DESIGN AND CONSTRUCTION OF A RECOMBINANT LY-49-FC FUSION PROTEIN

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Table of Contents:

List of abbreviations and acronyms:

bp	base pair
EtBr	ethidium bromide
cDNA	coding DNA
DNA	deoxyribonucleic acid
ECD	extracellular domain
EDTA	ethylenediaminetetraacetic acid
Fc	fragment crystallizable
FU	fluorescence units
GC	guanin-cytosine (content)
HMCV	human cytomegalovirus
ITAMs	immunoreceptor tyrosine-based activation motifs
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
IgG	immunoglobulin G
IL	interleukin
kb	kilo base pairs
KIR	killer cell immunoglobulin-like receptors
MCMV	murine cytomegalovirus
MCS	multiple cloning site
mRNA	messenger RNA
nt	nucleotides
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

qPCR	quantitative PCR
RIN	RNA integrity number
RNA	ribonucleic acid
rRNA	ribosomal RNA
rSAP	shrimp Alkaline Phosphatase
s	seconds
UTR	untranslated region

1. Introduction

The Ly49 receptors are transmembrane proteins of the C-type lectin superfamily primarily expressed on natural killer (NK) cells of mice, but also on other lymphocyte subpopulations such as T cells, mononuclear phagocytes, invariant NKT cells or γδ T cells (1). They play a critical role in the regulation of NK cell activity and immune modulation.by binding to their ligands – self-major histocompatibility complex (MHC) class I molecules. (2) The Ly49 receptor family encompasses at least 23 members, termed Ly49A through Ly49W, with either inhibitory or activating functions mediated through their structural features (3). Ly49C belongs to the class of inhibitory NK cell receptors and plays a role in the prevention of NK cell activation and subsequent lysis of target cells (3)

1.1 Function of Ly49 receptors

Being integral components of the immune system Ly49 receptors are fundamental in the regulation of NK cell activity, balancing activation and inhibition to ensure appropriate immune responses. (4)

Inhibitory Ly49 receptors are crucial for maintaining self-tolerance and preventing autoimmunity. (5,6) These receptors possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within their cytoplasmic domains. (3) Upon binding to MHC I molecules presented on the surface of healthy cells, these receptors transmit inhibitory signals that prevent NK cell activation. This interaction ensures that NK cells do not attack normal, healthy cells, thereby maintaining immune homeostasis. (4) The recognition of self-MHC class I molecules by inhibitory Ly49 receptors is essential for the "missing-self" hypothesis, according to which NK cells recognise and eliminate cells that have downregulated MHC class I molecules, a frequent strategy employed by virally infected or malignant cells to evade T cell-mediated immunity. (5,7)

In contrast, activating Ly49 receptors are designed to recognize abnormal cells, including those undergoing stress, infection, or transformation (8) These receptors often associate with adaptor proteins such as DAP12, which contain immunoreceptor tyrosine-based activation motifs (ITAMs). (3) When activating Ly49 receptors engage their ligands, typically stress-induced or altered MHC class I molecules on target cells, they transmit activating signals through the ITAMs, leading to NK cell activation, cytokine production, and cytotoxicity. This mechanism

enables NK cells to eliminate potentially harmful cells, including tumour cells and cells infected by viruses. (4)

The dynamic balance between signals from activating and inhibitory Ly49 receptors determines the overall activation state of NK cells. This balance is crucial for effective immune surveillance and response. An imbalance, such as an overactive inhibitory signal, can lead to inadequate immune responses, allowing pathogens or tumour cells to evade detection and destruction. Conversely, excessive activating signals can result in autoimmunity and tissue damage. (8)

1.2 Nomenclature of Ly49 gene family

There exist two major gene nomenclature systems for the Ly49 gene family - Ly49 and Killer Cell Lectin-like Receptor subfamily A (Klra), coded by the Klra gene cluster located on mouse chromosome 6, in a region termed NK gene complex (NKC). (7) In the case of Ly49C, the corresponding nomenclature is Klra3. (9)

1.3 Ly49 receptor structure

Ly49 receptors differ in their molecular structure depending on whether they are inhibitory or activating. Both inhibitory and activating Ly49 receptors share a prevalent extracellular structure featuring the C-type lectin-like domain, which is pivotal for ligand binding. (3) This structural feature ensures that both receptor types can recognize and respond to changes in MHC class I molecule expression. The primary difference lies in their cytoplasmic domains and their ability to interact with signalling molecules. The cytoplasmic tail of inhibitory Ly49 receptors contains one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Upon ligand binding, these ITIMs become phosphorylated and recruit phosphatases such as SHP-1 and SHP-2. These phosphatases dephosphorylate signalling molecules, transmitting an inhibitory signal that reduces NK cell activation and prevents cytotoxic responses against normal, healthy cells (3). Activating Ly49 receptors do not possess intrinsic signalling motifs in their cytoplasmic tails. Instead, they associate with adaptor proteins such as DAP12, which contain immunoreceptor tyrosine-based activation motifs (ITAMs). The interaction between the receptor and DAP12 is mediated through their respective transmembrane domains. The cytoplasmic ITAMs of the adaptor proteins become phosphorylated upon ligand binding. These

phosphorylated ITAMs initiate a several downstream signalling events that culminate in NK cell activation, cytokine production, and target cell lysis. (10)

1.4 Comparison to killer cell immunoglobulin-like receptors

Just like in mice, human immune cells are equipped with an extensive collection of cell surface receptors to identify intact and infected cells. Although structurally different, killer cell immunoglobulin-like receptors (KIRs) fulfil a similar function in humans as Ly49 do in mice, since they are also involved in MHC-I recognition and modulation of immune responses. (11,12) Just like Ly49 in rodents, different KIRs have either an activating or inhibitory role in regard to an immune response. (12) This makes the Ly49 family of receptors to a subject of interest, as they can be used as a model to investigate the function of KIRs and immune modulation in human NK cells.

1.5 Ly49-Fc fusion protein

Fusion proteins are constructed by the joining of two separate genes which originally code for different proteins. (13) In the case of this thesis, the cDNA of the extracellular domain of the Ly49C receptor was inserted into a plasmid designed for the fusion to an IgG Fc domain, called pFUSEN-hG1Fc (14), in order to further the research and understanding of Ly49 receptors and their role in immune modulation. This thesis intents to outline the objectives, used materials and methods, results and possible applications of this fusion protein.

2. Aims and objectives

Experimental work described in this thesis was carried out with the aim of constructing a recombinant eukaryotic expression vector, which can then be transfected into a suitable cell line to produce a fusion protein containing the extracellular domain of the mouse Ly49C receptor and the C_H2 and C_H3 domains of the Fc region of the human IgG1 heavy chain (i. e. Ly49C ECD – Fc fusion protein). Ly49C ECD – Fc fusion proteins can be used to generate monoclonal antibodies against mouse Ly49C receptors. The aforementioned antibodies, together with the fusion protein itself, can be used to detect and quantify either the surface levels of Ly49C receptors on target cells, such as NK cells, or the surface levels of Ly49C interaction partners on cells of interest. As such, both the Ly49C antibodies and Ly49C ECD – Fc fusion protein can serve as effective tools for investigating the role of Ly49C receptors in the context of NK cell biology.

To achieve the above-stated aim, the specific objectives that needed to be accomplished included the in-silico planning and simulation of the recombinant plasmid construction procedure, the isolation of total RNA from splenic lymphocytes, cDNA synthesis using the isolated RNA as a template, amplification of the Ly49C ECD coding sequence using PCR, the isolation of vector pFUSEN-hG1Fc from *E. coli*, digestion of the insert and the vector using appropriate restriction enzyme, ligation of the insert and the vector, transformation of *Escherichia coli* competent cells, and molecular analysis of transformants, which all culminated in the construction of the desired recombinant vector named pFUSEN-hG1Fc-Ly49ECD.

3. Materials and methods

3.1 Materials

3.1.1. Bacteria

Transformation experiments were performed using the HST08 strain of *Escherichia coli* (Stellar™ Competent Cells, Takara Bio, #2012869A, genotype: *F⁻, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ⁻*).

3.1.2. Lymphocytes

Previously prepared lymphocytes (courtesy of Jelena Železnjak, PhD) from a single, 8-week-old C57BL/6 female mouse (The Jackson Laboratory) were used as a source of total RNA.

3.1.3. Plasmids

Plasmid pFUSEN-hG1Fc (Invivogen #pfcn-hg1), propagated and maintained in *E. coli* HST08 cells (chapter 3.1.1), was used as a starting vector for the construction of the recombinant plasmid pFUSEN-hG1Fc-Ly49ECD. pFUSEN-hG1Fc-Ly49ECD contains the sequence of the mouse Ly49C receptor ECD fused to the 3' end of the sequence encoding the human IgG1 constant region. Both plasmids, together with features and restriction sites relevant to this work, are shown in Figure 1.

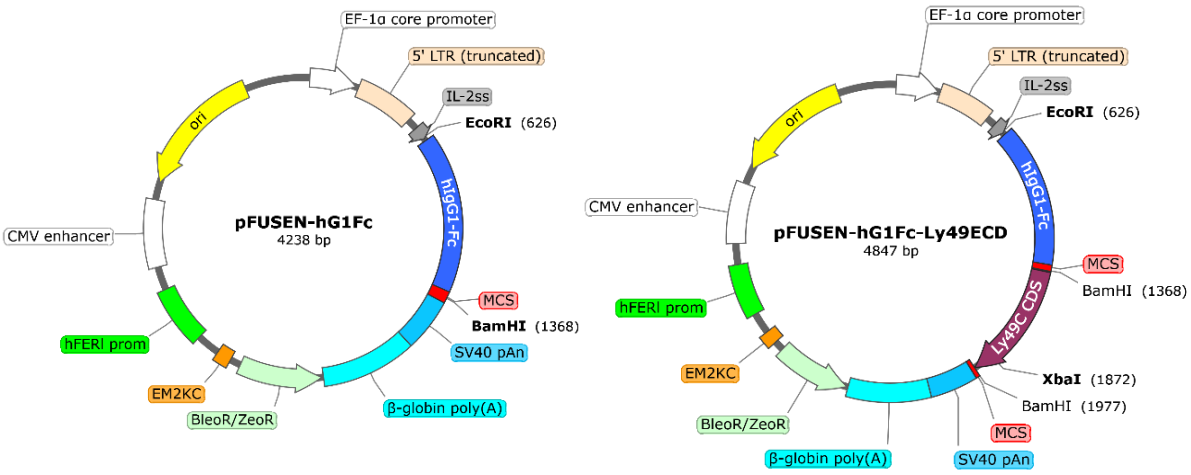

Figure 1. Maps of the plasmids pFUSEN-hG1Fc and pFUSEN-hG1Fc-Ly49ECD. Plasmid pFUSEN-hG1Fc (left) is routinely used for the construction of fusions between a protein of interest and an Fc domain of the human IgG1, such as the hG1Fc-Ly49ECD fusion constructed in this work (right). Labels: ***ori*** – the origin of replication in *E. coli*, ***CMV enhancer* + *hFERI prom*** – hybrid promoter driving the expression of the BleoR/ZeoR gene in eukaryotic cells, ***EM2KC*** **–**promoter driving the expression of the BleoR/ZeoR gene in *E. coli* cells, **EF-1α core promoter+5' LTR (truncated) –** hybrid promoter driving the expression of the transgene fused to the human IgG1 Fc region, **IL-2ss** –interleukin 2 signal sequence, enables the secretion of the Fc fusion protein, **hIgG1-Fc** – sequence encoding C_H2 and C_H3 regions of the human IgG1 heavy chain, **MCS** – multiple cloning site, **Ly49C CDS** – sequence encoding the extracellular domain of the mouse Ly49C receptor, **SV40 pAn** – simian virus polyA signal sequence, **β-globin poly(A)** - 3'-UTR from the human β-globin gene (14).

3.1.4. Primers

All used primers are listed in Table 1 below. Primers C-Bam-F and C-Bam-R were used to amplify the sequence encoding the extracellular domain of the Ly49C receptor using the total lymphocyte cDNA (chapter 3.2.5) as a template for PCR. In contrast, primers Ly49Check-F and Ly49Check-R were used to screen for bacterial transformants that contain the recombinant plasmid molecules, containing a single copy of the Ly49C ECD sequence fused in the proper orientation with the human IgG1 Fc sequence (pFUSEN-hG1Fc-Ly49ECD, Figure 1, right). Primers have been purchased from biomers.net GmbH.

Table 1. Primers used in this work (BamHI restriction site is indicated in **bold** typeface)

Primer name	Length (nt)	Sequence (5' -3')
C-Bam-F	34	CGGC**GGATCC**AAGATTTTTCAGTATAATCAACAC
C-Bam-R	32	ACTC**GGATCC**TTAATCAGGGAATTTATCCAGT
Ly49Check-F	22	TAGCGGTGGTGGCGGATCCAAG
Ly49Check-R	33	GGCCAGCTAGCTCGAGATATCGGATCCTTAATC

3.1.5 Chemicals, reagents, specific and general laboratory equipment

3.1.5.1. Reagents for isolation, extraction, and purification of nucleic acids

Isolation and purification of total RNA from mouse lymphocytes were performed using the NucleoSpin® RNA Plus kit (Macherey-Nagel, #740984.50). A recombinant RNase Inhibitor (Takara Bio, #ST0792) was used to protect purified RNA samples from degradation by RNases. Plasmid DNA from *Escherichia coli* (HST08 strain, Stellar™ Competent Cells, Takara Bio, #636763) was isolated and purified using the NucleoSpin Plasmid EasyPure kit (Macherey-Nagel, #740609.50). Finally, restriction enzymes, nucleotides, primers, buffers, and other contaminants were removed from restriction enzyme-digested plasmid suspensions or PCR reaction mixtures using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, #740609.50).

3.1.5.2. Reagents and equipment for capillary gel-electrophoresis

Capillary gel-electrophoresis using the Bioanalyzer 2100 system (Agilent, #DEDAE01120) has been used to evaluate the integrity of total RNA isolated from mouse lymphocytes. Total RNA samples for capillary electrophoresis have been prepared using the Agilent RNA 6000 Pico Reagents (#Agilent, 5067-1513), RNA Pico Chips (Agilent, #BN10BK30), and Chip Priming Station (Agilent #G2938-68700).

3.1.5.3. Reagents and equipment for standard agarose gel-electrophoresis

Separation of various RNA and DNA molecules according to size has been performed by agarose gel electrophoresis, using either the HVD miniGel Electrophoresis System (HVD, #W101-1016) or the VWR Electrophoresis System (VWR, #130919 031). Purple SDS Gel Loading Dye (New England Biolabs, #B7024A) was mixed with DNA or RNA samples to facilitate sample loading into wells of the gel, and the 1 kb DNA Ladder (NEB, #N3232) was used as a size standard. Agarose gels were prepared using the Roth Agarose Standard (Roth, # 3810.4) and in-house made 1× TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM EDTA). Separation of nucleic acids during gel-electrophoresis has been powered by the ELPHO power supply (HVD, #W100-1006), and following electrophoresis and EtBr (ethidium bromide) staining, DNA and RNA bands in gels have been visualized using the UV transilluminator (UVP Transilluminator, Analytik Jenam #H212520) operating at a wavelength

of 302 nm. Gel images have been documented and stored using the Uvitec gel imaging system (Uvitec, #06 15363).

3.1.5.4. Reagents for first-strand cDNA synthesis and PCR

Using total isolated mouse RNA as a template, first-strand cDNA synthesis has been performed using the reagents available in the ProtoScript® II First Strand cDNA Synthesis Kit (NEB, # E6560S). PCR synthesis of all amplicons in this work has been carried out using the appropriate primers listed in Table 1 (chapter 3.1.3), molecular grade sterile water (Sigma, #W4502-1L), Phusion® High-Fidelity DNA Polymerase (NEB, #M0530), Phusion® HF Buffer (NEB, #B0518S), and dNTPs (NEB, #N0447S).

3.1.5.5 Restriction enzymes

Before ligation, vector and insert DNA was digested using the restriction enzyme BamHI-HF (NEB, #R3136S), whereas the enzymes XbaI (NEB, # R0145S) and EcoRV (NEB, #R0195S) were used for restriction analysis of recombinant plasmid DNA. All restriction reactions were performed according to the instructions provided by the manufacturer, New England Biolabs.

3.1.5.6 Enzymes for 5'-phosphate removal and ligation

Dephosphorylation of the BamHI-digested vector was performed using the Shrimp Alkaline Phosphatase (rSAP) (NEB, # M0371S) to prevent vector recircularization and increase the efficiency of ligation of the vector and the insert. Prior to transformation, PCR-amplified, BamHI-digested fragment encoding the Ly49C ECD was ligated to the BamHI-digested and dephosphorylated pFUSEN-hG1Fc, using the T4 DNA ligase (NEB #M0202S).

3.1.5.7 Growth media and antibiotics

E. coli cells were grown in an in-house made lysogeny broth (LB) medium containing 10 g/L tryptone, 5 g/L sodium chloride, and 5 g/L yeast extract. After transformation, the bacteria were spread on a solid LB medium, which was made by adding 15 g/L agar to the liquid LB. Solid or liquid LB media were supplemented with the antibiotic Zeocin (Invivo Gen, #ant-zn-05) when necessary.

3.2 Methods

3.2.1 In-silico planning and simulation of the molecular cloning procedure

To facilitate the construction of the recombinant plasmid pFUSEN-hG1Fc-Ly49ECD (Figure 1), the entire molecular cloning procedure has first been planned and simulated using SnapGene v.7.1.2, and an overview of the predicted (expected) results, is shown in chapter 4. As a first step, the nucleotide sequence of the starting vector, pFUSEN-hG1Fc, obtained from the technical data sheet provided by InvivoGen (14), was imported into SnapGene. In the second step, a file containing the 1194-bp-long Ly49C receptor cDNA sequence was downloaded from the Ensembl genome database using the Ensembl browser, (15,16) and imported into SnapGene. Since the objective of this work was to construct a fusion between the sequence encoding the human IgG1 constant region and the sequence encoding the extracellular domain of the mouse Ly49C receptor, the sequence encoding the extracellular domain of the Ly49C was annotated using the information about the Ly49C receptor available in the UniProt database (7), according to which the extracellular domain (ECD) of the Ly49C receptor is encoded by amino acids 70 to 266, corresponding to bp 330 - 920 in the downloaded cDNA sequence. On the other hand, Dam et al. have shown that the extracellular domain of Ly49C spans amino acids 67 to 262 of the Ly49C receptor, corresponding to bp 321 – 908 in the downloaded cDNA sequence. (18) Both results were considered to ensure that the desired insert (Ly49C ECD) contains the entire extracellular domain of the Ly49C receptor. The sequence spanning nucleotides 321-920 was therefore annotated to encode the Ly49C ECD and used as a template for planning PCR amplification of Ly49C ECD from mouse cDNA. Primers chosen for amplification of Ly49 ECS are listed above in Table 1. In addition to flanking the entire Ly49C ECD coding region, additional nucleotides corresponding to the BamHI recognition sequence were present at the 5'-ends of each primer to enable subsequent digestion of the PCR product with BamHI and its ligation with the BamHI-digested vector. The PCR reaction was then simulated using SnapGene's built-in option to ensure that the amplified sequence for the Ly49 ECD can be ligated in frame with the sequence encoding the human IgG1 constant region located in the BamHI-digested starting vector pFUSEN-hG1Fc. As a last step, the structure of the desired final plasmid, pFUSEN-hG1Fc-Ly49ECD, was predicted/drawn using the restriction cloning option within SnapGene.

3.2.2 Total RNA Isolation from mouse lymphocytes

Previously obtained lymphocytes suspended in PBS (phosphate-buffered saline) (courtesy of dr. sc. Jelena Železnjak) were used as a source of RNA encoding the Ly49C receptor. Total RNA was isolated with the help of the NucleoSpin RNA isolation kit from Macherey-Nagel, according to the protocol recommended by the manufacturer, and the isolation was done in an RNase-free environment and under a sterile hood to prevent contamination of samples or RNA degradation. To lyse the cells, they were centrifuged, the resulting PBS supernatant was removed , and 350 µl of LBP lysis buffer was added to the cells, which were then gently resuspended. Next, the lysate was transferred onto a DNA removal column and centrifuged for 30 seconds at 11,000 g to eliminate the genomic DNA. The column was discarded, and 100 µl of binding solution was added to the flowthrough and mixed using a pipette. The lysate was then transferred onto a new RNA binding column in a collection tube and centrifuged for 15 seconds at 11,000 g. An amount of 200 µl of the included washing buffer WB1 was then added to the column, and the tube was again centrifuged for 15 seconds at 11,000 g. The flowthrough was again discarded, and 600 µl of the WB2 buffer was placed onto the column matrix, followed by another centrifugation under the same conditions. The flowthrough was discarded again, and 250 µl of the second washing buffer was added to the column, but now the tube was centrifuged for 2 minutes to ensure the column matrix was dry and free of any residual buffer solution. To elute the RNA, the column was transferred into a sterile, RNase-free 1.5 mL Eppendorf tube, and 30 µl of RNase-free water was added to the column matrix before centrifuging at 11,000 g for 1 minute. Finally, an additional elution step was performed with another 30 µl of RNAse-free water, followed by the addition of the RNase inhibitor and storage at -80 °C.

3.2.3 RNA and DNA visualization and quantification

To examine whether the RNA isolation was successful, standard agarose gel electrophoresis in TAE buffer and RNA capillary electrophoresis using the Agilent RNA 6000 Pico Bioanalyzer were used. For agarose gel electrophoresis, a standard 1% agarose gel in a TAE buffer (1×) was used. In the case of RNA electrophoresis, bleach in a concentration of 2% was added to molten agarose before the gels were poured. 100 ml of buffer and 1g of agarose were heated using a microwave until the agarose was completely dissolved. After the molten agarose cooled to approximately 45-55 °C, it was poured into a gel-casting tray and left at room temperature for

~20 minutes to allow the agarose to solidify. Once prepared in this manner, the tray with agarose gel was placed into an electrophoresis tank filled with 1X TAE buffer, the well-forming comb was removed, and each RNA/DNA sample was loaded into a separate well of the gel. Following sample loading, all gels were run at a constant voltage of 120 V and 400mA. After electrophoresis, the gels were incubated in the ethidium bromide solution (0.5 μg/ml) before visualization of nucleic acid bands on a UV transilluminator. The capillary electrophoresis was performed according to instructions in the Agilent Bioanalyzer 2100 User Manual and RNA 6000 Pico Kit for 2100 Bioanalyzer Systems manuals. (19)

3.2.4 First-strand cDNA synthesis

First-strand cDNA synthesis was accomplished using the ProtoScript® II First Strand cDNA Synthesis Kit by New England Biolabs, according to the manufacturer's recommendations. Briefly, in a microfuge tube, 5 μl the isolated total mouse RNA was combined with 2 μl the included oligo dT primers and 3 μl of sterile water before heating the mixture at 65 °C for 5 minutes, followed by brief centrifugation and cooling on ice. Next, 10 μl of the ProteoScript II reaction mix, as well as 2 μl of the ProtoScriptII enzyme mix, were added to the mixture before incubating the sample at 42 °C for one hour. Finally, reverse transcriptase was inactivated by heating at 80 °C for 5 minutes, and the cDNA synthesis product was stored at -20 °C. In parallel, a negative control, lacking the addition of the enzyme mix, was prepared under the same conditions.(20)

3.2.5 Polymerase chain reaction (PCR)

To amplify the sequence encoding the extracellular domain of the Ly49C sequence, PCR was performed using primers C - Bam - F and C – Bam – R (chapter 3.1.4) and mouse cDNA as a template. In total, 5 identical reactions, together with additional negative control, lacking the synthesized cDNA as a template, were prepared according to Table 2 below.

Table 2: Composition of PCR reaction mixture used to amplify Ly49C ECD.

Component	Volume in each individual reaction mixture (µL)
Sterile, demineralized, RNAse- and DNase-free H_2O	31.5
5× HF PCR buffer	10
Primer C - Bam - F (10 mM)	2.5
Primer C - Bam - R (10 mM)	2.5
dNTPs (10 mM each)	1
Mouse cDNA	2
Phusion Polymerase	0.5

In the case of the negative control, the cDNA was replaced by 2 µl of water. The PCR was performed using the Phusion high-fidelity polymerase from New England Biolabs according to the manufacturer's recommendations. The PCR program consisted of the steps and conditions listed in Table 3, with steps 2-4 being repeated 30 times (21).

Table 3: PCR program used for the amplification of the Ly49C ECD sequence:

Step No.	Description	Temperature	Duration
1.	Initial denaturation	98° C	5 min
2.	Denaturation	98° C	10 sec
3.	Annealing	57° C	30 sec
4.	Elongation	72° C	30 sec
5.	Final extension	72° C	10 min
6.	Hold	12° C	Until further sample processing

Template DNA for colony PCR, used for screening of bacterial clones, was prepared by scraping a part of the colony from an agar plate and transferring it into 2 mL of liquid LB medium supplemented with Zeocin. Following overnight incubation at 37 °C, 1 µL of the bacterial cell suspension was used as a template in a PCR reaction mixture additionally containing 12.4 µl of sterile water, 4 µl of 10× HF buffer, 1 µl each of the forward (Ly49Check-F) and reverse (Ly49Check-R) primer, 0.4 µl of 10 mM dNTPs and 0.2 µl of 2000 U/mL Phusion polymerase (NEB). The PCR program used for colony PCR is shown in Table 4 below:

Table 4: PCR program used for colony PCR:

Step No.	Description	Temperature	Duration
1.	Initial denaturation	98° C	3 min
2.	Denaturation	98° C	10 sec
3.	Annealing	72° C	30 sec
4.	Elongation	72° C	25 sec
5.	Final extension	72° C	5 min
6.	Hold	4° C	Until further sample processing

3.2.6 PCR cleanup procedure

Following PCR amplification of the Ly49C ECD, described in chapter 3.2.5, the added reagents had to be removed because they can affect downstream reactions. PCR clean-up was performed using the NucleoSpin® Gel and PCR Clean-up kit, according to the protocol provided in the kit. Briefly, following PCR, samples were mixed in a 1:2 ratio with the included NTI buffer, transferred onto the column, placed within a collection tube, and centrifuged at 11,000 g for 30 seconds. Afterward, the flowthrough was discarded, and 700 µl of the NT3 washing buffer was loaded onto the column before centrifuging again at 11,000 g for 30 seconds. The flowthrough was again discarded, and the collection tube with the column was centrifuged for 1 minute at 11,000 g to dry the silica membrane. The collection tube was placed inside a heating block at 70 °C for 5 minutes, with the lid open, to remove any remaining ethanol. As a final step, the column was transferred into a new tube, and 20 µl of Buffer NE from the kit was loaded onto the membrane and centrifuged at 11,000 g for 1 minute to elute the DNA bound to the column. In order to maximize the yield of DNA, this step was repeated by transferring the eluate onto the column matrix, followed by another round of centrifugation under the identical conditions as in the preceding step. (22)

3.2.7 Plasmid isolation from *E. coli* cells

All plasmid isolation procedures were performed using the NucleoSpin Plasmid EasyPure kit, as instructed by the manufacturer. Briefly, 2 ml of an overnight culture of *E. coli*, strain HTS08, was transferred into Eppendorf tubes, and bacterial cells were collected by centrifugation at

12,000 g for 30 seconds. The cells were then resuspended in 150 µl of Buffer A1 and lysed by adding 250 µl of Buffer A2 and gently mixing the contents of the tubes by inverting them several times. Next, 350 µl of Buffer A3 was added to the cell lysate to precipitate the proteins present in the lysate. The contents of the tubes were centrifuged at 12,000 g for 3 minutes to allow their separation from the remainder of the suspension containing plasmid DNA. Following centrifugation, the resulting supernatant was transferred into the DNA binding column and placed within a collection tube. After another cycle of centrifugation at 2,000 g for 30 seconds, the flowthrough was discarded, and the column with the bound DNA was placed back in the collection tube to be washed. For this, 450 µl of the ethanol Buffer AQ was evenly applied onto the column matrix, and the samples were centrifuged at 12,000 g for one minute. To reduce ethanol carry-over, the samples were then shortly incubated at 37 °C in a heating block with the lid open to give any residual ethanol some time to evaporate. To elute the bound DNA in a final step, the columns were placed in a new tube, and 50 µl of Buffer AE was applied to the column matrix, followed by centrifugation at 12,000 g for one minute. The extracted DNA was then stored at +4 °C. (23)

3.2.8 Restriction digestion of DNA molecules

Digestion of DNA molecules in this work has been performed according to the instructions given by the manufacturer of restriction enzymes (New England Biolabs) (24,25). All components required for a particular reaction (DNA, enzyme(s), buffer, and sterile, deionized RNase-free and DNase-free water) were mixed in an Eppendorf tube, with the restriction enzyme being added to the reaction mixture last. The reaction mixtures were then mixed, briefly spun down in a centrifuge, and incubated at 37 °C for up to one hour. Reaction mixtures for digestion of vector and insert with the restriction enzyme BamHI prior to ligation were prepared according to Table 5:

Table 5: Composition of reaction mixtures for digestion of vector and insert in µl

	Ly49C ECD PCR	pFUSEN-hG1Fc
H2O	21	36
rCutSmart Buffer (10×)	5	5
BamHI-HF (20000 U/µL)	4	4
DNA	20	5
Total	**50**	**50**

The volumes of DNA listed in Table 5 correspond to 3.3 µg of plasmid DNA and 0.5 µg of insert DNA. According to the manufacturer, 10 units of an enzyme are sufficient to digest approximately 1 µg of DNA, yet a considerably higher enzyme amount was added to reaction tubes in an effort to overcome any variability in the DNA material, quantity, or purity and to ensure complete digestion of the DNA. An analogous approach, employing a surplus of restriction enzymes, was applied to digest plasmid DNA isolated from positive bacterial clones (transformants that putatively contain the desired recombinant plasmid pFUSEN-hG1Fc-Ly49ECD), using the enzymes EcoRI and XbaI (chapter 4.5).

3.2.9 Removal of phosphates from the 5'-ends of the digested vector molecules

To prevent recircularization or dimerization of empty vector molecules during ligation with the insert, plasmid DNA was treated with Shrimp Alkaline Phosphatase (rSAP, New England Biolabs) to remove 5'-phosphate groups from the DNA ends obtained by digestion of vector with restriction enzyme BamHI. To that end, five units of rSAP were added after the vector digestion reaction, followed by an additional incubation period at 37 °C for 30 minutes. Following the 30-minute incubation period, the phosphatase was inactivated by heating the reaction mixture at 65 °C for 5 minutes. Enzymes present in vector and insert samples were then removed using the Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit.

3.2.10 Quantification of insert and vector DNA

To effectively ligate the plasmid with the insert, they must be combined in a specific molar ratio. For this, the concentration of BamHI-digested dephosphorylated and purified vector, as well as the concentration of BamHI-digested and purified insert, need to be evaluated. The evaluation of vector and insert concentrations was performed either by manually estimating the intensities of vector and insert DNA and comparing them to the intensities of bands present in

the ladder DNA following electrophoresis or by using Implen NanoPhotometer, as instructed by the manufacturer, to spectrophotometrically determine the DNA concentration in the samples.

3.2.11 Ligation

Ligation of the BamHI-digested, dephosphorylated linearized pFUSEN-hG1Fc plasmid and the linear, BamHI-digested insert dsDNA encoding the extracellular domain of the Ly49C receptor was performed by mixing vector and the insert in the 1:3 molar ratio and addition of the T4 DNA ligase (New England Biolabs) according to ligation protocol provided by NEB (26). The necessary volumes of the vector and insert suspension were estimated using the NewEngland Biolabs online calculator (27). In a microcentrifuge tube on ice, 11.6 µl of sterile water, 2 µl of T4 ligase buffer, 1.4 µl of plasmid solution (equivalent to 50 ng), 3.9 µl of insert solution (equivalent to 22 ng) and 1 µl of T4 ligase were combined. The reactants were mixed using a pipette and briefly spun down using a centrifuge before incubation at 16 °C overnight. The next day, the ligase was inactivated at 65 °C for 10 minutes in a heating block.

3.2.12 Media for growth and propagation of *E. coli* cells

A standard liquid LB medium was used to grow *E. coli* cells. The required components were dissolved in distilled water to prepare the liquid LB medium, and the solution was autoclaved at 121 °C for 20 minutes. The solid LB medium was prepared by the addition of agar to the broth before autoclaving and pouring the autoclaved medium into appropriate Petri dishes. When required, media were supplemented with 50 mg/L of antibiotic Zeocin.

3.2.13 Transformation of *E. coli* cells

Transformation of bacterial cells with ligation mixture was performed according to the standard bacterial transformation protocol (28). First, the competent *E. coli* cells, strain HST08, were thawed on ice for 20 minutes. Then, four samples in microcentrifuge tubes were prepared, each containing 100 µl of competent cell suspension: one with an additional 8 µl of the ligation mixture containing both the vector and the insert, one with 8 µl of the dephosphorylated and digested vector only, one with 0.5 µl of the undigested vector and one negative control, containing only competent *E coli* cells. After preparation and mixing, all samples were

incubated on ice for 15 minutes. Next, the cells were heat shocked by placing the tubes in a water bath prewarmed to 42 °C for 60 seconds and then immediately transferred back on ice for another 2 minutes. Following the brief incubation on ice, 900 µl of LB medium was added to each sample, and the tubes were incubated for 1 hour in a shaking thermoblock at 37 °C. Following incubation at 37 °C, the contents of the tubes were centrifuged for 5 minutes at 4000 g, 900 µl of the supernatant was discarded, pelleted cells resuspended in the remaining LB medium and evenly spread onto LB agar plates supplemented with Zeocin using glass beads. The Petri dishes were then incubated at 37 °C overnight until the growth of bacterial colonies could be clearly observed.

4. Results

As stated earlier, the main objective of this work was to construct a recombinant plasmid pFUSEN-hG1Fc-Ly49ECD (Figure 2, chapter 3.1.3), which contains the sequence encoding the human IgG1 constant region fused to the extracellular domain of the mouse Ly49C receptor. Construction of the plasmid pFUSEN-hG1Fc-Ly49ECD, outlined in Figure 2 below, was performed in a series of steps, which included the isolation of total RNA from mouse splenocytes (chapter 4.1), reverse-transcription of total mouse RNA and PCR amplification of the linear dsDNA fragment encoding Ly49 ECD (chapter 4.2), isolation of starting vector pFUSEN-hG1Fc and digestion of the vector and PCR-amplified Ly49ECD insert with restriction enzyme BamHI (chapter 4.3), vector and insert ligation, transformation of *E. coli* HST08 competent cells and colony screen of transformant colonies by PCR (chapter 4.4), and additional restriction analysis of plasmid DNA from positive clones identified previously by PCR (chapter 4.5)

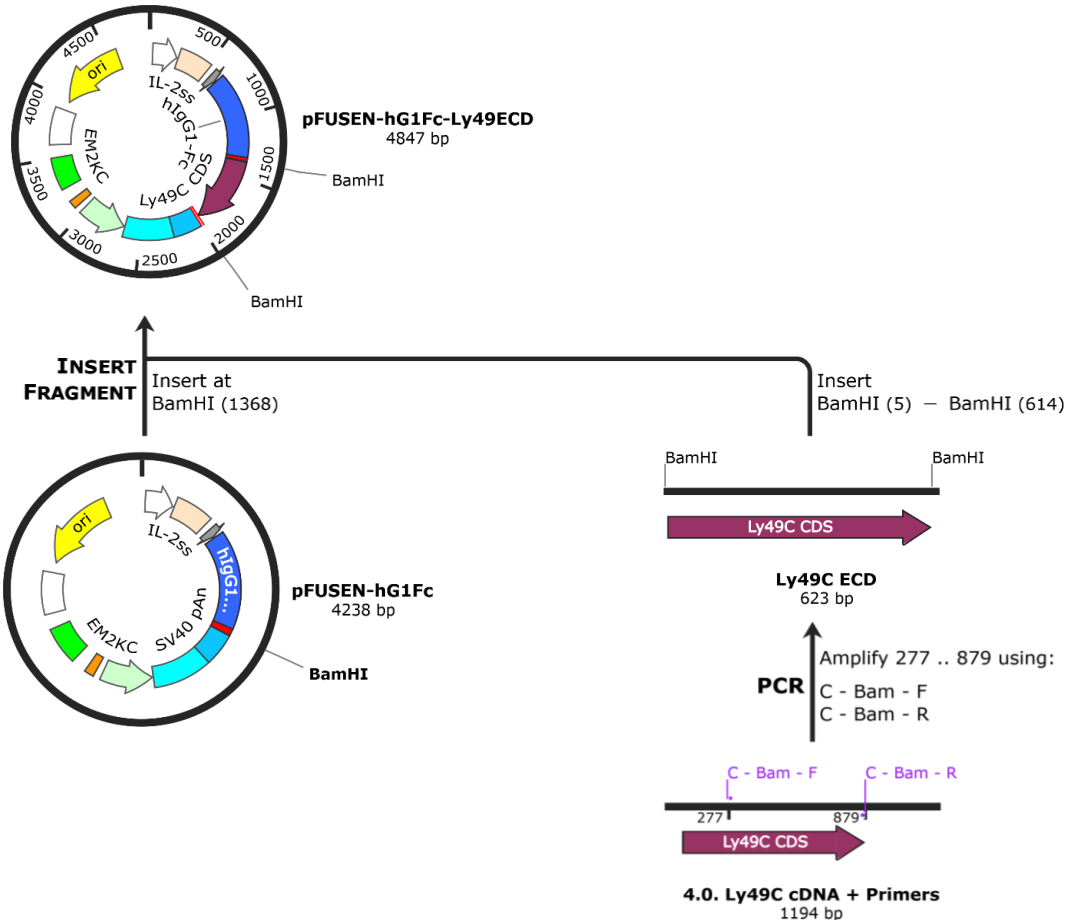

Figure 2. Outline of the construction of the pFUSEN-hG1Fc-Ly49ECD, showing the main steps in constructing the recombinant plasmid pFUSEN-hG1Fc-Ly49ECD. For details, see the main text. The image was drawn using SnapGene v.7.2.1.

4.1 Isolation of total mouse RNA from lymphocytes

Inhibitory Ly49 receptors, such as Ly49C, are primarily expressed in NK cells. (11) As NK cells are predominantly found in splenic tissue, lymphocytes were chosen as the source of transcripts encoding the Ly49C receptor. Following isolation and purification of total lymphocyte RNA from two samples containing approximately 8 x 10^7 cells, the efficiency of the RNA isolation procedure was evaluated by agarose gel electrophoresis (chapter 3.2.3), and the obtained results are shown in Figure 3.

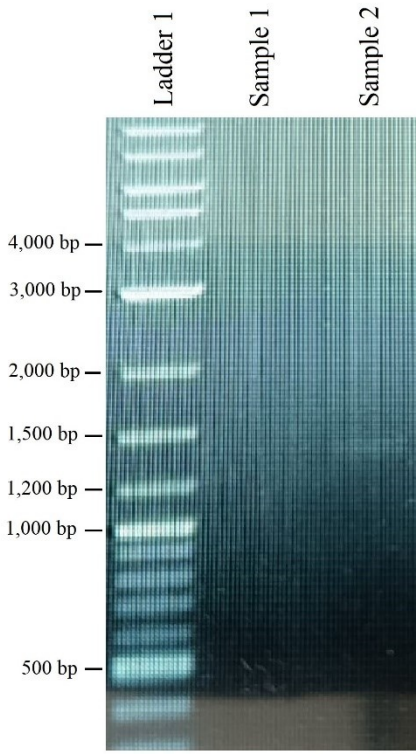

Figure 3. Verification of the total lymphocyte RNA quantity and integrity. Labels Ladder 1 = NEB 1kb Plus ladder, Sample 1 = a 2 µL aliquot of total lymphocyte RNA from Sample 1, Sample 2 = 2 µL aliquot of total lymphocyte RNA from Sample 2, bp = base pairs. (Note: Unfortunately, the gel capturing system was malfunctioning, leading to the poor quality of this figure.)

As can be seen from the gel image shown in Figure 3 above, not a single band was visible in the lane loaded with an aliquot of total RNA from Sample 1, whereas one band was visible in the lane loaded with an aliquot of total RNA from Sample 2. Intact, highest quality total RNA samples separated on agarose gels commonly display two distinct bands corresponding to the 28S rRNA and 18S rRNA species, whereby the fluorescence of the larger, 28S rRNA band is approximately 2-fold stronger compared to the smaller, 18S rRNA band. Based on the presence

and intensities (or lack thereof) of bands in gel lanes labeled Sample 1 and Sample 2 in Figure 3, it is possible that the RNA underwent (partial) degradation during or after the isolation procedure and/or that the insufficient quantities of total RNA have been loaded into wells of the gel in the first place.

Nonetheless, RNA samples with RNA concentrations below the detection level in EtBr-stained agarose gels might still contain sufficient RNA for downstream applications. Therefore, the quantity and quality of RNA in Sample 1 and Sample 2 have additionally been evaluated using a more sensitive approach based on capillary electrophoresis and determination of the so-called RNA Integrity Number, or RIN, which provides a more objective, standardized way to assess RNA quality. The RIN spans values between 1 and 10, with an RIN value of 10 indicating the highest RNA integrity and an RIN value of 1 indicating severely degraded RNA. The RIN scale is based on the analysis of RNA electropherograms produced by microcapillary electrophoresis apparatus, and the RIN number itself is calculated based on various parameters, including the ratio and height of the 28S and 18S rRNA peaks, the area under these peaks, and additional metrics reflecting the presence of degraded RNA fragments (13).

Having previous information in mind, total RNAs in Sample 1 and Sample 2 have been prepared for and analysed by capillary electrophoresis according to the procedure described in Materials and Methods (chapter 3.2.3). In addition to the undiluted total RNA samples, electrophoresis was also performed using samples diluted in ratios 1:10, 1:100, 1:1000, and 1:10000. Electropherograms obtained for undiluted and diluted samples are shown in Figure 4 below.

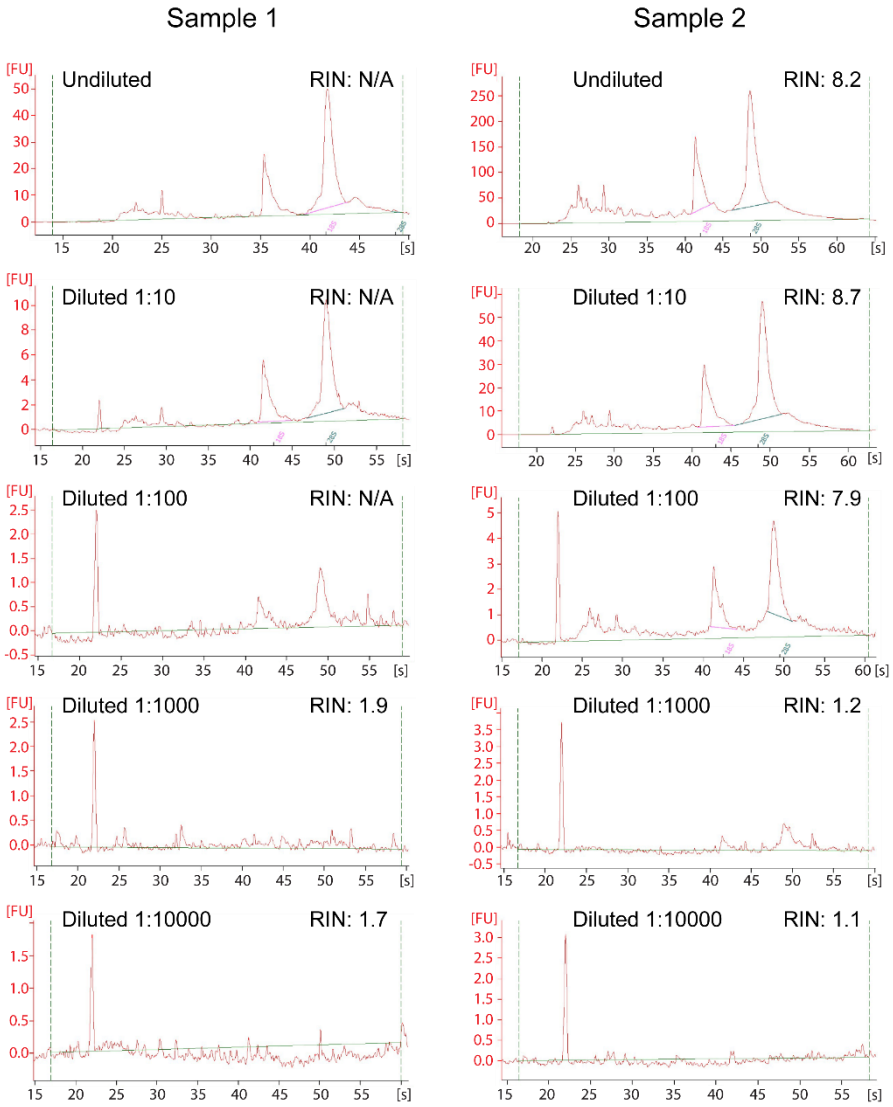

Figure 4. Verification of the total lymphocyte RNA quantity and integrity using capillary gel-electrophoresis. Shown are the electropherograms for undiluted and diluted total lymphocyte RNA Sample 1 (left) and Sample 2 (right), in decreasing order of RNA concentrations. The y-axis displays the fluorescence units (FU) as a measure of RNA concentrations, while the x-axis shows the migration time, in seconds (s), of detected RNA fragments, which is proportional to their length. RIN values and dilution ratio for each sample are indicated within the corresponding electropherogram.

As can be seen from Figure 4, two distinct peaks are visible in the right half of electropherograms corresponding to undiluted and 10-fold diluted total RNA samples of both RNA preparations (Sample 1 and Sample 2). The accompanying Agilent Bioanalyzer 2100 Expert Software has called these peeks as representing 28S rRNA and 18S rRNA species only in a 10-fold diluted preparation of Sample 1. In contrast 28S rRNA and 18S rRNA peaks were called in undiluted, as well as in 10-fold and 100-fold diluted preparations of Sample 2. Such an occurrence is not unusual since the migration of nucleic acids through a capillary gel can be strongly affected by the concentration of salts in the sample. Moreover, correct fragment

migration times are one of the factors required for the proper identification of RNA species, and the instrument cannot correctly identify fragments with aberrant migration times, even though the peak has the expected shape. Indeed, as can be seen in the above figure, diluting the sample apparently brings the salt concentration within the accepted range, which enables proper peak identification. Therefore, it is likely that the peaks displayed in the electropherogram for the undiluted preparation of Sample 1 also represent rRNA molecules, suggesting, in turn, that the total RNA isolation from both lymphocyte suspensions (Sample 1 and Sample 2) has been successful. Moreover, capillary electrophoresis demonstrated that, in addition to quality, the quantity of RNA in Sample 2 was also superior to the quantity of RNA in Sample 2 (Sample 1: 2,258 pg/µL vs Sample 2: 22,100 pg/µL). This result was consistent with the results of standard agarose gel-electrophoresis shown in Figure 3 and prompted us to use total RNA from Sample 2 for downstream applications.

4.2 Reverse-transcription of total mouse RNA and PCR synthesis of dsDNA fragment encoding Ly49C ECD

Following isolation, the two samples of total RNA isolated from mouse lymphocytes were combined, and the resulting mixture was used as a template for reverse-transcription/first-strand cDNA synthesis according to the procedure described in Materials and Methods (chapter 3.2.4). Following cDNA synthesis, a 2 µL aliquot of the newly synthesized mouse cDNA was used as a template for synthesis of the linear dsDNA fragment encoding the extracellular domain of the Ly49C receptor by PCR, as described in chapter 3.2.5. Following the amplification of the Ly49C ECD insert, 2 µl of an aliquot of two of the five samples of the PCR reaction were analysed using agarose gel electrophoresis. The analysis of these two aliquots (Sample 1 and Sample 2) revealed that a single amplicon, slightly larger than 600 bp was obtained in both PCR reactions, whereas no amplicons were detected in the negative control (Figure 5).

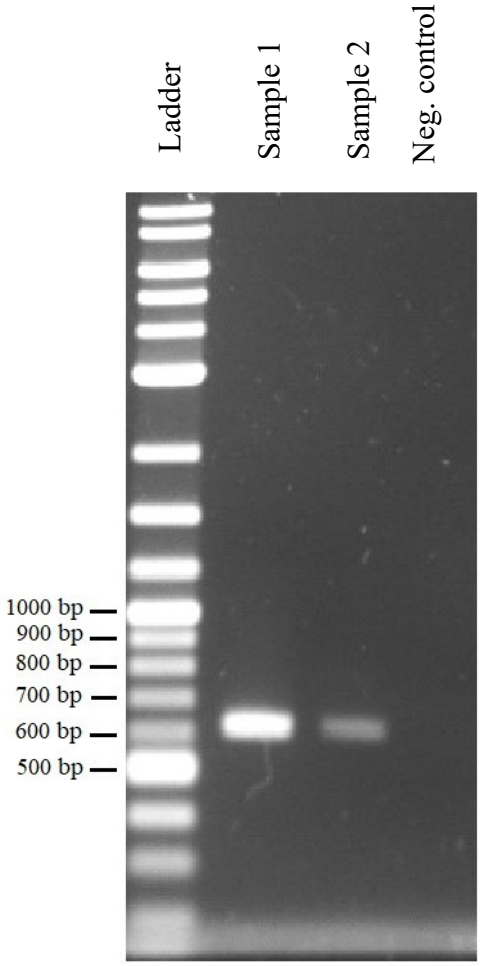

Figure 5: Verification of PCR amplification of dsDNA fragments encoding Ly49C ECD, bp = base pairs Labels: Ladder = 2 µl of NEB 1kb Plus Ladder, Sample 1 and 2 = 2 µl each of aliquot of Ly49C ECD insert from two of the five samples which underwent amplification, Neg. control: negative control containing sterile water and dye only

Since the expected size of the linear dsDNA fragment encoding Ly49C ECD is 623 bp (Figure 2), gel-electrophoresis results strongly suggest that the amplification and purification of the desired amplicon were successful.

4.3 Vector isolation and preparation of vector and insert for ligation

Vector pFUSEN-hG1FC was isolated from a small volume of overnight *E. coli* culture, as described in chapter 3.2.7. Following plasmid DNA isolation, the concentrations of plasmid (~668 ng/uL) and insert (~25 ng/uL) DNA prior to digestion with BamHI-HF were determined as described in chapter 3.2.10. Vector and insert were then digested with BamHI-HF (chapter 3.2.8), whereby the vector linearization was followed by alkaline phosphatase treatment. Following digestion and dephosphorylation, enzymes were removed from the reaction mixtures, as described in chapter 3.2.6. The concentrations of the BamHI-digested vector and insert preparation following the clean-up procedure were evaluated by gel electrophoresis and the obtained results are shown in Figure 6.

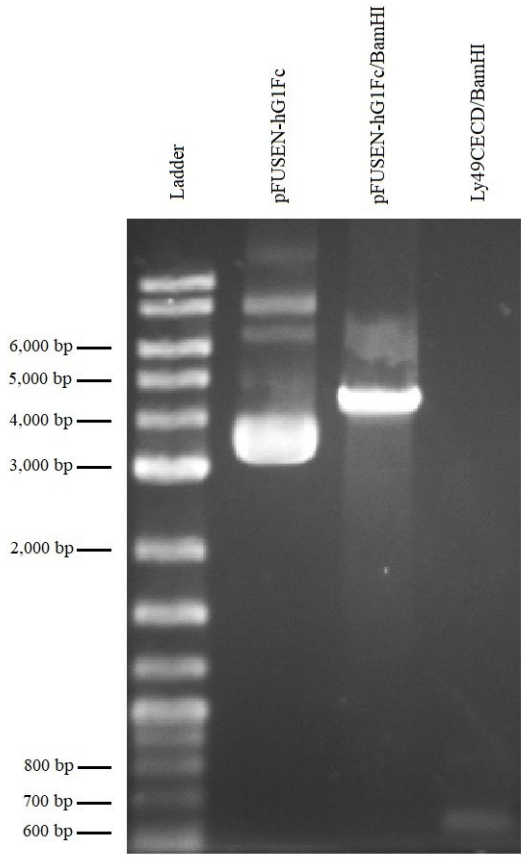

Figure 6: Quantification and control of digestion of pFUSEN-hG1FC and Ly49C ECD insert using gel electrophoresis. Labels from left to right: NEB 1kb Plus ladder, 1 µl of undigested isolated pFUSEN-hG1Fc plasmid, 2 µl of the digested and dephosphorylated isolated pFUSEN-hG1Fc plasmid after clean-up process and 2 µl of the digested DNA encoding for Ly49C ECD after clean-up.

The undigested isolated pFUSEN-hG1Fc plasmid showed an intense band between 3,000 and 4,000 bp, while the digested and dephosphorylated isolated pFUSEN-hG1Fc plasmid showed a band between 4,000 and 5,000 bp. This can be explained due to the smaller circular structure of the undigested pFUSEN-hG1Fc, which allows it to travel faster through the gel, thus indicating a successful plasmid digestion by BamHI. Furthermore, in lane 2 (undigested pFUSEN-hG1Fc) multiple other bands with longer bp sequences are visible. This is most likely due to the formation of secondary structures, as these bands are not visible anymore after digestion (lane 3, pFUSENFc/BamHI). As the used pFUSEN-hG1Fc plasmid has a length of 4,238 bp, the formation of the two intense bands around the 4,000 bp mark in lane 2 and 3 was anticipated. For the sample of digested DNA encoding for Ly49C ECD a single band around the 600 bp mark can be seen, corresponding to its expected length.

The concentration of DNA in each sample can be determined by comparing the intensity of the described band with the markings with a known mass left by the ladder.

For the concentration calculation, it was estimated that the fluorescence intensity of the band representing the BamHI-digested pFUSEN-hG1FC (lane 3, Figure 6) was five times higher than the fluorescence intensity of the 4,000 bp band of the ladder. Since the 4,000 bp band of the ladder has a mass of 16 ng, the mass of the digested pFUSEN-hG1FC was estimated to be 80 ng. Given that 2 µl of the digested pFUSEN-hG1FC was loaded into the well of the gel, the concentration of the digested vector was estimated to be approximately 40 ng/µl. Analogously, it was estimated that the band corresponding to the BamHI-digested Ly49C ECD insert (lane 4, Figure 6) had the same intensity as the 11.5 ng of the 600 bp band of the ladder. Since 2 µl of the BamHI-digested insert suspension were loaded into the gel, its concentration was estimated to be approximately 5.75 ng/µl. In addition to the visual estimation of DNA concentrations, the Ly49C ECD insert and pFUSEN-hG1Fc vector were quantified using spectrophotometry. Table 6 displays the combined results obtained using these two approaches.

Table 6: Determined concentrations of vector and insert (in ng/µl)

Method	**Ly49C ECD**	**pFUSEN-hG1Fc**
Visual estimation of band intensities	5.7	40.0
Spectrophotometry	5.5	28.9
Mean	**5.6**	**34.5**

Based on the mean values shown in Table 6, the appropriate amounts of the BamHI-digested vector and BamHI-digested insert were ligated in a reaction catalysed by T4 DNA ligase, as described in chapter 3.2.11.

4.4 Transformation and screening of transformants by colony PCR

Following ligation of the BamHI digested vector (pFUSEN-hG1Fc) and insert (linear dsDNA fragment encoding Ly49C ECD), an aliquot of the ligation mixture was used to transform competent *E. coli* cells, strain HST08, as described in chapter 3.2.13. The individual suspended *E. coli* cells were then spread on LB agar plates supplemented with Zeocin.

In order to screen for clones that contain a plasmid with a single copy of the insert ligated with the vector in the correct orientation, colony PCR and gel electrophoresis were performed. For this purpose, 14 Eppendorf tubes with liquid LB medium were inoculated with cells from separate colonies and incubated at 37 °C overnight before performing PCR using the pre-designed primers Ly49Check-F and Ly49Check-R. As described previously under 3.1.3, the primers used for colony PCR were designed in such a way that the 649-bp PCR amplification product can be obtained only if a single copy of the BamHI-BamHI Ly49C ECD fragment was ligated with the BamHI digested vector pFUSEN-hG1Fc in the correct orientation. Following colony PCR, an aliquot of each PCR reaction was analysed by agarose gel electrophoresis, and the obtained results are shown below in Figures 7 and 8

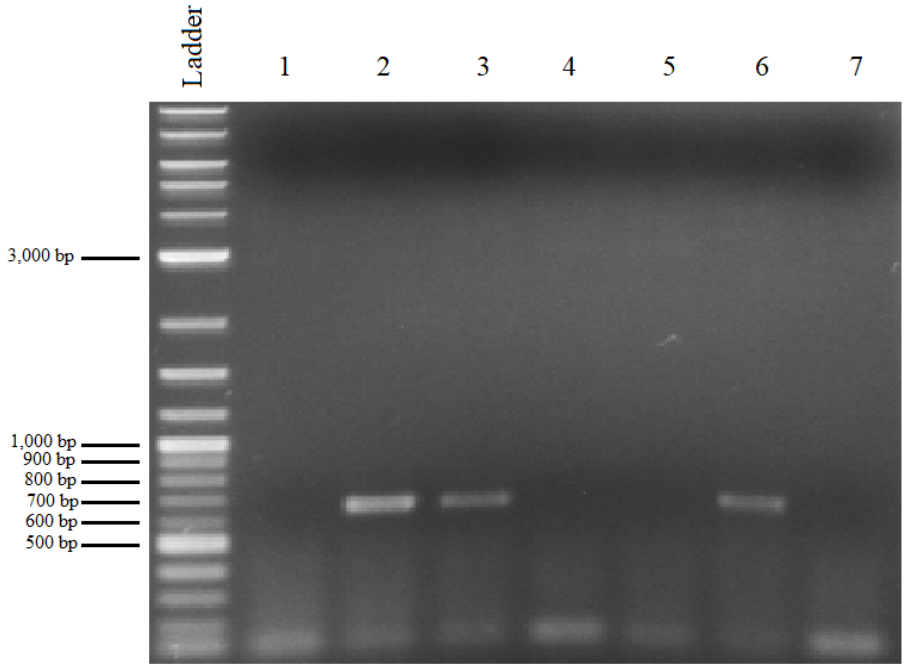

Figure 7: Gel electrophoresis of 1 kb Plus NEB ladder and 3 µl each of samples 1-7, obtained from separate colonies after PCR amplification using Ly49Check-F and Ly49Chek-R primer

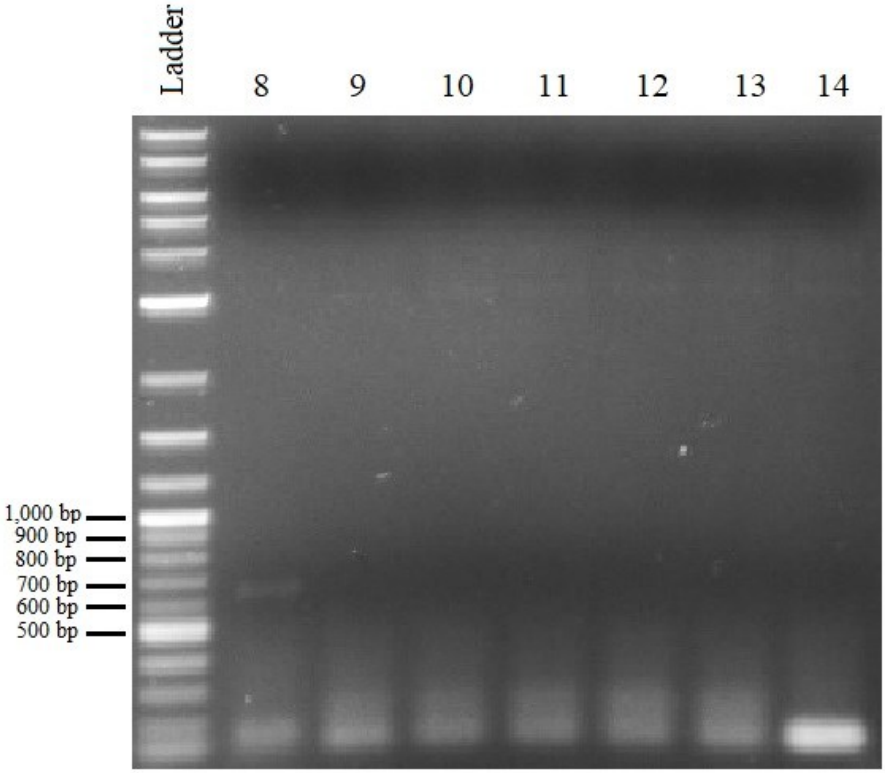

Figure 8: Gel electrophoresis of 1 kb Plus NEB ladder and 3 µl each of samples 8-14, obtained from separate colonies after PCR amplification using Ly49Check-F and Ly49Chek-R primer.

The results shown in Figures 7 and 8 demonstrate that a band between 600 bp and 700 bp was successfully amplified using colonies labelled with numbers 2, 3, 6, and 8 as a source of template DNA in colony PCR. In other words, these results indicate that 4 out of 14 analysed transformants contain a plasmid with a structure consistent with the desired final construct, plasmid pFUSEN-hG1Fc-Ly49ECD. To further confirm the presence of the desired final construct, plasmid DNA from these colonies was additionally analysed by restriction analysis, as described in the next chapter.

4.5 Restriction analysis of plasmid DNA isolated from positive clones

As described in the previous chapter (chapter 4.4), colony PCR indicated that 4/14 bacterial clones contained the desired recombinant plasmid pFUSEN-hG1Fc-Ly49ECD. In order to additionally confirm that these clones do contain a plasmid molecule with a Ly49C ECD insert in the correct orientation and to exclude the possibility that the 649-bp PCR fragment detected by colony PCR is not a consequence of contamination of PCR reaction mixture by other templates containing Ly49C ECD sequence (such as, for example, total mouse lymphocyte cDNA), additional restriction analysis of plasmid DNA isolated from positive clones 2, 3 and 6 was performed. Restriction analysis of plasmid DNA is based on the fact that the total size of the plasmid, as well as the orientation of the insert, can be estimated using the appropriate enzyme or combination of enzymes. Such an approach is illustrated in Figure 9, which shows the excepted structure of the desired recombinant plasmid pFUSEN-hG1Fc-Ly49ECD as well as a plasmid in which the BamHI-BamHI fragment ligated in the wrong orientation.

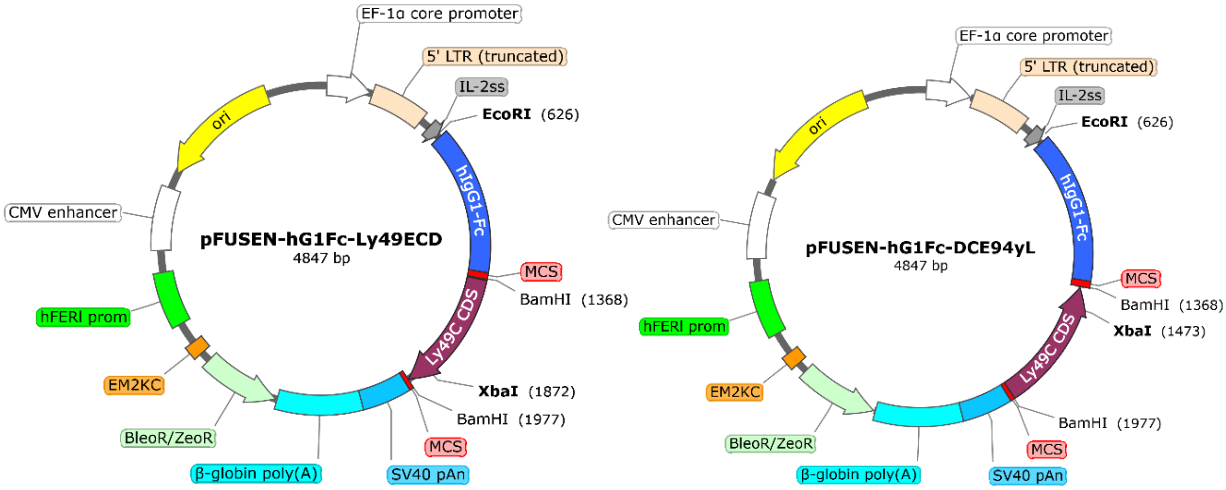

Figure 9: Maps of potential recombinant plasmid with Ly49C ECD insert in correct orientation on the left (pFUSEN-hG1Fc-Ly49ECD) and Ly49C ECD in incorrect orientation on the right (pFUSEN-hG1Fc-DCE94yL). The enzymes used for restriction analysis, EcoRI and XbaI, are indicated in bold.

As can be seen from Figure 9, ligation of the Ly49C ECD insert in the wrong orientation (right) changes the position of the XbaI restriction site, located in the insert, relative to the EcoRI restriction site, located within the vector. Consequently, fragment sizes obtained after digestion of plasmid DNA isolated from clones 2, 3, and 6 can not only indicate whether the total size of the plasmid corresponds to the expected size (4847 bp), but also which clones contain the desired construct that harbors the Ly49C ECD insert in the correct orientation (Fig 9. left). To perform the described analysis, plasmid DNA from clones 2, 3, and 6 was isolated and double-digested using restriction enzymes EcoRI and XbaI, as described in Materials and Methods (chapter 3.2.8). Following digestion, an aliquot of each restriction reaction mixture, together with a sample of undigested empty vector, was analysed by gel electrophoresis. Obtained results are shown in Figure 10.

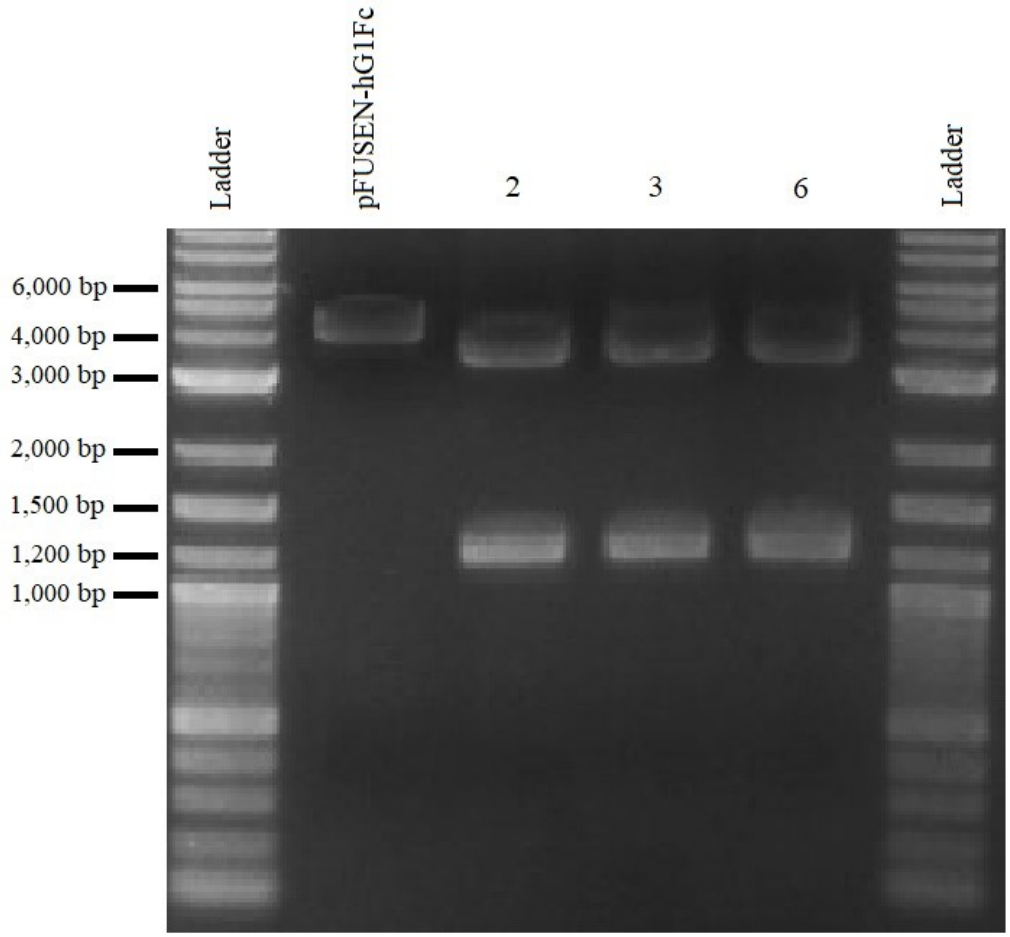

Figure 10: Restriction analysis of positive samples 2, 3, and 6. 2.5 µl of the isolated pFUSEN-hG1Fc plasmid was used as a negative control. After digestion using XbaI and EcoRI, 5 µl of samples 2, 3, and 6 were loaded into the corresponding wells.

As can be seen, for all three samples, a clear band around 4,000 bp and one around 1,200 bp was visible. These lengths correspond to the expected lengths left by EcoRI and XbaI in case of a successful ligation of the pFUSEN-hG1Fc plasmid and the Ly49C ECD insert in the correct orientation. In the case of a missing insert, a single band should only be visible around the 4,000 bp mark, which is approximately the length of the pFUSEN-hG1Fc plasmid without the insert, as can be seen in the case of the negative control. If the Ly49C ECD insert had been ligated in the wrong orientation, the two resulting bands should have different lengths, as depicted in Figure 10, of approximately 850 bp and 4,000 bp.

The results of colony PCR and restriction analysis of plasmid DNA from transformants 2 and 3 suggest that they contain a recombinant plasmid whose structure is consistent with the anticipated structure of the desired final recombinant plasmid, pFUSEN-hG1Fc-Ly49ECD.

5. Discussion

5.1 Expectations of results

In general, the interim and final results coincided with the expectations. An exception are the results of the RNA isolation described under 4.1, as the bands for the 28S and 18S subunit of sample No 1 were not at the anticipated lengths and the total quantity of isolated RNA was significantly lower than in sample No 2. Possible reasons for this could be the formation of secondary structures, causing the RNA to run faster and hence appear shorter than expected. Another possibility could be a partial degradation of the RNA, which could also lead to shortened bands.

Furthermore, the differences in the results of the quantification of the plasmid by spectrophotometry and gel electrophoresis were not expected. This is most likely due to the subjective comparison of band intensity. To determine the concentrations of DNA more precisely, fluorometry or qPCR could have been utilized. Since a moderate deviation from the real value does not affect the ligation process, this was not done.

5.2 Potential errors

Several steps were taken to minimise the risk for errors while creating the fusion protein, but it is impossible to eliminate the chance completely.

To avoid contamination of any of the used samples or materials with foreign genetic material or RNases, all work was performed in sterile environments and under laminar flow hoods while wearing gloves and lab coats. Nonetheless, it is still possible that contamination at some point occurred. This can happen by various means, like improper handling of samples, unsterile equipment, the use of already contaminated materials or incorrect storage of samples. Besides the mentioned countermeasures, the samples were inspected visually, and intermediate screening with negative controls as described were performed to identify a possible contamination as early as possible. The use of specific primers and selective growth medium with antibiotics was also done to decrease the risk of contamination.

The introduction of foreign genetic material could produce mixed populations or erroneous results, which would be invalid, as well as non-specific amplification during PCR which can lead to the misidentification of clones or strains, affecting downstream analysis and

interpretation. The contamination by RNases would cause the degradation of the samples which would hinder the ligation and transformation process.

Another possible source of errors are mutations either in the plasmid, insert or fused construct after transformation. For example, DNA polymerases used in PCR can cause point mutations. To reduce the risk for this, high-fidelity polymerases were chosen. Mutations can also occur during restriction digestion, ligation, transformation or in the host cell during replication. Depending on the kind of mutation and its position, it can change the function and activity of the fusion protein, its structure or expression.

To exclude any mutations, samples of the cloned DNA will be sequenced and compared to the in-silico model.

5.2 Possible applications of the fusion protein

As aforementioned, Ly49C plays an integral role in the inhibition of NK cell activity by binding to MHC I-self molecules (1) The created Ly49C-Fc fusion protein can be utilised to investigate the relevance of the Ly49C receptors for the immune response. For example, it can be used to directly assess Ly49C binding to its ligands and/or factors or proteins affecting this binding. Multiple conditions, like viruses, autoimmune disorders or cancer influence the surface MHC I expression and subsequently the binding of inhibitory receptors to their ligands. This is especially significant in regard to mouse cytomegalovirus (MCMV) that can manipulate binding of inhibitory Ly49 receptors to MHC I on infected cells, thus evading an immune response and eradication (10). It does so by encoding two proteins, m04 and MATp1, which allow the expression of small proportion of altered MHC-I complexes on the surface of infected cells, thus simultaneously providing ligands for inhibitory Ly49 receptors (29,30) which leads to NK cell inhibition, but also avoiding fast recognition of CD8+ T cells .Furthermore, with the help of the Ly49C-Fc fusion protein, monoclonal antibodies against the Ly49C receptor could be devised with the purpose to detect and quantify levels of Ly49C expression on the surface of cells of interest.,

Although the family of Ly49 receptors does not exist in humans, this strategy can provide a model to study KIR-MHC I interactions in steady-state conditions, during virus infection such as human cytomegalovirus (HCMV), as the virus uses similar methods to modulate immune responses and other pathologies, including tumours models. (31)

6. Conclusion

Ly49 receptors play an important role in the regulation of immune responses in mice. As similar receptors are important for the course of pathologies like CMV or cancer in humans, receptors like Ly49C are a subject of interest in research, as they can be used as models to investigate the impact of receptors like KIRs on the course of certain diseases in the human population.

For this reason, a Ly49C-Fc fusion protein was created. This was achieved by isolating the necessary sequences from a CL56/BL6 mouse and the plasmid from E. coli. All necessary steps for constructing the fusion protein were successful and validated. It has been established that three samples of *E. coli* contain plasmid DNA consistent with the structure of the desired final recombinant plasmid pFUSEN-hG1Fc-Ly49ECD.

7. Summary

Ly49C is an inhibitory receptor found of NK cells in mice, regulating their activity. To further investigate their impact on immune modulation and response to pathogens like MCMV, a fusion protein of its extracellular domain and the Fc region of a human IgG antibody was successfully constructed. This was carried out on the base of an in-silico model created in SnapGene. The fusion protein was constructed using a commercial plasmid for cloning called pFUSEN-hG1Fc, which was extracted from *E. coli* and contained the sequence coding for the Fc region.

The insert was isolated from the spleen of a C57BL/6 mouse. For this, the spleen was excised and its lymphocytes isolated, before the cells were lysed to extract the RNA. Using reverse transcription and oligo dT primers, cDNA was synthesised from the isolated RNA. With the help of specific primers and PCR, the desired sequence was amplified.

To facilitate ligation, both plasmid and insert were digested using BamHI-HF and their concentrations were determined using spectrophotometry and gel electrophoresis. After dephosphorylation of the plasmid, they were ligated and transformed to E. coli. To confirm the successful construction of the desired fusion protein, colony PCR and restriction analysis was performed. Out of the 14 analysed colonies, at least three of them contained the desired fusion protein. This protein will be used as a tool to further the research on inhibitory receptors on the immune response and diseases like CMV.

8. Literature Cited

1. Mir Munir A. Rahim, Andrew P. Makrigiannis. Ly49 receptors: evolution, genetic diversity, and impact on immunity. John Wiley & Sons Ltd Immunological Review. 267/2015.

2. Scarpellino L, Oeschger F, Guillaume P, Coudert JD, Lévy F, Leclercq G, et al. Interactions of Ly49 Family Receptors with MHC Class I Ligands in trans and cis1. J Immunol. 2007 Feb 1;178(3):1277–84.

3. Nazzareno Dimasi, Roberto Biassoni. Structural and functional aspects of the Ly49 natural killer cell receptors. 2005. 83, 1–8.

4. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. 2008. 5:495–502.

5. Kim S, Poursine-Laurent J, Truscott SM, Lybarger L, Song YJ, Yang L, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. Nature. 2005 Aug 1;436(7051):709–13.

6. Kärre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H–2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986 Feb 1;319(6055):675–8.

7. Brown MG, Scalzo AA, Stone LR, Clark PY, Du Y, Palanca B, et al. Natural killer gene complex (Nkc) allelic variability in inbred mice: evidence for Nkc haplotypes. Immunogenetics. 2001 Sep;53(7):584–91.

8. Vivier E, Raulet DH, Moretta A, Caligiuri MA, Zitvogel L, Lanier LL, et al. Innate or Adaptive Immunity? The Example of Natural Killer Cells. Science. 2011 Jan 7;331(6013):44–9.

9. Alan Rowe Schenkel, Luke C. Kingry, et al. The Ly49 Gene Family. A Brief Guide to the Nomenclature, Genetics, and Role in Intracellular Infection. 2013.

10. Jelena Zeleznjak, Branka Popovic, et al. Mouse cytomegalovirus encoded immunoevasins and evolution of Ly49 receptors – Sidekicks or enemies? 09/2017. 189:40–7.

11. Mir Munir A. Rahim, Megan M. Tu, et al. Ly49 receptors: innate and adaptive immune paradigms. 02/04/2014. 5–2014.

12. Carlos Vilches, Peter Parham. KIR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity. 2002. Vol. 20:217-251.

13. Czajkowsky DM, Hu J, Shao Z, Pleass RJ. Fc-fusion proteins: new developments and future perspectives. EMBO Mol Med. 2012 Oct;4(10):1015–28.

14. InvivoGen. pFUSEN-hG1Fc data sheet, Version 20K09-v35 [Internet]. Available from: https://www.invivogen.com/sites/default/files/invivogen/products/files/pfusen_hg1fc_tds.pdf

15. Yates AD, Achuthan P, Akanni W, Allen J, et al. Ensembl 2020. Available from: https://pubmed.ncbi.nlm.nih.gov/31691826/

16. ensembl.org entry for Ly39C coding sequence [Internet]. Available from: https://www.ensembl.org/Mus_musculus/Gene/Summary?db=core;g=ENSMUSG00000067591;r=6:130300252-130314537

17. UniProt database, sequences encoding for Ly49C domains [Internet]. Available from: https://www.uniprot.org/uniprotkb/Q64329/entry#structure

18. Dam et. al. Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by crystal structure of Ly49C bound to H-2Kb. Novemb 2003. 4:1220.

19. Agilent RNA 6000 Pico Kit Quick Start Guide [Internet]. Agilent Technologies; Available from: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90049_RNA6000Pico_QSG.pdf

20. ProtoScript® II First Strand cDNA Synthesis Kit Instruction Manual [Internet]. Available from: https://www.neb.com/en/-/media/nebus/files/manuals/manuale6560.pdf?rev=4075495cd88f496b93b7322bad92c506&hash=798F44D162C4AA153313DC5A40C1F2A3

21. NewEngland Biolabs. NewEngland Biolabs Tm Calculator [Internet]. Available from: https://tmcalculator.neb.com/#!/main

22. Macerey-Nagel Instructions for PCR clean -up Kit [Internet]. Available from: https://www.mn-net.com/media/pdf/02/1a/74/Instruction-NucleoSpin-Gel-and-PCR-Clean-up.pdf

23. Macherey-Nagel Plasmid Easy Pure Kit instructions [Internet]. Available from: https://www.mn-net.com/media/pdf/0c/20/aa/Instruction-NucleoSpin-Plasmid-Easypure.pdf

24. New England Biolabs. BamHI-HF restriction enzyme [Internet]. Available from: https://www.neb.com/en/products/r3136-bamhi-hf#Product%20Information

25. New England Biolabs. Optimizing Restriction Endonuclease Reactions [Internet]. Available from: https://www.neb.com/en/protocols/2012/12/07/optimizing-restriction-endonuclease-reactions

26. New England Biolabs. Ligation Protocol with T4 DNA Ligase [Internet]. Available from: https://www.neb.com/en/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202

27. New England Biolab Ligation calculator [Internet]. Available from: https://nebiocalculator.neb.com/#!/ligation

28. addgene. Bacterial transformation protocol [Internet]. Available from: https://www.addgene.org/protocols/bacterial-transformation/

29. Babić M, Pyzik M, Zafirova B, Mitrović M, Butorac V, Lanier LL, et al. Cytomegalovirus immunoevasin reveals the physiological role of “missing self” recognition

in natural killer cell dependent virus control in vivo. J Exp Med. 2010 Nov 15;207(12):2663–73.

30. Železnjak J, Lisnić VJ, Popović B, Lisnić B, Babić M, Halenius A, et al. The complex of MCMV proteins and MHC class I evades NK cell control and drives the evolution of virus-specific activating Ly49 receptors. J Exp Med. 2019 May 29;216(8):1809–27.

31. Barbara Seliger, Ulrike Koehl. Underlying mechanisms of evasion from NK cells as rationale for improvement of NK cell-based immunotherapies. 12/08/2022.

8. CV

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