuspended pellet was made up to 35 ml with 10% FBS DMEM HG media, centrifuged at $300 \times g$ for 5 min. This washing step was repeated until clear supernatant was observed. Resuspended cells were counted and noted for total cell count - 1:2 or 1:4 cell ratio is desired for fusion (1x for splenocyte - 2x or 4x F0 myeloma cells). F0 Myeloma cell and Splenocytes were transferred to the same falcon tube and centrifuged at 300xg for 5 minutes (+4 °C), supernatant was discarded, pellet was removed. 1 ml incubated PEG 4000 was slowly poured dropwise over the cell pellets for 1 min, pipetted for homogenization. 4 ml Serum-free DMEM HG media was poured gently, cells were resuspended for 2 min. 20 ml Serum-free DMEM HG media was poured gently, cells were resuspended for 2 min. 25 ml 20% FBS DMEM HG media was slowly poured in, cells were resuspended for 1 min, pipetted for homogenization. The resuspended fusion cells were incubated at 37°C for 1 hour, then centrifuged at 300xg for 5 minutes (+4°C), the supernatant was discarded, the pellet was removed. The pellet was resuspended with the help of 20% DMEM HG media (50X HAT Supplementation -) supplemented with 2% HAT, seeded in h 96-well plates previously seeded with feeder cells. 100 μ l of cell suspension was used for each well. 10 plates are required for each mouse fusion used. Following 10 days of incubation, a media change was made to 20% DMEM HG supplemented with 1% HT. Additionally, colonies in each well were screened and observed for healthy cells. Wells

with periodic color changes were checked by ELISA scanning to check the presence of antibody and colony functionality.

2.3.11 Hybridoma fusion with clona cell kit

Table 6. Reagents and media used from the ClonaCell™-HY Hybridoma kit

Hybridoma Kit Products	Description and Purpose
Medium A	Culture medium for hybridoma and myeloma cells
Medium B	Medium used to wash cells before and during the fusion process
Medium C	Medium used to promote the viability of hybridoma cells before selection with HAT
Medium D	Semi-solid methylcellulose-based medium used for hybridoma selection and cloning (includes HAT supplement and serum)
Medium E	Media used for growth and expansion of hybridoma cells after selection by HAT
PEG	Reagent for the fusion process

1 day before the hybridoma process, culture mediums were taken from -20 C and placed into the 4C fridge for them to be thawed and ready. 3 days before the process F0 myeloma cells were sub-cultured for them to be in the log phase in order to increase the efficiency. Mice was sacrificed by cervical dislocation. Spleen and the lymph nodes of the mice were collected and kept in 50 uM supplemented SFM DMEM media. Collected tissues were transferred to a cell strainer which was placed on a 50 ml centrifuge tubes and mashed with the back of a 10 ml syringe. Strainer was washed with 30 ml Medium B and centrifuged for 10 minutes at 400 x G. Supernatant was discarded and the washing process was repeated for 3 times. After the washing process was completed cell pellet was re-suspended with 25 ml Medium B and counted in 9/10 3% acetic acid solution. 100 million B cells were separated to be used in the process.

F0 myeloma cells were harvest with a cell scraper and centrifuged for 10 minutes at 300 x G, supernatant was discarded and the pellet was resuspended with 30 ml

medium B for washing process and centrifuged at 300 x g for 10 minutes, washing process was repeated for 3 times. After the washing process was completed cell pellet was re-suspended with 25 ml Medium B and counted in 9/10 3% acetic acid solution. 20 million B cells were separated to be used in the process.

B cells and myeloma cells were added into a 50 ml centrifuge tube and centrifuged for 10 minutes at 400 x G. Supernatant was discarded. 1 ml of PEG-400 was added onto the pellet during a 1 minute duration while stirring. After that 4 ml of medium B was added over a 4 minute period, following that 10 ml of medium B was added slowly and the cells were incubated at 37 C water bath for 15 minutes. 30 ml of Medium A was added and the cells were centrifuged for 7 minutes at 400 x G. Supernatant was discarded and re-suspended at 40 ml Medium A, centrifuged for 7 minutes at 400 x G. Pellet was re-suspended in 10 ml of medium C, 20 ml of medium C was added to a T-75 tissue culture flask. Cells which were re-suspended in 10 ml medium C were transferred to the flask and incubated for 24 hours.

After 14 days of incubation with HAT added medium C, grown colonies has been isolated from the semi solid medium with a 10 ul micropipette and transferred into 200 ul medium E. Cells were incubated for 4 days in medium E. After 4 days, 100 ul of the supernatant has been taken and transferred into a BSA-CX3CR1 peptide coated 96 well plate. Indirect sandwich ELISA method was performed and the colonies that showed the highest antibody production wwere detached from the plate and diluted into single cells in different wells of another 96 well plate in order to find the perfect clone that produces the antibody of interest. After 4 days of incubation, another ELISA test was used and this went on until the production reached to the desired amount. As the perfect cell colony has been found after several attempts, cells were transferred into a 24 well plate and incubated for 4 days in medium E medium B mixture. Another ELISA test has been performed to make sure that cells are still producing the desired antibody. As the tests showed that cells were adjusted to medium B, all of the processes after this step was performed in medium B. This process went on until reaching to T75 cell culture flask. After cells were transferred into a T75 flask and incubated for 4 days, 20 ul of the supernatant was and mixed with 1% BSA in PBS. The mixture was added

into an isostrip tube and mixed by pipetting 10 times and vortexed for 30 seconds. After the mixing is done, the tube has been left to incubate for 30 seconds RT and isostrip has been added to the tube. The isostrip was incubated for 10 minutes. As the incubation time was over results were checked and it was seen that the tested colony was positive for IGG g1 Kappa light chain. Due to this, protein A column was selected for purification.

Supernatant was collected and the antibodies were purified with protein A column with the help of Cytiva affinity chromatography. The purified antibody was tested in a western blot assay and it was seen that the size of the produced antibody was just a little under 30 kDa.

After every 4 days of incubation, supernatant was collected and went through ultrafiltration process with 100 kDa, 30 kDa and 3 kDa ultrafiltration tubes. First the supernatant was transferred into a 100 kDa ultrafiltration tube and centrifuged for 13 minutes at 2700 xG. The concentrated part at the top and flow through samples were collected. After that, the flow through was transferred into a 30 kDa ultrafiltration tube and centrifuged the same way. Again, the concentrated part and flow through was collected. The flow through was transferred into a 3 kDa ultrafiltration tube and centrifuged, samples from concentrated part and flow through was collected. All the collected parts has been kept at 4C until the end of the ELISA assay. 96 well plate was coated with BSA-CX3CR1 peptide. Different samples from the ultrafiltration process was added into the wells and tested for the highest reactivity. It was seen that the highest measurement was seen at 3 kDa concentrated part which confirmed the western blot results and it was determined that the size of the antibody is between 3 kDa and 30 kDa. For further uses the same protocol was used and the 3 kDa concentrated part samples were collected to be purified.

The purification was performed with a peristaltic pump and protein A column with 0.02 M sodium phosphate pH 7.0 as washing buffer and 0.1 M glycine-HCl as the elution buffer. First, 3 column volumes of washing buffer was passed through the column then 4 ml of concentrated supernatant was passed through, 3 column volumes

of washing buffer and 1 column volumes of elution buffer was used in the purification process.

2.3.12 Concentration determination by SRB cell viability assay

For the SRB assay, HSC, SU.86.86. and T3M4 cell lines were used. Cells were counted and seeded into the 96 cell plate as 3.000 cells/plate and incubated for 1 day for them to attach to the surface. After the incubation, 10, 5 and 1 μ m of the antibody was added into the media and cells were incubated for 1 more day. Supernatant was discarded and wells were washed with 100 μ l PBS. Cells were fixed on the the plate with the use of 50% (wt/vol) TCA in 4C for 1 hour. Wells were washed under running water for 3 times. 50 μ l 0.04% SRB solution was added into the wells and the palte was incubated for 1 hour RT. SRB solution was discarded and the wells were washed under running water 3 times. 200 μ l 1% acetic acid solution was added into the wells and cells were washed with it to get rid of the unbound dye. Wells were washed under running water 3 times. 50 μ l of 10 mM tris base ph 10.5 was added to the wells and placed on a shaker for 10 minutes. The absorbance measurement was performed with a spectrophotometer at 510 nm.

2.3.13 Chronic pancreatitis

Chronic pancreatitis experimental procedure was performed in order to determine the correct antibody concentration under in vivo conditions. According to the literature a range between 10 μ m to 200 μ m was chosen. Before the starting of injections mouse were numbered from the ear and baseline von frey pain test was performed. 8 week old C57BL6 mouse were injected with 75 μ g/kg cerulein for 3 days a week 6 times a day with 1 hour intervals. After the second week of injections, antibody treatment of 50 μ g and 25 μ g was started to be injected intraperitonally. After every 2 weeks of the course, von frey was performed to determine effect of the treatment. After 8 weeks of the injections a final von frey was performed and mouse were sacrificed with the help of guillotine. Following the sacrifices following samples were collected in 4% PFA: Blood, brain, pancreas, spinal cord and DRG. Spinal cord and DRG's were kept

in 4% PFA for 2 hours and then transferred to 25% (w/v) sucrose solution and kept at 4 C. Brain and pancreas were kept in PFA overnight then transferred into PBS RT. Blood was collected into separation tubes. The tubes were centrifuged for 10 minutes at 16.000 xG. After the centrifuge plasma was collected and kept at -20 C.



3 RESULTS

3.1 Mouse Immunization ELISA

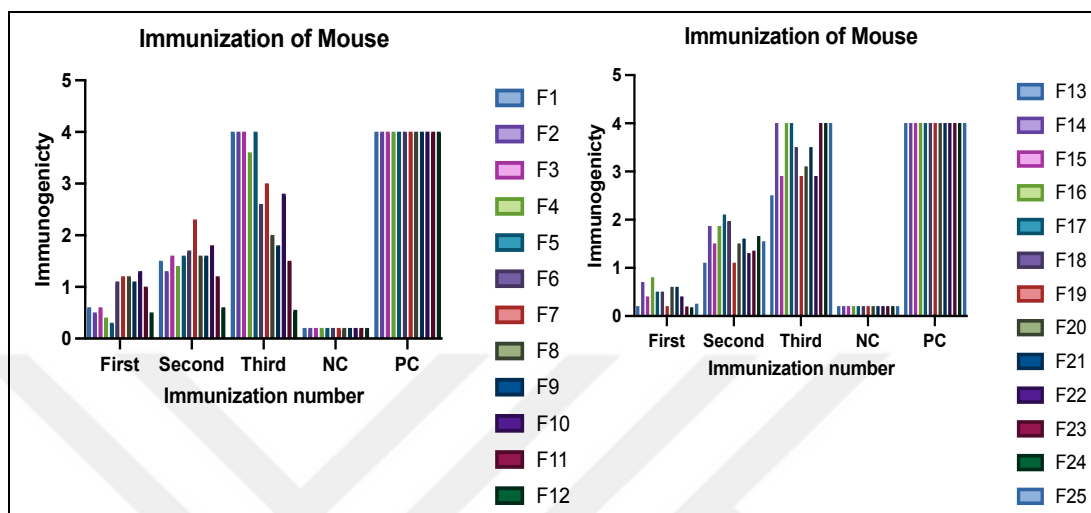


Figure 24. The effect of the immunizations over the mice's immunogenicity can be seen. As the number of injections are increasing, the IgG of the mice starts to build up.

The graph indicates that with every injection, the immunization of the mice increases. When it reaches to the highest absorbance limit possible, it is decided that the mice is ready for the hybridoma procedure.

3.2 Fusion

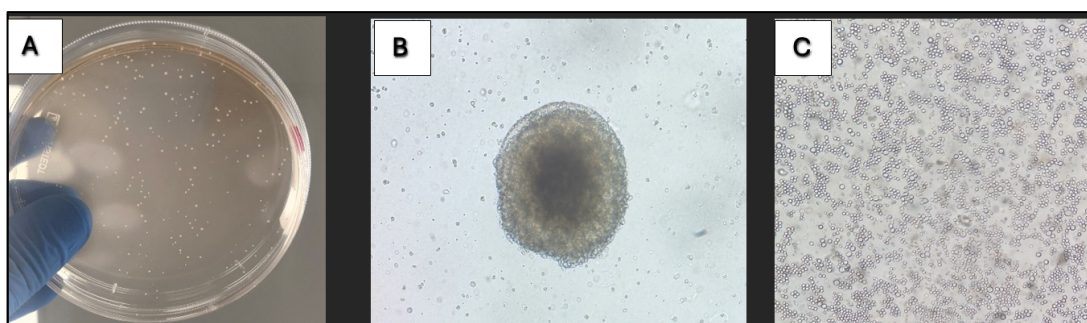


Figure 25. (A) Positive clones 14 days after fusion in semi-solid media, (B) Positive Clone in semi-solid media at 10X magnification, (C) Fused cells in Medium C 1 day after fusion process, before transferred to semi-solid media. As the incubation and culturing continues, these cells form into hybridoma colonies waiting to be checked for the antibody production capability.

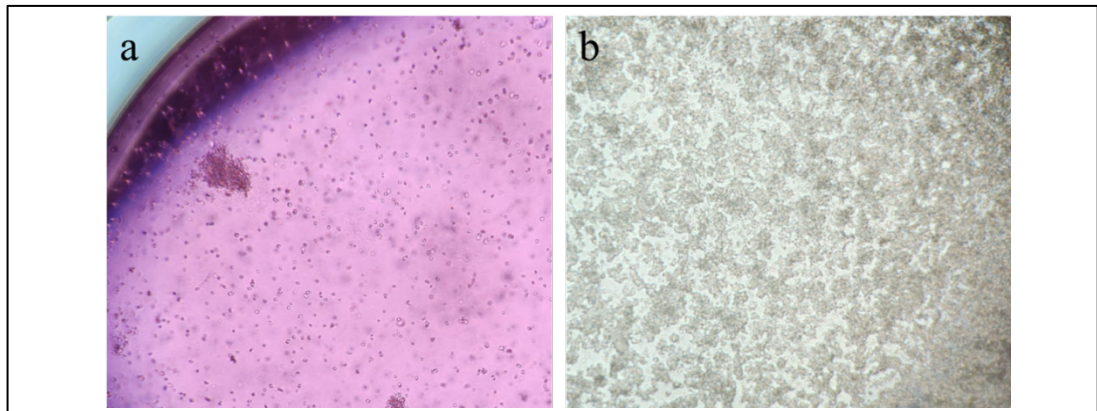
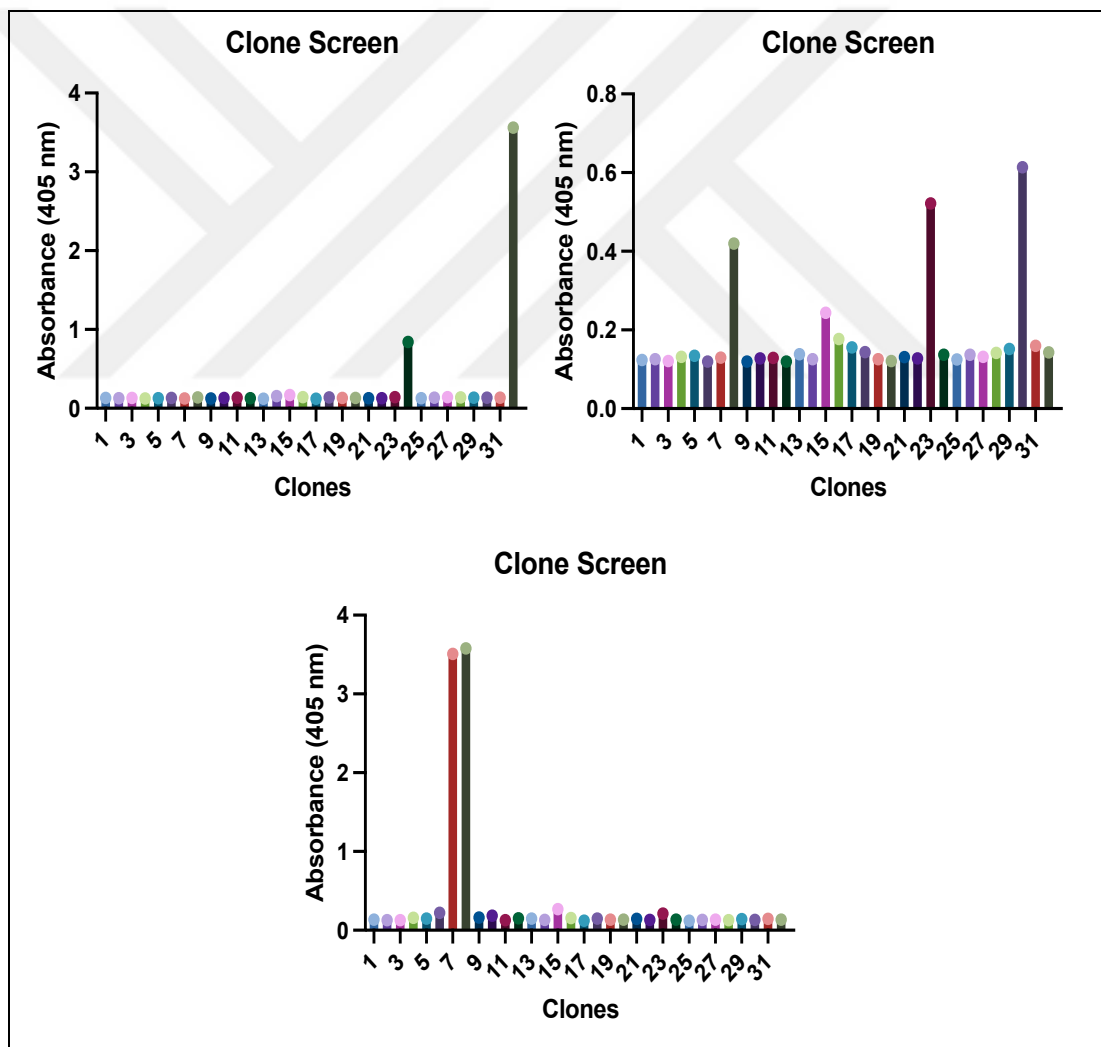


Figure 26. Microscopic control of active colony formation. 1 day after collection from semi-solid media (A), 3 days after transfer (B).



In the ELISA test performed on the 11th day, 10 positive clones were identified and these clones were transferred to 24-well plates. Figure 13 shows the morphology of the cells 4 days after transfer to a 24-well plate. In the ELISA test performed 4 days later, it was seen that 2 out of 10 clones responded positively. Part of these 2 clones were frozen and stored, and the rest was transferred to a 6-well plate. Clones that continue to grow are continued to be checked with ELISA testing.

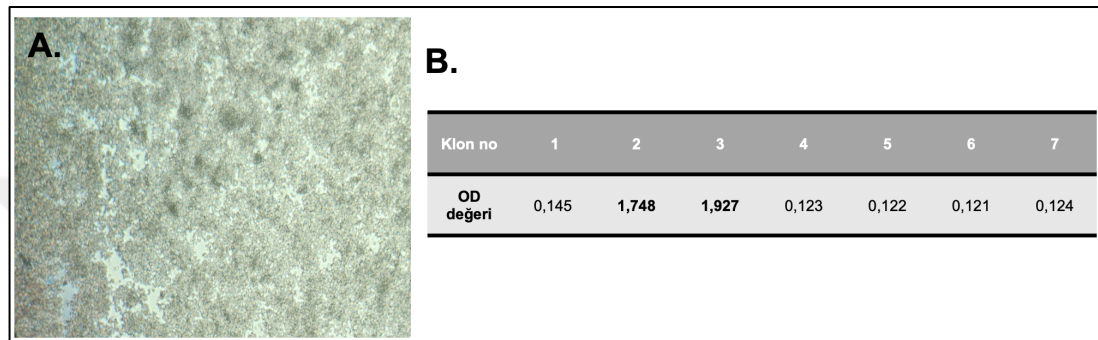


Figure 28. (A) Morphology of the hybridoma cells 4 days after transferred into 24 well plate. (B) ELISA result of the 10 positive clones that were tested before.

As seen in the figure, there is positivity in the kappa part, which indicates that antibodies of the IgM subtype with the kappa light chain are produced (Figure 14).

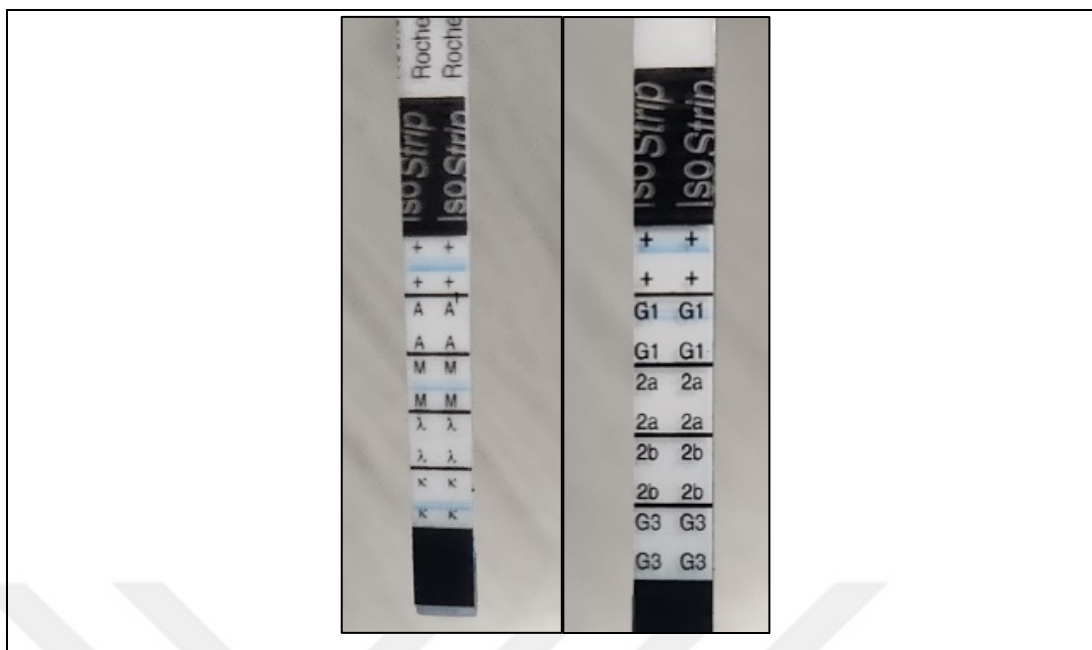


Figure 29. Isostrip antibody subtype determination assay

In addition, subcloning is required to prevent the risk of overgrowth of cells that do not produce antibodies and the possibility of polyspecificity. Therefore, in the next stage, the cells that give positive results in indirect ELISA will be diluted with serial dilutions to the amount of 1 cell per well and left to grow in a carbon dioxide incubator for 10 days. Then, it will be examined under a microscope and tested with indirect ELISA in wells containing single and several clones. Thus, specific antibody production will be completed.

As a result, promising results were obtained as a result of synthesizing a new peptide and administering the peptide with a carrier protein during the 6-month pause period. At this stage, cells with positive results grown in 24-well plates have been transferred to flasks, and studies are continuing to select the positive clone with the 28-day ELISA test. Then, the specific clone will be selected by distributing it as a single cell and the process will be completed. After antibody production, after the sensitivity and specificity of the antibody are determined, studies will be carried out to purify and multiply the antibody on a large scale.

3.3 Colony ELISA

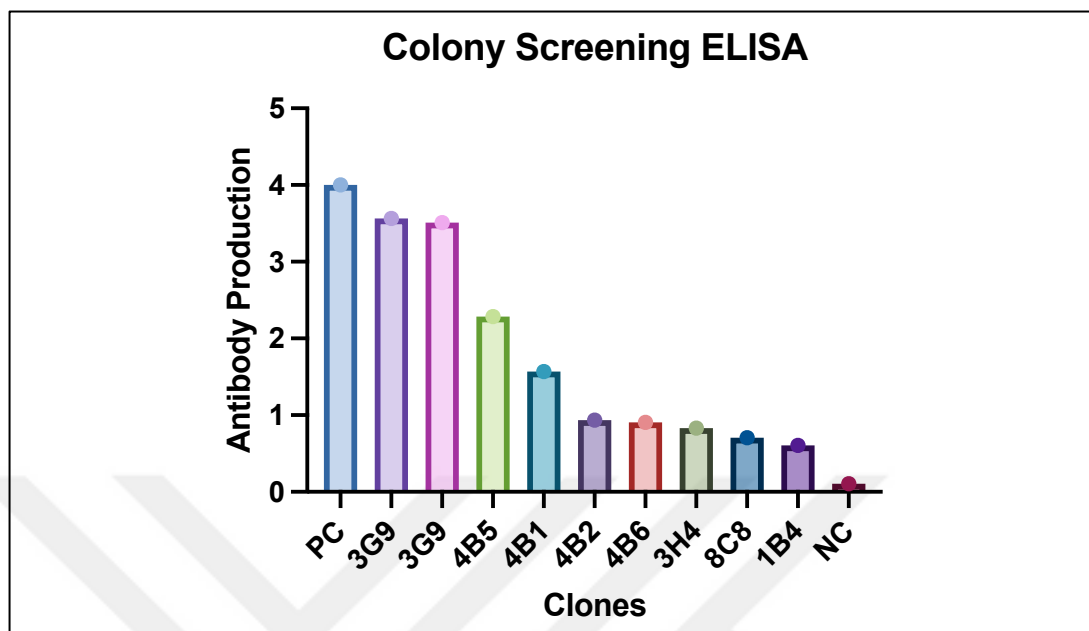


Figure 30. Positive clones detected after in the screening process

From over 1500 clones, 9 of them showed positive results. 3G9 and 3H4 colonies were transferred into 24 well plates for mass production. Other colonies have been splitted into single-cell colonies in order to find the cell responsible for the desired antibody production.

3.4 Isostrip Antibody Isotype Control



Figure 31. Isostrip antibody subtype determination assay

From the clone 3G9, cell culture supernatant was collected and tested with isostrip. It can be seen that the clone is positive for IgG1 with a Kappa light chain.

3.5 Affinity Chromatography Purification

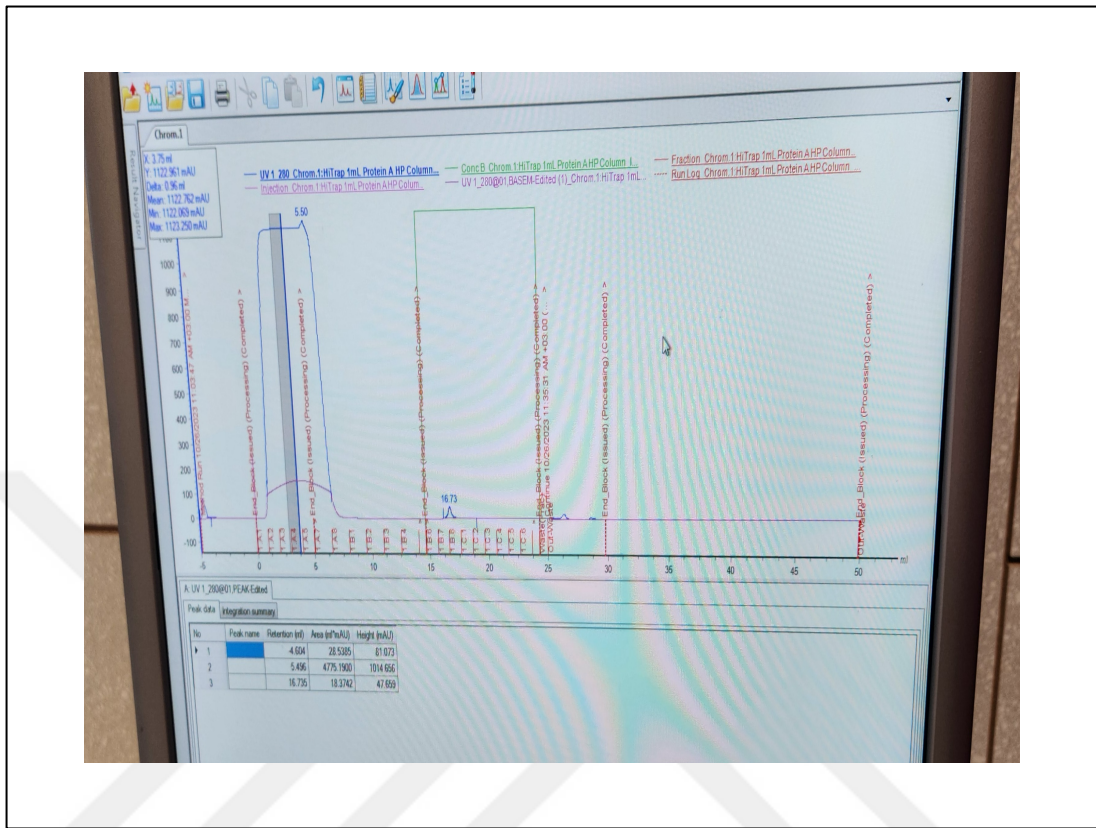


Figure 32. Affinity chromatography chromatogram of the purified antibody

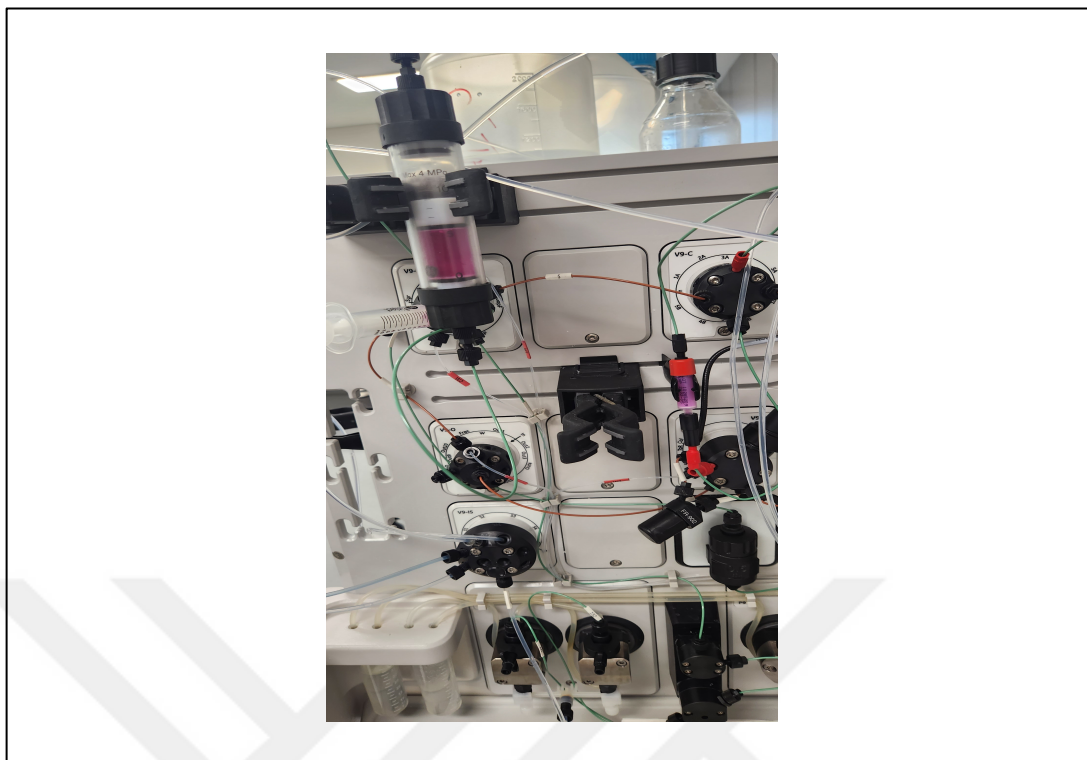


Figure 33. Insertion of the column to the affinity chromatography

$$0,015 = 1,65 * 0,2 \text{ mg} / 1,36 \text{ ml}$$

$$0,24$$

$$0,06 \text{ mg/ml} = 60 \text{ ug/ml}$$

The purification of the cell culture supernatant was performed with cytiva affinity chromatography. From the peak shown by arrow, the concentration can be calculated by beers lambert law. For the purification process, protein A column was used due to the information obtained from isostrip isotype prediction test.

3.6 Western Blot

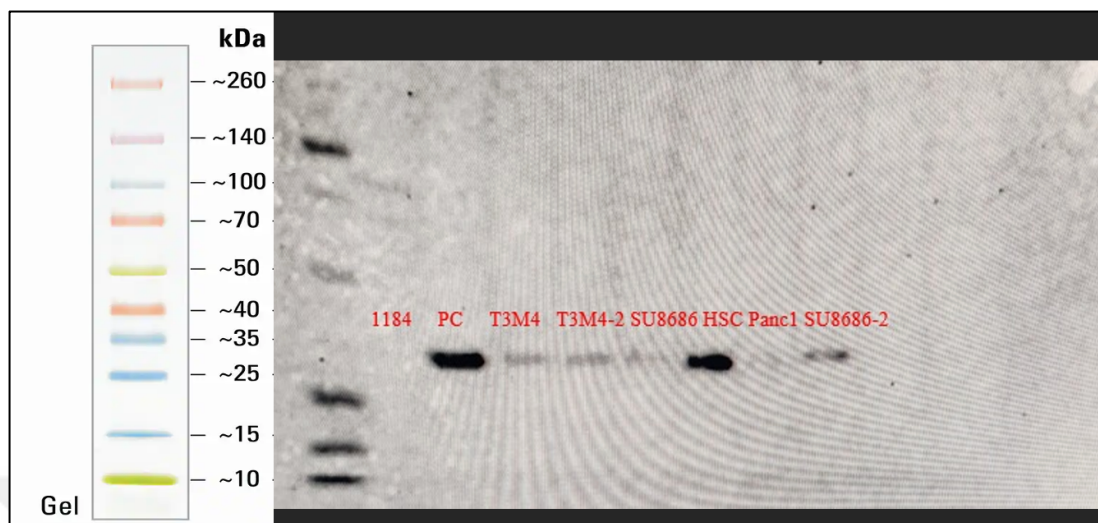


Figure 34. Western blot results for the characterization and mass determination of the antibody

The western blot performed with 1184: mouse fibroblast cell line, PC: hybridoma cell protein, PCa cells and HSC. It can be seen that the size of the produced antibody is just a little under 30 kDa.

3.7 Dosage Determination by SRB

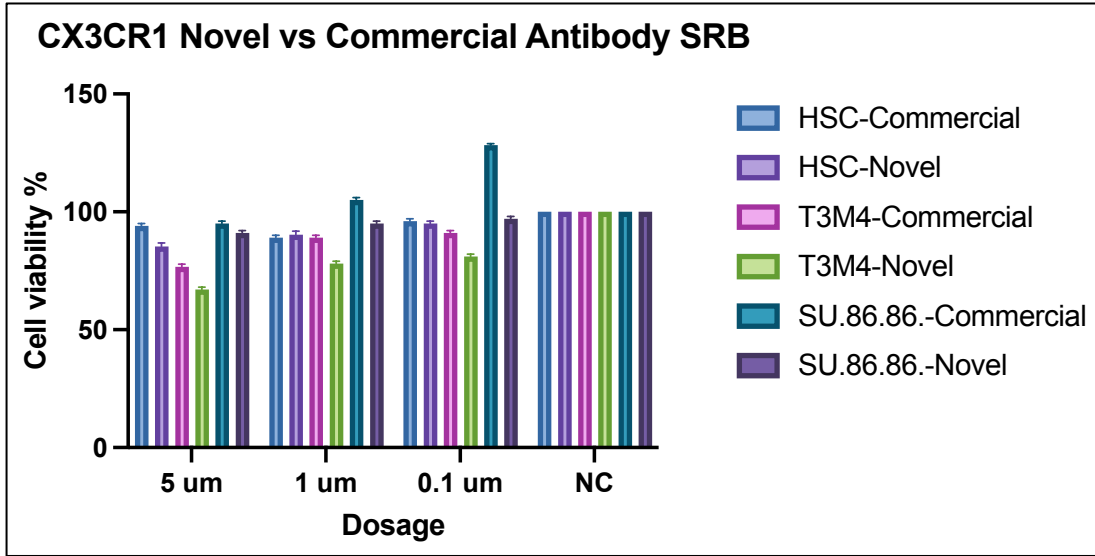


Figure 35. SRB cell proliferation results of the antibody compared with the commercial one

3.8 Ultrafiltration ELISA

Table 7. ELISA results that was performed on the samples obtained from ultrafiltraion

CX3CR1	1/10 Dilution
H4	1,296
H4 100K FT	0,274
H4 30 K Conc	0,265
H4 30 K FT	0,225
G9	0,249
G9 100 K FT (Cons)	0,633
G9 30 K Conc	0,32
G9 30 K FT	Over
NC	0,494

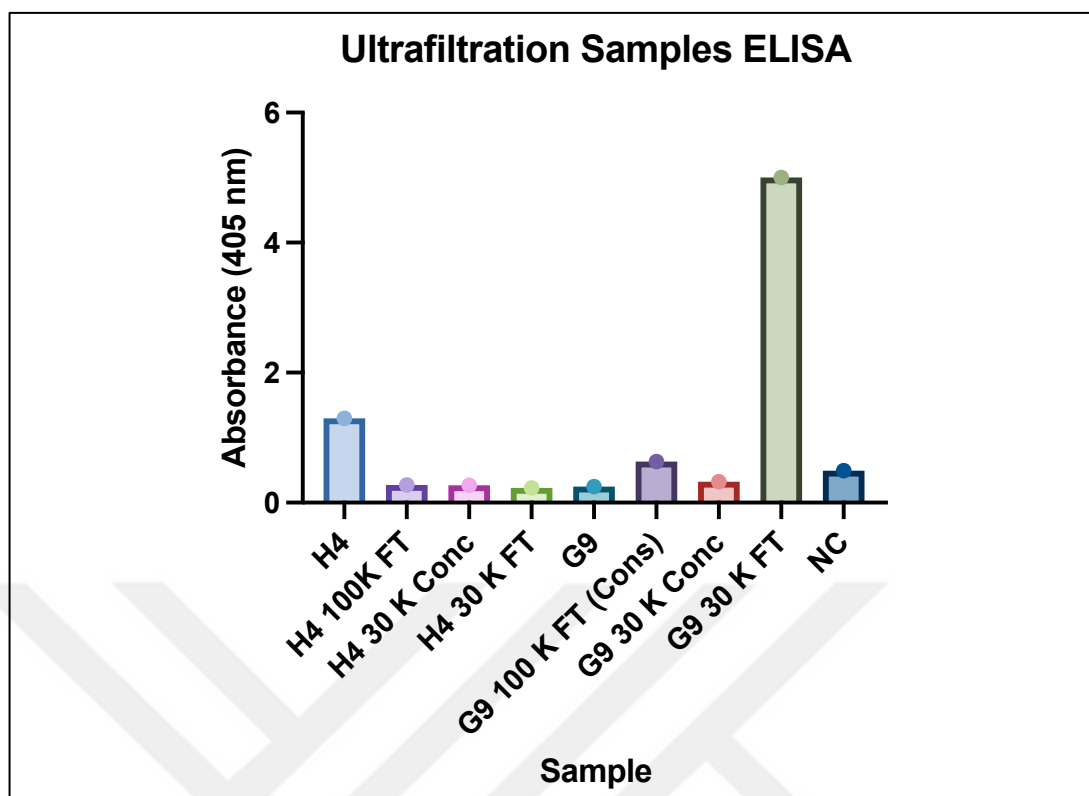


Figure 36. ELISA results that were performed on the samples obtained from ultrafiltration process

From the ELISA performed with ultrafiltration samples, it can be seen that the highest reactivity occurred on 30 kDa flow through which correlates with western blot results.

3.9 Chronic Pancreatitis

3.9.1 Von frey

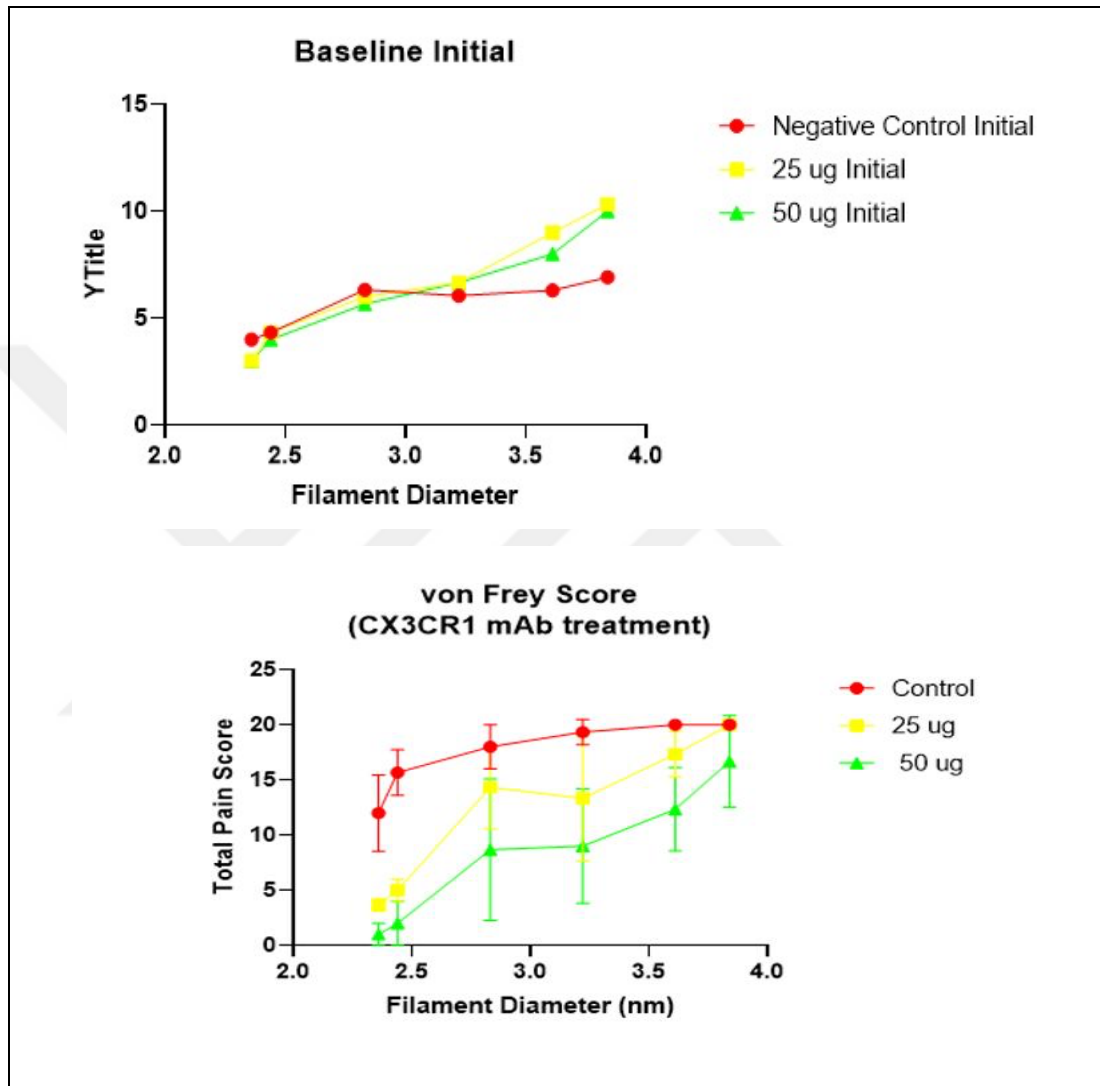


Figure 37. Results of the Von Frey pain test, baseline vs final week (8 weeks)

The baseline and final von frey data can be seen. From the data it is safe to say that mouse who were treated with antibody feels less pain.

3.9.2 Open field

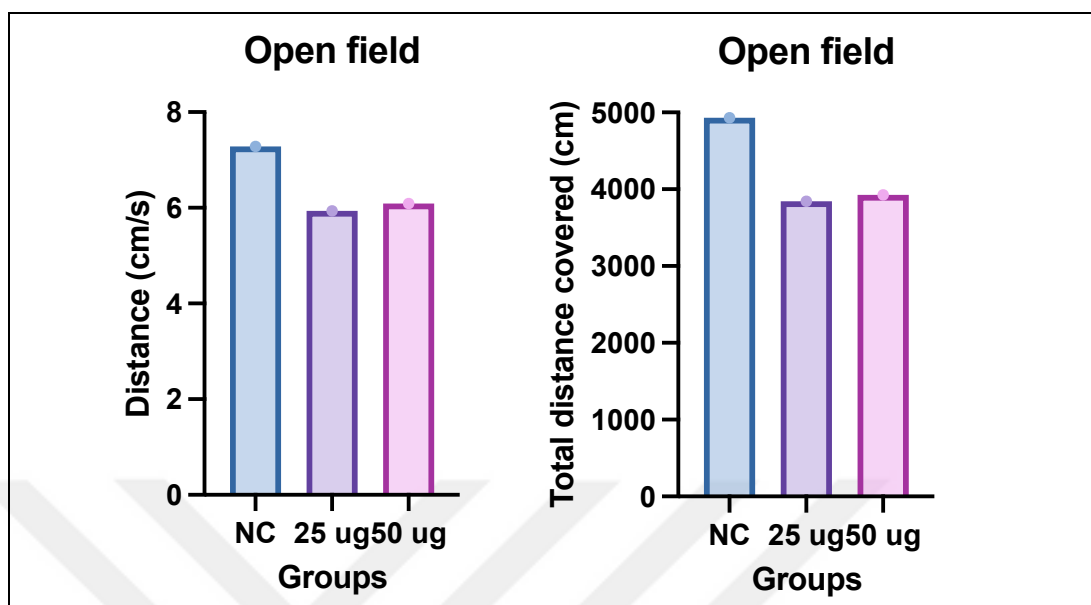


Figure 38. Results of the open field test as Distance covered (cm) and velocity (cm/s)

The data obtained from the open field assay shows that the control group which was injected with saline is more mobile.

4 DISCUSSION

As it was previously shown by our group that Fractalkine is responsible for the neural invasion in chronic pancreatitis and pancreatic cancer, this study aimed to inhibit the function of Fractalkine chemokine by inhibiting its main receptor CX3CR1 and, therefore, decrease the pain felt by patients. For this reason, the 3D structure of the CX3CR1 receptor has been mapped, and docking analyses were performed in order to determine the binding sequence. It was seen that the N-terminus of the 3rd loop is mainly responsible for the chemokine-receptor interaction. With the information obtained from this data, a peptide has been synthesized. These peptides were mixed with adjuvants and were injected into immunodeficient Balb/C mice until they reached the desired immunogenicity. Following this, the spleen and the lymph nodes of the mice were isolated as they are the responsible organs for antibody production in the mice. The collected organs were used in the hybridoma process. The colonies that tested positive with indirect sandwich ELISA for the desired antibody were cultured, and the supernatants were collected. The collected supernatants were purified by affinity chromatography and used in in vitro and in vivo assays.

Early characterization of the isotype of a monoclonal antibody is important for many reasons. First, the isotype of an antibody can determine the simplest purification method. For example, IgG2a and IgG2b bind to Protein A, and thus, Protein A can be purified by affinity chromatography; however, this method may not be suitable for the IgG1 subtype because they do not bind well to Protein A under standard conditions. Second, some isotypes are more amenable to certain immunological techniques than others. For example, IgG2b antibodies most effectively stimulate complement, making them the best isotype for in vivo immunotherapy studies and complement-mediated killing of cells bearing the epitope in vitro (Steplewski et al., 1988). Similarly, purification and enzyme conjugation of IgG3 and IgM antibodies can be difficult, making one-step applications unlikely. Third, isotype characterization also reveals the structure of an antibody, making it possible to generate information about antibodies that are undesirable for some applications. IgMs exist as pentamers consisting of five 180 kD subunits; therefore, these unstable IgM monoclonals are often too large for

applications requiring monomers, such as IgG isotypes or the majority of IgAs. Fourth, the isotype of an antibody determines the best method to prepare Fab fragments by proteolysis (e.g., using papain enzymes for IgGs) (Zola & Roberts Thomson, 2001).

The usage of complete and non-complete Freund's adjuvant is necessary for the immunization process. In essence, they both contain the same ingredients, paraffin oil and mannide monooleate, as allergens. The complete adjuvant also contains heat-killed and dried *Mycobacterium tuberculosis* to enhance the immune system by attracting macrophages and other cells to the site. As Balb/C mouse model is immunocompetent, the boost with complete adjuvant is required. After awakening the immune system, the usage of a complete adjuvant is not necessary. The other reason to use these adjuvants is they have lipophilic structure. When mixed with the antigen 1/1 ratio and injected to the mice it takes longer to be dissolved in the body. As it slowly dissolves over the course of around 10 days the immune system gets triggered constantly. Rather than triggering the immune system with the high dosage initially, the constant triggering causes the body to produce antibodies all the time (Stils Jr, 2005).

Submandibular blood was collected from the mouse 3 days after every injection. Three days is suitable for the body to react to the antigen that has been given to it, and 3-4 days is ideal for the production of IgG in the body. After the first injection, it takes around a week for the IgG to be produced by B cells. Therefore, it is irrelevant to collect blood and test it in the first week (Uhr & Möller, 1968).

The main defense line of the body consists of IgM. The higher avidity of this molecule makes it better for recognizing different antigens. However, the half-life and affinity of these molecules are less compared to IgG. Therefore, after the body recognizes the antigen, it starts to produce IgG to fight. Every time the injection to the mice occurs, the binding ability and the blood antibody levels are increasing which results in a higher immunoreactivity on ELISA assay (Vieira & Rajewsky, 1988).

22 different attempts were performed with unconjugated peptide and manual hybridoma method. In these trials, positive colonies have been collected however, the culturing of these colonies into mass production was not successful. The reason for this has been proven in graph X. The colonies were cultured in manually prepared DMEM HG supplemented with 20% FBS and clonacell medium B. As can be seen, both the morphology and antibody production of the cells changed. If it was known what the difference was in this step, the procedure should have been successful.

In most of the trials, it wasn't possible to see positive colonies. This may be due to the small size of the peptide as it wasn't enough to stimulate the B cells into producing the antibody of interest. Therefore, KLH and BSA conjugated peptides have been synthesized. The higher weight of these proteins was ideal for immune cell stimulation. The BSA conjugated peptide has been used for ELISA assays, and KLH conjugated peptide has been used for immunization. The reason for this can be seen in Figure 1; the immunogenicity index of KLH is higher compared to BSA. Another fact to be mentioned in this step is the peptide sequence has been altered due to the fact that KLH might cross-react with Cysteine (Kudryashov et al., 1998).

Feeder cells collected from the mouse before the hybridoma process are useful to present cells with a suitable environment. Feeder cells provide the necessary growth factor release for hybridomas. Aggregated feeder cells also support hybridomas for better attachment and growth as a monolayer. Feeder cells can be taken from any healthy untreated mouse – Balb/C - C57BL/6 mice can be used. The preferred genotype to be used for feeder cell isolation is wild type. If the peritoneum is punctured or damaged, isolation may fail due to contamination of the visceral cavity. Cultivation of feeder cells should be applied at least 3 days before the fusion procedure (Hoffmann et al., 1996).

As mentioned, the hybridoma procedure is mainly creating a single cell by fusing 2 different cell types. The normal chromosome number of a mouse is 42 (2n). After the fusion process, the resulting cells contain 84 (4n) chromosomes. It is unnatural, and with every sub-culturing or day in the culture environment, the risk of some

chromosomes being thrown out is present even though the selected method of hybridoma technology is homohybridoma, which is known to be more stable. Therefore, the hybridoma cells need to be checked regularly for antibody production. It is also important to cryopreserve at every step to reduce the risks (Köhler, 1980).

After the hybridoma procedure, there are 3 different possibilities: unfused B cells, unfused myeloma cells, and hybridoma cells. Unfused B cells die after 14 days of incubation because they are not resistant enough to survive in culture conditions not designed for them. Unfused myeloma cells die due to HAT supplements. Myeloma cells perform DNA replication through the de novo pathway. Aminopterin in the HAT supplement inhibits the de novo pathway, resulting in the death of these cancer cells. The fused hybridoma cells contain the properties of both myeloma and B cells. As B cells have the capability to perform DNA replication through the salvage pathway, hybridoma cells survive. H and T, Hypoxanthine, and Thymidine in the supplement are used for DNA replication promoters (Hnasko & Stanker, 2015).

Ultrafiltration tubes were used in order to concentrate the supernatants collected from the hybridomas. Western blot indicated that the target antibody has a weight of less than 30 kDa. To confirm this result, 100 kDa, 30 kDa, and 3 kDa ultrafiltration tubes have been used, and both concentrated and flow-through samples have been collected and tested with ELISA. It was also seen in ELISA that the highest reactivity was observed in a 3 kDa concentrated sample. From the data observed in these 2 assays, it was confirmed that the final product had a weight of 3 kDa – 30 kDa. In the first steps of the purification process, it wasn't possible to obtain high amounts of concentrated antibodies, which showed the need for more concentrated samples; therefore, concentrating the samples with ultracentrifuge tubes was chosen.

For the purification process, a protein A column has been chosen. Hybridoma cells were positive mainly for IgG1 and less for IgG3. As the half-life and binding capability of IgG1 are higher, using a protein A column was more logical. Because the affinity of the protein A column is high for IgG1 and less for IgG3, on the other hand, the

protein G column is not the best at separating these immunoglobulins as its affinity is considered to be high for both types (Sheoran & Holmes, 1996).

The SRB assay was used for in vitro concentration determination purposes before following with the in vivo assays. In this setup, a commercial inhibitory antibody and the antibody produced in the lab were compared as a control. The results indicate that the antibody produced by our group is much more efficient in affecting the proliferation capacity of cancer cells and HSC, which are known to have high levels of Fractalkine.

For the in vivo assays, again concentration determination was performed on chronic pancreatitis mouse models induced with 75 mg/kg cerulein injection. Mouse has been separated into 3 groups with 3 individuals. The first group was the control group, which received saline. The second group was injected with 25 ug antibody intraperitoneally, and the third group was injected with 50 ug antibody intraperitoneally for 3 times a week after the cerulein injections. The von Frey data indicates that the antibody-injected groups have less sensation of pain, which shows the efficiency of the produced antibody. In order to look into why, blood, brain, pancreas, and DRG were isolated from these groups and fixated to be paraffin-embedded. The main expectance is to observe fewer macrophage infiltrations in the tissues with antibody injection. Also, it is expected to see more fractalkine expression in blood as it could not get into the cells through its receptors, the body would release more fractalkine (Demir et al., 2011; Ulmasov et al., 2013).

The open field data was expected to be reversed. As the mice felt less pain, they should have moved around more and faster compared to the control group. However, results indicate a 20% decrease in distance covered and a 15% decrease in speed. This might have been caused by a cross-reaction caused by the Fractalkine inhibition. The inhibition of Fractalkine can result in the inhibition of the 5-hydroxytryptamine-2a receptor, which is one of the receptors mainly responsible for dopamine release. So, as mice feel depressed and scared, they tend to move less (Pei et al., 2024).

The effectiveness of this novel antibody will also be tried on pancreatic cancer genetic-knock-in mouse models (p48-Cre; LSL-KrasG12D, p53lox/+, “KPC”) and Acute pancreatitis mouse models. Further tests and analyses are needed to be sure that this antibody is suitable for clinical trials. The results are promising and change the course of the treatment of mentioned diseases.

Fractalkine signaling seems to be crucial in initiating and maintaining pancreatic hyperalgesia in acute pancreatitis, most likely by triggering of the local inflammatory reaction and activating spinal cord microglia via the highly selective receptor CX3CR1. Therefore, these novel findings reveal CX3CR1 as a promising new target for the treatment of acute pancreatitis.

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5 CURRICULUM VITAE

