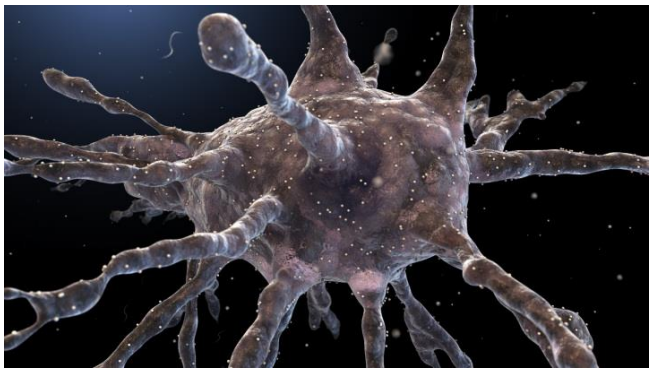

INDUCTION OF THE IMMUNE SYSTEM RESPONSE THROUGH THE ACTIVATION OF ANTIGEN PRESENTING CELLS MEDIATED BY CONJUGATED APTAMERS

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PRESENTATION PLACE

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1. Contact

I have been in The Life & Medical Sciences Institute (LIMES) at the University of Bonn. LIMES Institute is an internationally oriented centre for biomedical research and higher education. The main scientific focus of the institute is to explore the regulation of lipid metabolism and the immune system in health and disease, and decipher the signalling processes that take place both within and on biomembranes.

I worked at the Department of Chemical Biology & Chemical Genetics, in Mayer lab. In this department, the aptamer technology is studied to understand biological phenomena and develop diagnostic and therapeutic applications. The aim of this research group is to identify sophisticated molecules that exert a specific function and utilize them to study biological systems.

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2. Resume

During the last years, hundreds of treatments against pathological affections have been developed. One of the innovators fields in research is the use of our own organism to fight these diseases by the activation of the immune system.

In this study, the binding of conjugated aptamers with an antigenic peptide to dendritic cells (DCs) in order to induct their activation in a direct way was investigated. In consequence, T cells, and other type of effector cells from the immune system, would also be activated because of the binding with the activated DCs. The aim of this study was to validate the

aptamer D#7, which had been already tested in other studies (Silvana Haβel, 2016). This means that, DCs must be evaluated with and without the aptamer (conjugated and non-conjugated) in order to know if DCs were being properly activated and to discard that non-specific bindings were not occurring.

It also wanted to be determined if aptamers FN#9, FN#12, FN#14, FN#15 and FN#20, which are derived from the DC protein Fc-FN, could be used as specific binding molecules for the development of immunotherapies against several diseases, for instance, against cancer. These aptamers were tested in two cell lines: THP1 and J774A1 cells, which are human monocytes and mice macrophages, respectively.

The aptamers had a fluorophore attached, that way the fluorescence emitted by the fluorophore could be measured by flow cytometry and determine if the aptamers were bound to the cell surface or not.

In the D#7 validation, 5 different cell receptors (CD40, CD86, MHC-I, MHC-II and CD80) were analysed in order to determine if they were upregulated or downregulated when DCs were activated by LPS (lipopolysaccharides) or MHC-I peptide (Major Histocompatibility Complex) by using conjugated antibodies with a fluorophore.

DC-binding aptamers were selected by two different SELEX strategies, protein-SELEX approach and cell-SELEX approach. Protein-SELEX approach was used to identify aptamer CTL#5 by addressing recombinant proteins originated from the murine mannose receptor (MR), which is an endocytic receptor involved in the recognition, uptaking and processing of the antigens by DCs. The control used in this experiment is a derivative of CTL#5. Protein-SELEX approach was also used to identify aptamers FN#9, FN#12, FN#14, FN#15 and FN#20.

Cell-SELEX process was used to identify aptamer D#7, which was selected by using murine bone marrow-derived DC as complex targets.

The validation of the D#7 aptamer was not completed because the binding of the aptamer was not reproducible and there was only one cell receptor that was upregulated (CD40) when the DCs were activated by LPS or MHC-I peptide. Consequently, the experiment could not be continued because of the incoherence of the obtained results.

Concerning to the testing of the aptamers FN#9, FN#12, FN#14, FN#15 and FN#20, it was observed that aptamers FN#9 and FN#12 bound to the THP1 and J774A1 cells in the condition of the cells treated with Binding Buffer and cells treated with cell medium. FN#20 also bound to J774A1 cells. Although, in the case of cell medium, the fluorescence of the aptamer was more intense, so, probably, more quantity of aptamer bound. The best result was the binding of the aptamer F#12 in cell medium conditions.

3. Key words

- [Dendritic cells] AND [Aptamers]
- [Lymphocytes T]
- [Antigen presentation]
- [Immune system response]
- [Immunotherapy]
- [Tumour antigens]
- [SELEX]
- [lipopolysaccharides (LPS)]
- [Major histocompatibility complex (MHC)]

4. Introduction

Immunotherapy is a set of therapeutic strategies that consist in stimulate or reconstitute immunological functions in order to fight pathological affections, whether infectious, physiological (immunosuppression) or molecular (cancer). The development of the smallpox vaccine by Edward Jenner in 1796, can be considered as the first immunotherapeutic strategy. It was developed from exudates produced by infection with the vaccine virus, although the practice of deliberately inoculating healthy individuals with smallpox as prevention, was already carried out in China in the 10th century [1]. Since then, and especially in the last decade with the advances in Molecular Biology, immunotherapeutic strategies have emerged as one of the most promising options in the fight against various human pathologies, as shown by recent successes in the treatment of different cancers [2].

4.1 Active and passive immunotherapy

There are two types of immunotherapies, active immunotherapy and passive immunotherapy. Active immunotherapy is known as the stimulation of the immune system itself to cope with pathological involvement. Several strategies can be found: through the administration of vaccines containing cells extracted from the patient himself and modified *ex vivo*, such as DCs to which a molecule of interest has been attached and which are then implanted again in the patient. Or by administering recombinant DNA, vectors carrying a gene of interest, adjuvants, etc. [2],[3]

In contrast, passive immunotherapy involves principally the use of monoclonal antibodies that will target the marker of interest i.e., cancer antigens or antigens from pathogens, enhancing lytic mechanisms of the immune system such are phagocytosis, antibody mediated killing or activation of humoral components, among others [2],[3].

This study focuses on active immunotherapy. Long-term immunity requires activation of the immune system's effector cells, particularly T cells, by means of antigen-presenting cells (APCs). APCs are distributed throughout the body in order to capture antigens (Ag) and migrate to the lymph nodes in aim to activate T cells which in turn are responsible in developing effector responses and immune memory.

4.2 Antigen Presenting Cells (APCs)

APCs are cells that internalize antigens by endocytosis, process them and present them on their surface, bound to the MHC, to the T cells. In addition, they have co-stimulatory signals that complete the activation of T cells which will be explained later.

There are different types of APCs [4]:

- Dendritic cells. They are cells with cytoplasmic prolongations found in the lymphatic organs, the skin epithelium and the mucosa of the digestive and respiratory system. They are responsible for activating immature T-lymphocytes and promoting their clonal expansion as well as the differentiation of effector cells.
- Macrophages. They are phagocytic cells that usually contain antigens from bacteria (such as the LPS of the bacterial wall) and parasites. They produce co-stimulators for T-lymphocyte activation.

- B lymphocytes. It recognizes the antigens through the membrane receptor (BCR), internalises it, processes it and presents it to the T helper lymphocytes through MHC-II. Each BCR is specific to a single antigen.
- Endothelial cells. These are the cells that line the inside of blood vessels. They present antigens to T lymphocytes circulating in the blood or attached to the vascular endothelium through MHC-II. In this way, T lymphocytes are recruited at the site of infection.
- Epithelial cells of the thymus. They also present antigens for MHC-II to immature T lymphocytes.

4.3 Interaction between the T cell and the DC

Each T cell has a T cell receptor (TCR) that recognizes a specific antigenic structure attached to the MHC that is present in the activated APCs on its surface. MHCs are glycoproteins with highly variable peptide binding properties.

The binding of MHC to CD4+ or CD8+ lymphocyte TCR depends on the type of MHC. MHC-I presents intracellular antigenic peptides to CD8+ lymphocytes. While MHC-II presents extracellular antigenic peptides to CD4+ lymphocytes.

TCR is expressed on the cell surface in association with a signal transduction complex. In the plasma membrane the TCR is attached to a CD4+ or CD8+ co-receptor. According to the co-receptor, the activated T-cell may become a cytotoxic T-cell (Tc), causing apoptosis of the target cells. Or, become a T helper (Th) or T regulator (Treg). The Th can be Th1 or Th2, both trigger an immune response. Th1 triggers a cellular-type immune response through the action of phagocytic cells and granulocytes. On the other hand, Th2 triggers a humoral immune response by differentiating B-lymphocytes, which secrete the antibodies of interest and then activate the migration of the immune system's effector cells that best match the required immune response.

Two signals are needed for activation of the T-lymphocyte. The first one is the interaction described above between the TCR and the MHC. The first activation signal received by the T cell causes all receivers to become polarized (oriented) towards the area where it has occurred. When the T lymphocyte is activated, it secretes cytokines, which exert the effector mechanism: on the one hand to stimulate B lymphocytes to synthesize antibodies and on the

other hand, different cell types to carry out different processes (mainly macrophages and granulocytes), all depending on whether the response is humoral or cellular.

The second signal is the binding of CD28 co-stimulatory molecules with CD80 (B7-1) or CD86 (B7-2) which triggers a signalling cascade that triggers the activation, differentiation and survival of T cells. The CD80 and CD86 ligands are two ligands that can do the same function, which consist in the activation of the T cell by binding to CD28 receptor or inhibit the activation of the lymphocyte by binding to CTLA-4 (CD152) receptor. Other co-stimulatory molecules are CD2 or SLAM. The activated T cell expresses the CD40L receptor (binding of the CD40, which is found in APCs, such as DCs and B-lymphocytes), thus activating B cells in a T-dependent way[5].

Finally, the cytokine secretion will lead to one type of immune response or another, depending on the type of cytokines that are secreted. For example, if the APCs secrete inflammatory cytokines such as interleukin-12 (IL-12) or interferon gamma (INF-γ) and the T cell picks them up with the proper receptor, the activation and differentiation of the T cells will be cellular (Th1). However, if IL-12 is not secreted but another cytokine type, such as IL-4, IL-5, or IL-13, a humoral response (Th2) occurs. **(Figure 1)**

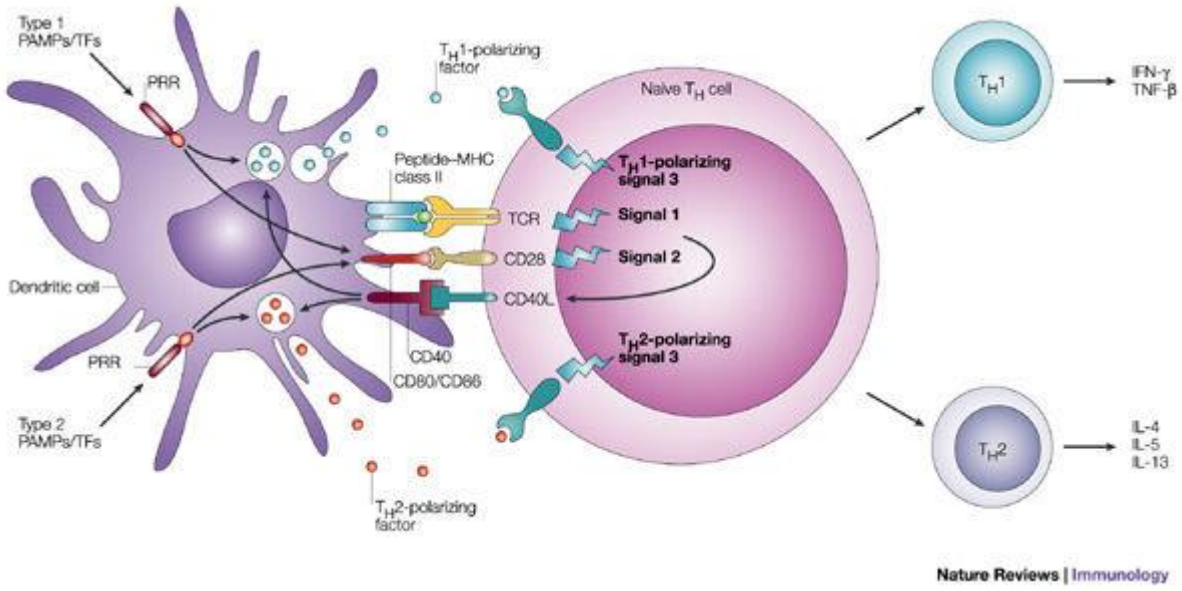


Figure 1. *Diagram of the interaction between DC and T cell.* The two main signals necessary for the activation of the T cells are observed. The first signal (TCR-MHC) and the second signal (CD28 / CTLA4-CD80 / CD86). Co-stimulators (CD40-CD40L) and cytokine secretion are also observed, leading to the maturation of the T lymphocytes to Th1 or Th2.

For a T cell to be effectively activated in the long term, a prolonged signalling of the two signals explained is necessary. If this is not possible, the activation of the T cells is incomplete and the T cell becomes tolerant and useless for the activation of the immune system.

4.4 Aptamers

Aptamers are single stranded nucleic acids with a specific three-dimensional structure, such as loops or hairpins, that can form complexes with the target protein, allowing a specific interaction (they can be DNA or RNA). These interactions are hydrogen bridge, van der Waals forces, pi interactions of the aromatic or electrostatic rings [6].

The first aptamers were isolated in 1990 by Larry Gold and Craig Tuerk consisting on RNA ligands that recognized the DNA polymerase of the bacteriophage T4. Parallel to and independent of Gold and Tuerk, Andy Ellington and Jack Szostak, through what they called *in vitro* selection, selected RNA-based ligands against various organic dyes.

Szostak's laboratory coined the term aptamer (from Latin *aptus*, which means to fit) for the oligonucleotides that selectively interacted with the dyes.

Since the discovery of the aptamers, different research groups throughout the world have researched the Aptamers. In 2001, the SELEX (Systematic evolution of ligands by exponential enrichment) process was automated in the laboratory led by Andy Ellington at the University of Texas, Austin, and SomaLogic, Inc (Bouder, CO), reducing the duration of the selection rounds from six weeks to just 3 days [7].

Aptamers used in the present work have been produced following the SELEX strategy, which will be explained later. Also, they are not as immunogenic as a monoclonal antibody might be. Aptamers are therefore a new and revolutionary tool for the development of alternative immunotherapies [8].

4.4.1 Mechanism of action of the aptamers

Antigenic peptide-conjugated aptamers bind to APCs, particularly to DCs, as they are the most potent APCs for activating T-cells. DCs internalize the conjugated aptamers by endocytosis. This way they are activated and migrate to the lymph nodes where they interact with an immature T cell and triggers the maturation and activation of the T lymphocyte. Therefore,

there is a fast and efficient induction of the immune response. The binding of DCs to T cell represents the passage from the innate immune response to the adaptive immune response.

Previous studies show that when DC has only one Aptamer attached (not conjugated with the Ag) it is not activated. Therefore, it would not generate any immunogenic response (Silvana Haßel, 2016).

When the antigen conjugated with the aptamer binds to the dendritic cells, two situations can occur:

- The dendritic cell develops tolerance for the antigen bound to the aptamer, becoming a tolerogenic DC that will convert the immature T cell into a T reg.
- The dendritic cell is activated and presents the antigen to the T lymphocyte, which is now activated.

Therefore, there are two possible immunotherapeutic directions to investigate, depending on whether the immune system is activated or suppressed. The first case could be used for allergy care, suppressing the immune system. The second case, for the treatment of cancer, as the immune system could be targeted to the tumour by presenting tumour antigens to the T cells. This study focuses on the activation of the immune system with the use of conjugated aptamers, therefore, for the development of immunotherapies.

The small size of the aptamers is a problem because they are easily eliminated in a renal way (Günter Mayer, *et al*., 2013). However, modifications can be made, such as adding molecules with higher molecular weight to increase their stability (Da Pieve *et al*., 2012).

5. Hypothesis

The DCs' receptors CD40, CD80 CD86 MHC-I and MHC-II are upregulated when they are activated. So, these receptors will be upregulated when DCs are activated by LPS or MHC-I peptide, showing this way, the correct activation of the DC.

Regarding to the searching of aptamers as new candidates for immunotherapies, they are derivatives from a recombinant protein (Fc-FM) that is found in DCs. Moreover, in previous studies (Silvana Haβel, 2016) aptamer D#7 bound to DCs, macrophages and monocytes, successfully. Thus, as all APCs have similar properties, the receptor in which D#7 bound might be the same in all APCs. So, there might be some FN# aptamers that bind to the selected APCs.

6. Objectives

The aim of this work is to confirm the conjugated aptamer D#7 with an antigenic peptide as a possible immunotherapeutic tool through the binding of monoclonal antibodies which were analysed by flow cytometry. The fluorescence of the fluorophores bound to different antibodies determined if the receptors were upregulated or downregulated and, in consequence, if the DC was activated by the conjugated aptamer.

If fluorescence was observed, it would mean that the antibody did not bind to its target. If the fluorescence was intense, there would be more receptors in activated DCs than in non-activated DCs, so the receptors would be upregulated when the conjugated aptamer was attached to the DC, meaning that the conjugated aptamer would have worked correctly, activating the DC. If the fluorescence was low, there would be less receptors in activated DCs than in non-activated DCs, so the receptors would be downregulated when the conjugated aptamer was supposed to be attached to the DC, which basically would mean that the conjugated aptamer was not really bounding to the DC or that it did not activate the DC, so it was not working.

On the other hand, there is another objective in this work which consists in searching for other aptamers as possible candidates for immunotherapies. The cell lines used in this experiment are THP1 and J774A1 cells, which are human monocytes and murine macrophages, respectively, and the aptamers are FN#9, FN#12, FN#14, FN#15 and FN#20. These aptamers were selected with a protein SELEX process. The FN# aptamers were derived from the recombinant Fc-FN protein which is present in DCs and its was provided by Prof. Sven Burgdorf from the LIMES Institute, University of Bonn, like the recombinant Fc-CTL protein from which the control sequence was also selected by protein SELEX approach. The rest of the details will be carefully explained later in the work.

The number of therapeutic applications is immense. Although, one of the most demanding fields is cancer treatment. Thus, the principal application of this work, besides immunotherapies, in general, would be to find aptamers that bound to DC and use them to fight the tumours by activating DCs with conjugated aptamers with tumour non- immunogenic antigens, like tumour exosomes from the non-immunogenic mesothelioma tumour, which are a source of tumour associated antigens (TAA) (Niken M. Mahaweni *et al.,* 2013).

7. Methodology

In this work the sources of information that has been used are mainly, NCBI and internet websites. The research period has been from 01/04/2018 until 11/06/2018. The selection criteria consisted in search for articles, mostly, with no more than years of antiquity and that showed studies related with the required topics. The key words for the selection of these articles were: [Dendritic cells] AND [Aptamers], [Lymphocytes T], [Antigen presentation], [Immune system response], [Immunotherapy], [Tumour antigens], [SELEX], [LPS] and [Major histocompatibility complex (MHC)].

7.1 Identification of Bone Marrow Dendritic Cells binding aptamers

Murine bone marrow dendritic cell-specific aptamers were made from oligonucleotides, in concrete D#7 and the control aptamer sequence CTL#5, have been identified by our research group using purified cell surface proteins in a protein-SELEX approach or using live cells in a cell-SELEX process. In protein-SELEX, specific membrane proteins can be chosen as they can facilitate presentation on MHC-I or MHC-II molecules. In cell-SELEX, membrane proteins maintain their native conformation, so epitopes are fully available (Shamah *et al*., 2008).

In order to future applicability on biomedicine, the aptamers must show binding specificity to the target, ability to be internalized by DCs and non-immunogenic. To test if the aptamers accomplish these requirements, techniques including flow cytometry binding assay (for specificity assay) and confocal microscopy analysis for BMDCs (Bone Marrow Dendritic Cells) uptake and cell localization, were used.

7.2 SELEX strategy

SELEX process is used for the selection of cell-specific aptamers. This strategy does not always work because the oligonucleotides that will bind to the target are unknown and it is also possible that none of them accomplish to bind correctly to the target. However, it is the strategy currently used.

The first step in SELEX strategy is the incubation of the target of interest (in our case the DCs) with a naïve oligonucleotides library which is composed of a random region embedded between fixed primer binding sites. In other words, the person who is doing the experiment design a primer according to the type of aptamer that must be used (RNA or DNA, in our case they are single-stranded DNA oligonucleotides) and to the type of cells (DCs) so all the

parameters can be optimised (melting temperature, %CG, etc.). Then, the primer is sent to a company and they put a random region of oligonucleotides in between the two primers. Therefore, the naïve oligonucleotide library is bought and the first step can be performed.

Next, the background and/or the sequences that have not bound to the target are removed and those that have bound are eluded of the target. This is achieved by fixing the target in a matrix or the unbound nucleic acids are removed by centrifugation, electrophoresis or flow cytometry (Nasim Shahidi Hamedani *et al*., 2015 and Marie-Sophie L. Raddatz *et al*., 2008). The eluted sequences are amplified by polymerase chain reaction (PCR) and single-stranded nucleic acids are generated by separating the double stranded nucleic acids by biotin streptavidin interaction (Günter Mayer *et al*., 2001 and Avci-Adali *et al*., 2010). Finally, the resulting nucleic acid sequence library is used in the next selection cycle. **(Figure 2)**

Therefore, this strategy will be repeated several times, adjusting the conditions and making them more specific in order to find the most specific aptamer for the target.

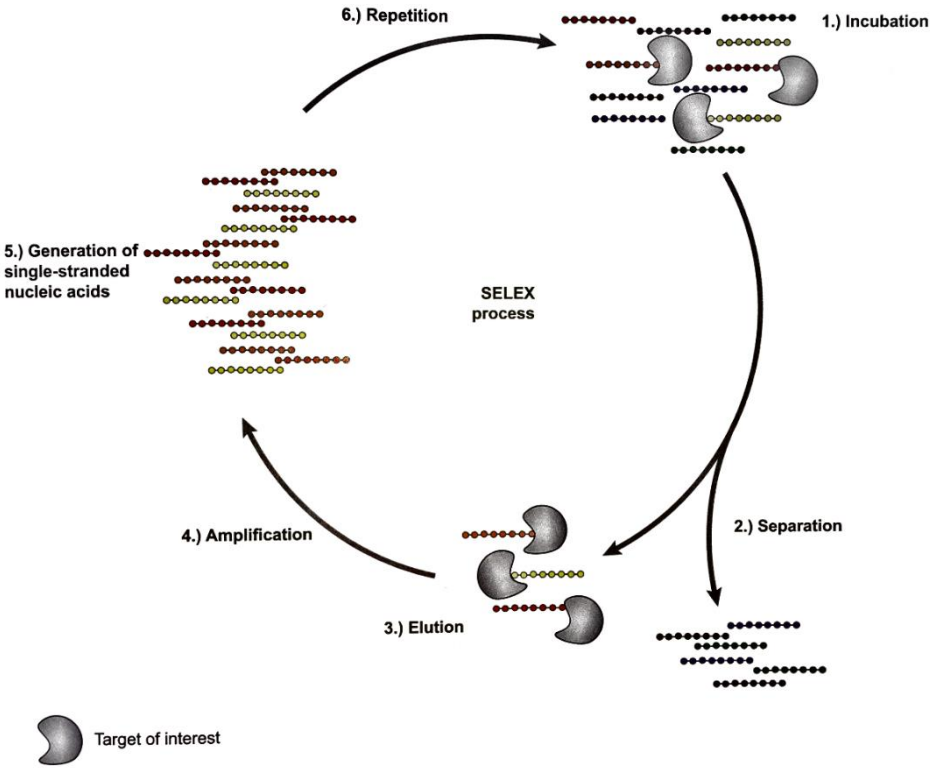


Figure 2. *Schematic representation of the SELEX process*. SELEX process is used to identify high affinity aptamers. The different steps are: 1) Incubate the target of interest with the naïve oligonucleotide library. 2) Sequences that have not been attached to the target are separated. 3) Elute oligonucleotides that have remained attached to the target. 4) Amplify them by PCR. 5) Generation of single chain oligonucleotides. 6) Use them in the next cycle. (Silvana Haβel, 2016).

7.3 Oligonucleotides

The 5'-ATTO 647N labelled oligonucleotides and control sequences (CTL), were purchased from Ella Biotech GmbH (Martinsried). The DNA was supplied HPLC-purified and lyophilized (Silvana Haβel, 2016).

Down below, the sequences of the aptamers and the CTL, from which the control used is derived, are written.

Name	*Sequence 5'-3'*
D#7	GCTGTGTGACTCCTGCAACGTGGGTGGGTTTATATTCGGTGGTGGTGGGGGTGGTACT GTTGCAGCTGTATCTTGTCTCC
CTL#5	GCTGTGTGACTCCTGCAATGCAATCTAGCTGACAATGGGGGGGAAGAATGTGGGTGGG GGCAGCTGTATCTTGTCTCC
F#9	CGGGTTTGCTCTTGGTTAGTGCTTGTGGTGGTGTGCGACTTGG
FN#12	GGGGATTCTGTTTTTTTTTTGTAACTCGGGGTTGGGTATCGTTG
FN#14	CCTGTTCTGTGTTTATGTATTGTTGTTATAGTTGTGTTTCCTG
FN#15	CGTGGGCTGGGATTTATTGGGGTTTGTGCTTGTTTGTTAGGCT
FN#20	CGGTGGCCGTGGTTTCTTCGTGTGGTTGTGTTTTTCGTCCTTG

7.4 Antibodies FACS test

As it has been explained previously, conjugated aptamers with an antigenic peptide bind to DCs and activate them. Then, DCs will activate T cells and the immune response starts. Thus, aptamers are potential tools for developing new immunotherapies.

The receptors CD40, CD86, MHC-I, MHC-II and CD80, which are the most involved receptors and co-receptors in the activation of the T cell, were supposed to be tested to observe if they were upregulated or downregulated when the DC was activated by the aptamer conjugated with an antigenic peptide. If the receptors were upregulated, it would mean that the DC was properly activated. So, basically, the aim of this experiment was to determine if the DCs were adequately activated when the conjugated aptamer with the antigenic peptide was bound to them.

However, before performing the experiment with the conjugated aptamer with an antigenic peptide, the conditions that were going to be used for the murine BMDC needed to be tested to know if they were optimum for all antibodies that were going to be analysed (anti-CD40,

anti-CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies). Only after checking these conditions, the experiment with the conjugated aptamer could be carried out. The antibody experiments had four different samples (cells treated with LPS + antibody, MHC-I peptide + antibody, untreated + antibody and untreated), which are explained extensively in the following point of the work *(7.4.1 Sample description)*. In **figure 3,** anti-CD40, anti-MHC-I and anti-CD80 antibodies have n=6 per each sample. Only the untreated group of anti-CD-40 antibody has n=4. Anti-CD86 and anti-MHC-II antibodies have n=4 per each sample.

The continuation of the experiment would be to treat the DCs with the D#7 conjugated with an antigenic peptide and after that, treat the cells with all the antibodies used in the first experiment (anti-CD40, anti-CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies) in order to determine if the DC were activated when they were exposed to the conjugated aptamer. If the receptors were upregulated would mean that the DCs are indeed activated.

The aptamer that has been selected for performing this experiment is D#7. During the analysis of the different samples, that are explained down below, it was needed to evaluate if D#7 was binding to the DC. As it was shown before, D#7 was supposed to bind to the DC (this was already tested using confocal microscopy and the internalization of the aptamer was observed as the aptamer fluorescence was present as punctual structures in the cytoplasm of BM-DCs) (Silvana Haβel, 2016). Thus, if D#7 did not bind to DCs, it could mean that there had been a mistake during the proceeding, that the protocol needs to be improved or that the DCs were defective or not optimum.

7.4.1 Sample description

Murine BMDC, which are primary cells, were extracted from mice in their 6th day of differentiation into DCs, and frozen until they were needed.

Frozen cells need to be in their 8^{th} day of differentiation into DCs to be cultivated, so they needed to be sown 48 hours before the antibody test and incubated at 37º in DC culture. In order to do that, *11.2. Annex 2: BMDC Culture, part 1* was followed.

Two days after the seeding of the BMDC, *11.2. Annex 2: BMDC Culture, part 2* was performed. The samples that were analysed in this protocol are the following ones:

1. Cells treated with the aptamer (D#7): the aptamer is a DNA sequence (80 nucleotides length) that binds to DC. If it bound to the DC, A647N fluorophore would emit a fluorescence that would be measured in FACS (with the APC channel).
   The receptor in which the aptamer is bound is unknown, but TLR can be discarded because if it was this receptor, the DC would be activated with the unconjugated aptamer, and this did not happen, so it is not possible.

To discard that the aptamer bound to a non-specific receptor there was the control sequence.

2. Cells treated with the control sequence (CTRL): The CTRL is a DNA sequence, similar to the aptamer sequence. They share the primers and the %CG (but not in the same order). This DNA sequence did not bind to DCs but it emitted a background fluorescence (A647N fluorophore). This fluorescence was also used to compare the fluorescence emitted by the aptamer sequence.

To activate the DC and see if the receptors were upregulated or downregulated, cells were exposed to LPS and MHC-I peptide and then were labelled with the correspondent antibody.

3. Cells treated with LPS + antibody: LPS are found in the outer membrane of Gram-negative bacteria, they are very immunogenic and they generate an important immune response in human beings. LPS was uptaken by TLR4 (Toll Like Receptors 4) of the DCs. This was how the DC was activated. LPS were used to know if the BMDC from mice were being activated, meaning that they were the positive control. They were labelled with an antibody because the receptors CD40, CD86, MHC-I, MHC-II and CD80 of the DC should be more expressed on the surface of the cell when it is activated. So, with the antibody, which had a fluorophore, it would be shown if the receptor was upregulated or downregulated. Moreover, it was used to compare the results of flow cytometry of the antibodies with other samples.
4. Cells treated with MHC-I peptide + antibody: Class I MHC molecules bind peptides generated mainly from degradation of cytosolic proteins by the proteasome. The epitope peptide was bound on extracellular parts of the class I MHC molecule and it activated the cell without the need of being internalized.

The purpose of using this peptide was to check if the antibody was binding to its receptor, which would be over expressed when MHC-I peptide was added. It was another positive control.

5. Untreated Cells + antibody: All the receptors are always expressed in a basal level and they should be overexpressed when the DC is activated (when it has incorporated an antigenic peptide and it is ready to present it to the T cell). This sample was used to see the basal level of the expression of the receptors in non-activated DCs. This was the negative control.
6. Untreated Cells: they did not emit any fluorescence as they did not have any antibody bound to them. They were used to make sure that the antibody bound to its receptor, that way its fluorescence could be compared with the UN Ab+. This was another negative control.

7.5 Aptamers as potential candidates for immunotherapies

In parallel, another experiment was performed, which consisted in searching aptamers that could be potential candidates for the development of new immunotherapies.

The aptamers that were tested were FN#9, FN#12, FN#14, FN#15 and FN#20 and the cells lines were THP1 (human monocytes) and J774A1 (murine macrophages). The control used in these experiments was the same as in the antibodies FACS test (CTRL derived from CTL#5).

Aptamers FN#9, FN#12, FN#14, FN#15 and FN#20 were derived from the recombinant protein Fc-FM, which is present in DCs. Aptamer D#7, as it has been shown, bound to DCs, but it also bound to other types of APC like macrophages and monocytes (Silvana Haβel, 2016). Probably because all the APC have a similar composition, so the receptor in which the D#7 bound might be present in these three different APCs. FN# aptamers, as they derivate from a DC protein, might also bound to macrophages and monocytes and this is the reason why these particular aptamers were tested.

Each aptamer was evaluated four times in two different conditions, twice per condition. One condition consisted in using Cell medium (RPMI + 10% FCS in the case of THP1 cells and DMEM + 10% FCS in the case of J774A1 cells) during the experiment and the other condition consisted in using Binding Buffer. The protocol details and the composition of the buffers can be found in the paragraph *11.4 Annex 4: THP1 and J774A1 cell culture* protocol. In the individual

graphics **(figure 4)** n=2 per each sample. Only in the "THP1 FN#9 and FN#12_1" graphic, the samples "Untreated Binding Buffer", "Untreated Cell Medium", "CTRL Binding Buffer" and "CTRL Cell Medium" have n=4.

In **figure 5,** the samples "Untreated" and "CTRL" of the MFI mean of THP1 cells have n=14 and FN# samples have n=4. The samples "Untreated" and "CTRL" of the MFI mean of J774A1 cells have n=12 and FN# samples have n=4.

The Binding Buffer was used as one of the conditions in this experiment because it was used in the cell-SELEX selection of the D#7 in previous studies (Silvana Haβel, 2016). Thus, there were probabilities that murine macrophages and human monocytes would be comfortable in this solution, meaning that their cell behaviour would be normal and the aptamers might be able to bind to them. On the other hand, cell medium was used because is the most similar solution to the one that THP1 and J774A1 cells would have in their natural environment. Henceforth, the experiments performed in cell medium are expected to have a major ratio of binding between the aptamers and the THP1 and J774A1 cells.

7.6 Statistical analysis

To describe the results of both types of experiments, the mean and median was used as a measure of centralization and the standard deviation as a measure of variability.

To establish the significant differences in the antibodies subjected to the different conditions, the nonparametric Mann Whitney test was used.

Two-factor analysis of variance (Two-way ANOVA) was applied to establish the differences between different treatments and different conditions, using the Tukey Test for post hoc testing. When the variable did not follow a Gaussian distribution, the nonparametric tests Kruskal-Wallis and Mann Whitney were utilized.

In all cases, a significant difference was considered when the significance was less than 5% ($p<0,05$). The SPSS V24.0 software was used for its implementation.

7.7 Flow cytometry

FACS sorting core facilities of the Department of Chemical Biology & Medicinal Chemistry at the University of Bonn was used for the determination of the binding of D#7 and FN# aptamers to the DCs. Flow cytometry was also used to determine the binding of the anti-CD40, anti-

CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies to their respective cell receptors in DCs. Aptamers and antibodies were labelled with their respective fluorophores, which are explained later in the work, untreated cells and CTRL aptamer sequence were used as control. For each sample 10.000 cells were analysed in the flow cytometer. Mean Fluorescence Intensity (MFI) of different samples was measured by flow cytometry, using the corresponding channel of measurement, depending on the fluorophore:

APC channel

- Anti-CD86 primary antibody (fluorophore Alexa-Fluor 647).
- Anti-MHC-I secondary antibody (fluorophore Alexa-Fluor 647).
- Anti-MHC-II primary antibody (fluorophore Alexa-Fluor 647).
- Aptamer D#7 and control sequence (fluorophore Atto647N).
- FN# aptamers (fluorophore Atto647N).

PE channel

- Anti-CD40 primary antibody (fluorophore eFluor PE).
- Anti-CD80 secondary antibody (fluorophore eFlour 450).

Flow cytometry data was analysed with FlowJo software package.

8. Results and discussion

8.1 Descriptive and statistical analysis of the results of the antibodies FACS test

The following results correspond to the experiment explained in *7.4 Antibodies FACS test.* The

Down below, there are the explanations for the results for each antibody. There are two experiment repetitions per antibody and each experiment is composed by two duplicates for each sample.

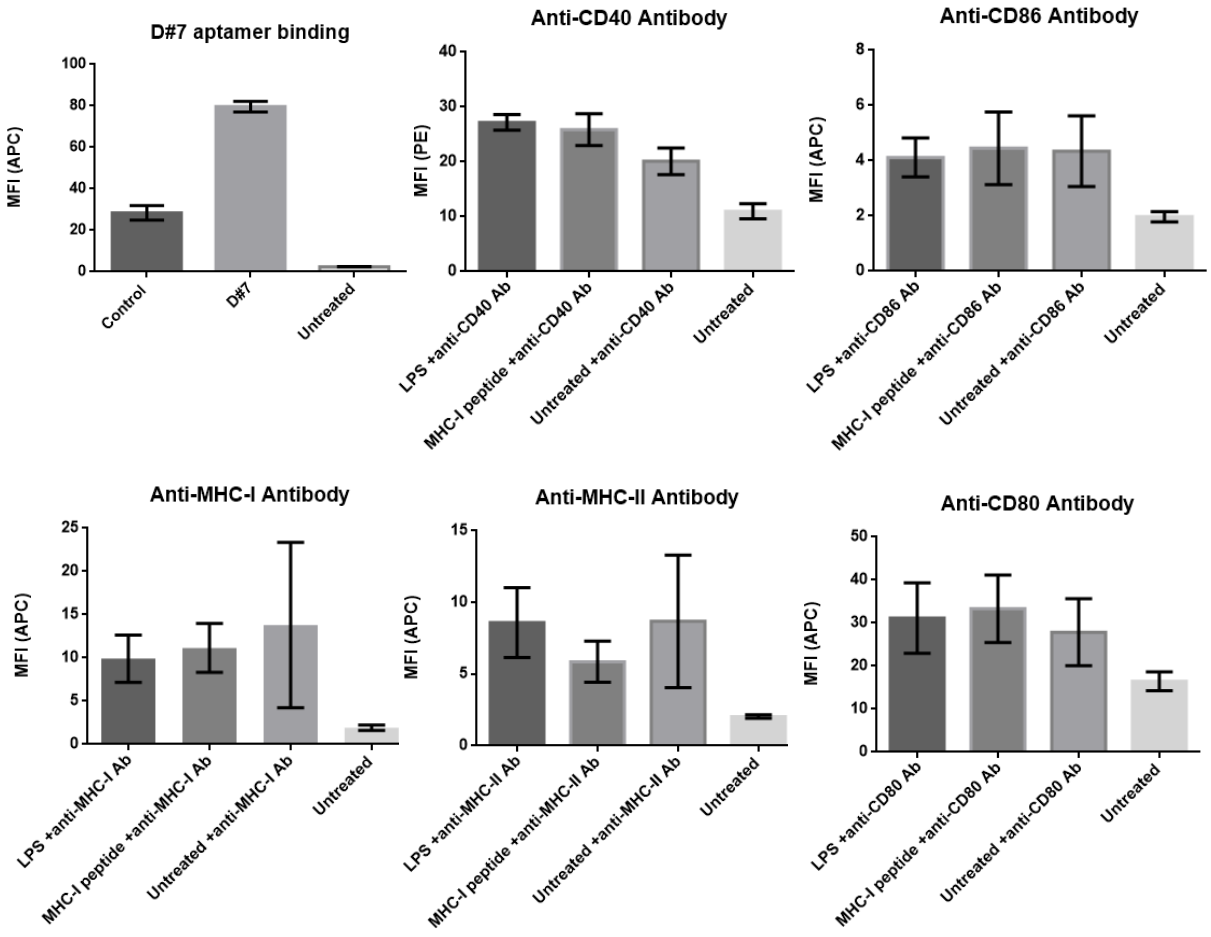


Figure 3. *Expression of CD40, CD86, MHC-I, MHC-II and CD80 receptors from activated DCs and binding of the aptamer D#7.* Analysis of the binding aptamer (D#7) and the expression of diverse cell receptors of DCs (CD40, CD86, MHC-I, MHC-II and CD80) under different conditions* and tested with the corresponding antibodies (anti-CD40, anti-CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies). BMDC were treated with 250nM/mL D#7 and control sequence or 1ng/µL LPS or 1nM/mL MHC-I peptide and incubated 10 minutes*. Then the DCs were incubated 4 hours with DC analysis medium and after 4 hours, expression of CD40, CD86, MHC-I, MHC-II and CD80 were analysed by flow cytometry *(mean ± SD)*.

In **figure 3,** the first graphic corresponds to the MFI of the aptamer (D#7), the control sequence and the untreated cells. The "D#7 aptamer binding" is a representative of the binding aptamer experiment, which was performed 20 times and it was not reproducible.

The wash buffer, which contained magnesium chloride, was supposed to be kept at room temperature, instead, it was kept in the fridge. That error might have had something to do with the reproducibility of the binding of the aptamer experiment, given that the chlorine might have precipitated. Thus, it only worked the first day, when the magnesium chloride was prepared freshly and stocked at room temperature.

There was no point in doing a statistical analysis of the binding of the aptamer as n=2, thus, the analysis consists only on the description of the graphic. The fluorescence of the aptamer D#7 (79,6 MFI) is more intense than the fluorescence of the control (28,41 MFI), which only has the background fluorescence, and that in turn, is more intense than the UN cells (2,45 MFI). That means that the aptamer has bound to the DCs effectively. In previous works the MFI of D#7 was 125 and 62,5 for CTL#5 (Silvana Haβel, 2016).

The rest of the graphics are grouped per antibodies (anti-CD40, anti-CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies).

CD40 receptor is supposed to be upregulated when the DC is activated. Moreover, CD40, CD80, CD86 and MHC-I receptors, are all supposed to be upregulated when the DC is activated by LPS as it was shown in other studies (Judith Rauen *et al*., 2014). In "anti-CD40 antibody" graph, it can be observed that the CD40 receptor was upregulated when the cells were treated with LPS as the MFI of this sample is significantly more intense than the MFI of the untreated cells + anti-CD40 antibody (p value<0,004).

MHC-I peptide binds to the MHC-I molecule and this starts a chain signalling that will end up activating the DC without internalising the MHC-I peptide. Consequently, the DC will upregulate the receptors mentioned previously. So, cells treated with MHC-I peptide should have the cell receptors CD40, CD80, CD86 and MHC-II upregulated as the peptide is supposed to activate the cell. MHC-I receptors is not as studied as MHC-II receptor and it is not clear if it should be upregulated or downregulated. Indeed, cells treated with MHC-I peptide had a significative difference compared to the untreated cells + anti-CD40 antibody, which have the background fluorescence (p value<0,013).

There were no significant differences between the cells treated with LPS and the cells treated with MHC-I peptide (p value<0,423).

In other words, CD40 receptor is upregulated when the DC is activated (with LPS and MHC-I peptide).

The cells treated with anti-CD80, anti-CD86, anti-MHC-I and anti-MHC-II antibodies did not show an upregulation or downregulation of none of the receptors that were being studied when the DCs were activated with LPS or MHC-I peptide and comparing the MFI with the untreated cells + antibody (p value>0.05).

In other studies, the LPS concentration used to treat de BMDC was inferior than the concentration used in this experiment but the time of incubation after the cell exposure to the antibodies was much superior. It was 24 hours instead of 4 hours (Judith Rauen *et al*., 2014). Thus, the incubation time of the cells might not have been enough for the cells to upregulate or downregulate any receptor. DCs could not have been fully activated by the LPS and, in consequence, the cell receptors might not have had time to be upregulated.

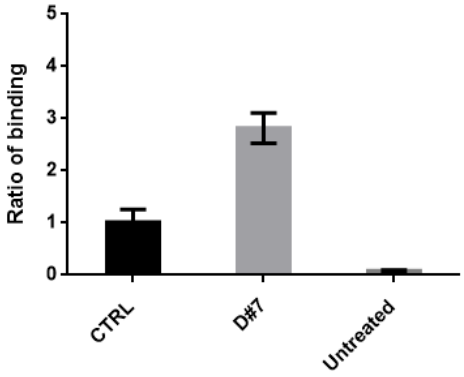


Figure 4. *Ratio of binding (CTRL/CTRL, D#7/CTRL and UN/CTRL).* BM-DCs were treated with 250 nM of ATTO 647N-labeled control sequence (ctrl) and aptamer D#7 *(mean ± SD)*.

In previous studies BM-DCs were treated with 500 nM of ATTO 647N-labeled control sequence (CTRL), the ratio of binding for D#7 in 500nM was 3,7 and the control was 1 (Silvana Haβel, 2016). In this experiment, BM-DCs were treated with 250 nM of ATTO 647N-labeled control sequence and D#7 and the ratio of binding for the control is 2,82 and 1,01 for the control. As the cells were treated with half of the concertation than in previous studies, it is normal that the ratio of binding in this experiment is lower than in the previous studies **(figure 4)**.

In conclusion, the experiments were not reproducible and the protocol should be readjusted in order to have better conditions, like increasing the time of incubation for the DCs in the DC

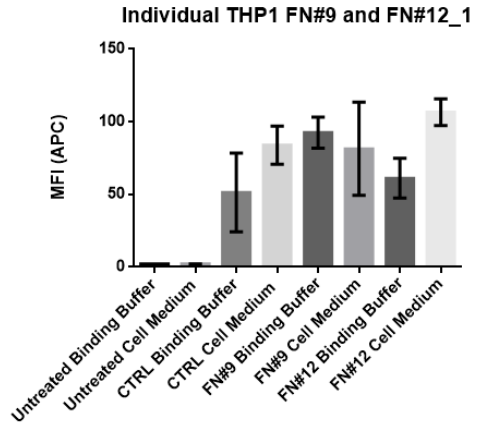
analysis medium. However, this readjustment would have needed another month so it was not possible to optimise the experiment and repeat it. This is the reason why the experiment could not be able to be continued, as it was supposed to, meaning that the conjugated aptamer with the antigenic peptide was never tested.

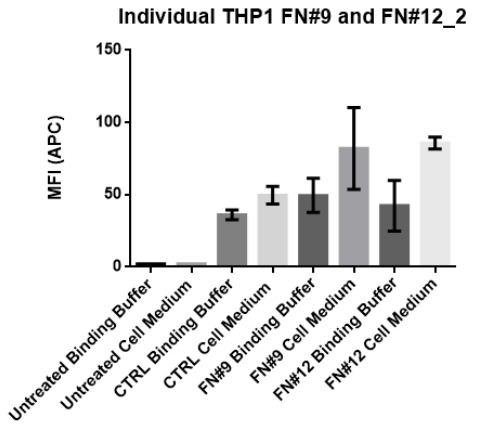
8.2 Descriptive analysis of the results of the aptamers as potential candidates for immunotherapies test

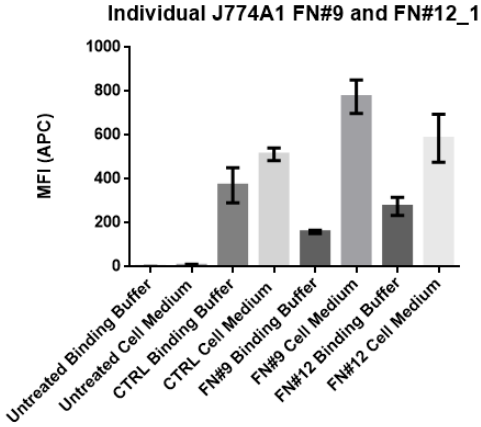
The following results correspond to the experiment explained in *7.5 Aptamers as potential candidates for immunotherapies.*

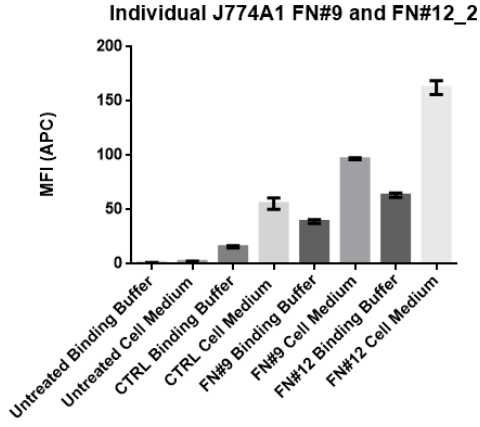
Down below the individual graphics with the MFI for each aptamer (FN#9, FN#12, FN#14, FN#15 and FN#20) are represented. The graphics are shown individually because the MFI values were too different in each experiment and it would have been confusing to just explain it all in the same graphic. Due to the great variability of the results and the low number of observations (n=2) per graphic, it was considered that no result of any hipothesis contrast would be reliable. So, hereunder there is the description of each individual graphic **(figure 5)**.

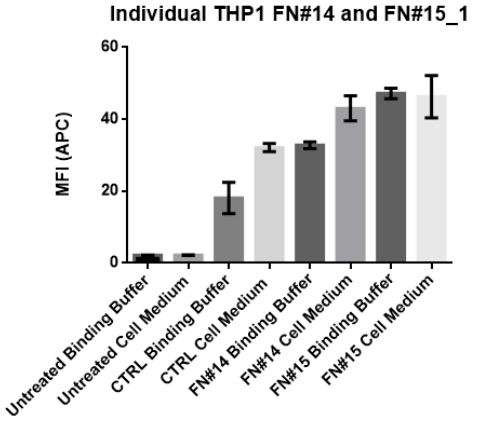
However, the mean of all the MFI of FN# aptamers is also represented and the statistical analysis is made from this graphic. Moreover, the ratio of binding for each cell type are represented as well to have a general look to the results **(figure 6)**.

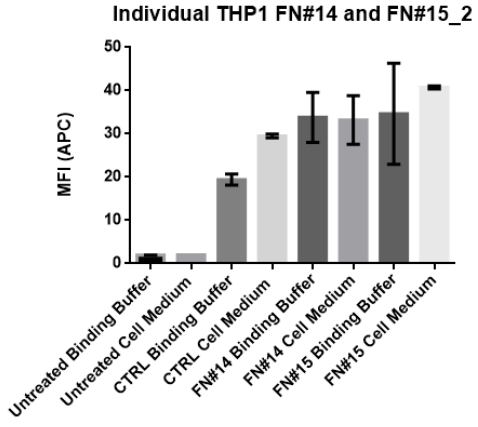

Individual THP1 FN#9 and FN#12_1
MFI (APC)
150
100
50
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#9 Binding Buffer
FN#9 Cell Medium
FN#12 Binding Buffer
FN#12 Cell Medium

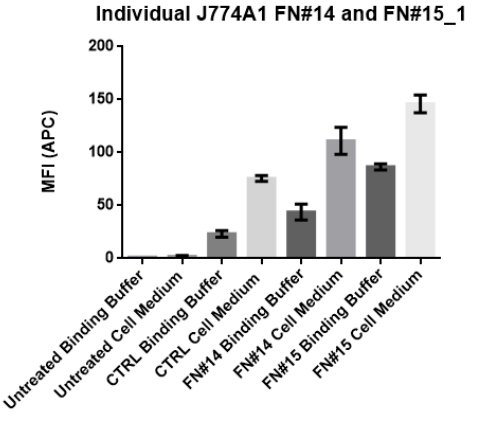

Individual THP1 FN#9 and FN#12_2
MFI (APC)
150
100
50
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#9 Binding Buffer
FN#9 Cell Medium
FN#12 Binding Buffer
FN#12 Cell Medium

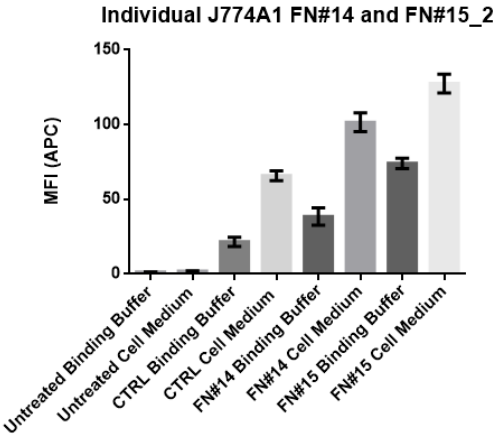

Individual J774A1 FN#9 and FN#12_1
MFI (APC)
1000
800
600
400
200
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#9 Binding Buffer
FN#9 Cell Medium
FN#12 Binding Buffer
FN#12 Cell Medium


Individual J774A1 FN#9 and FN#12_2
MFI (APC)
200
150
100
50
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#9 Binding Buffer
FN#9 Cell Medium
FN#12 Binding Buffer
FN#12 Cell Medium


Individual THP1 FN#14 and FN#15_1
MFI (APC)
60
40
20
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#14 Binding Buffer
FN#14 Cell Medium
FN#15 Binding Buffer
FN#15 Cell Medium


Individual THP1 FN#14 and FN#15_2
MFI (APC)
50
40
30
20
10
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#14 Binding Buffer
FN#14 Cell Medium
FN#15 Binding Buffer
FN#15 Cell Medium


Individual J774A1 FN#14 and FN#15_1
MFI (APC)
200
150
100
50
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#14 Binding Buffer
FN#14 Cell Medium
FN#15 Binding Buffer
FN#15 Cell Medium


Individual J774A1 FN#14 and FN#15_2
MFI (APC)
150
100
50
0
Untreated Binding Buffer
Untreated Cell Medium
CTRL Binding Buffer
CTRL Cell Medium
FN#14 Binding Buffer
FN#14 Cell Medium
FN#15 Binding Buffer
FN#15 Cell Medium

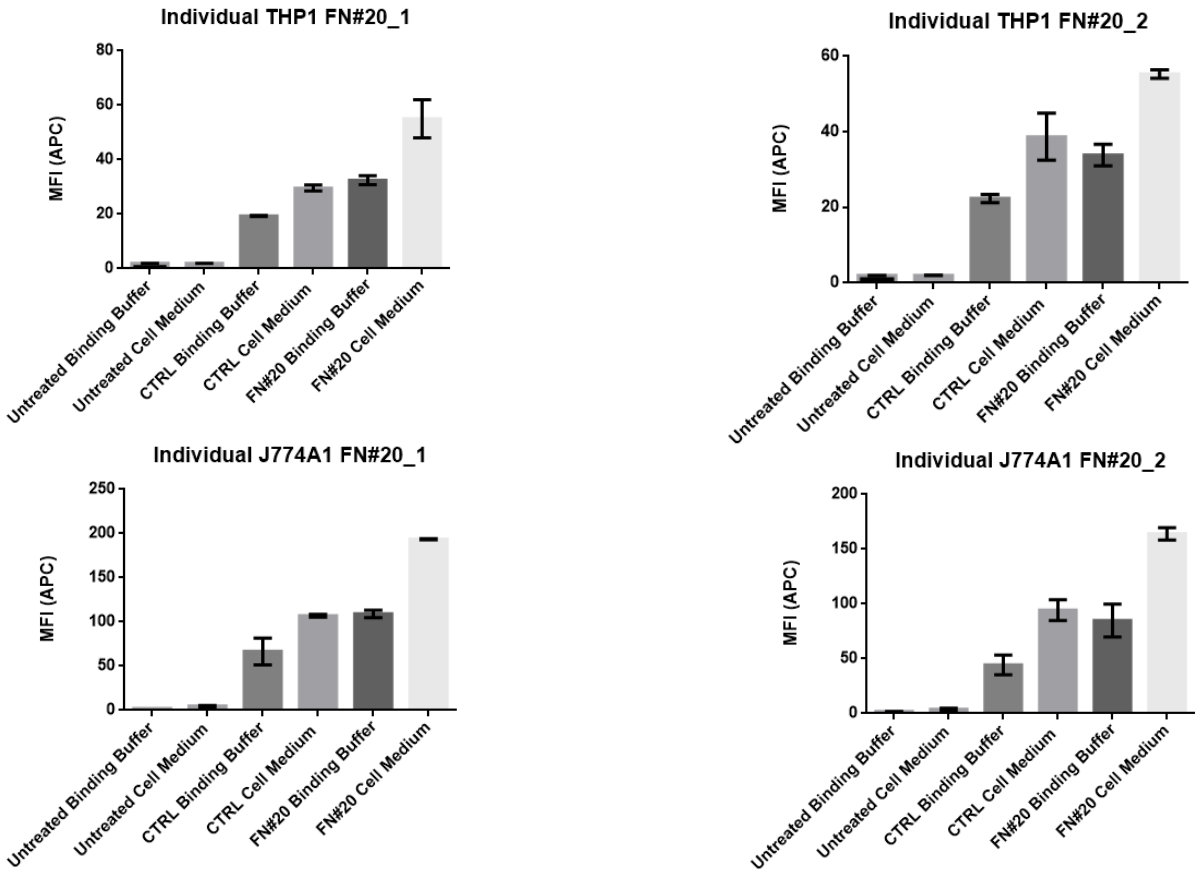


Figure 5. *Binding* of aptamers *F#9, FN#12, FN#14, FN#15 and FN#20*. THP1 and J774A1 cells were treated with 250nM/mL FN# and control sequence and incubated for 30 minutes. Then the MFI was measured with flow cytometry to see if FN# were candidates for the treatment of immunotherapies. *(mean ± SD)*.

In **figure 5,** the individual results for each apatamer and cell line are represented. At first sight, aptamers FN#9 and F#12 look like they did not bind to the THP1 cells, in the first graph, the aptamer MFI is not bigger than the controls' or has a SD overlapped with the control SD. Only in one of the duplicates, F#12 bound to the THP1 cells as the MFI of the aptamer is much bigger than the MFI of the control.

However, in the J774A1 cells, in both duplicates, both apatmers (FN#9 and FN#12) bound to the J774A1 cells when the cells were treated with the cell medium (DMEM + 10% FCS). In one of the duplicates, the cells treated with Binding Buffer also showed a MFI more intense when the aptamer was added. Altough, the most significant binding was when the cells were treated with the cell medium.

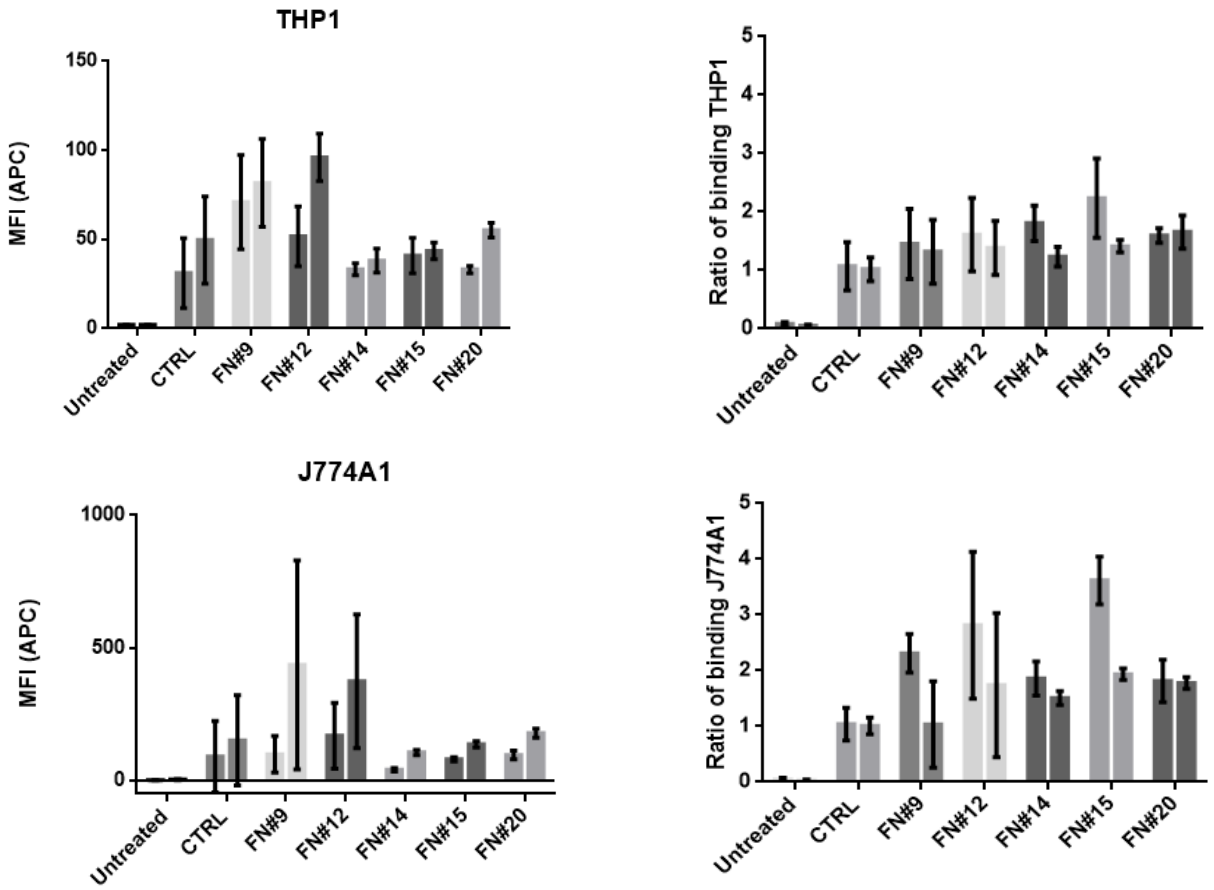


Figure 6. *On the left-hand side, mean of the MFI of all the aptamers FN# of the two cell lines (THP1 and J774A1) including the control and the untreated cells and on the right-hand side, ratio of binding (CTRL/CTRL, UN/CTRL and D#7/CTRL).* The first column of each group corresponds to the cells treated with Binding Buffer and the second column corresponds to the cells treated with cell medium (RPMI + 10% FCS for THP1 cells and DMEM + 10% FCS for J774A1 cells*). (mean ± SD)*.

Aptamers FN#14 and FN#15 bound in the first experiment carried on with THP1 cells. Although in the second one only the FN#14 bound to the cells treated with Binding Buffer and the FN#15 bound to the cells treated with cell medium.

In the case of J774A1 cells, both aptamers (FN#14 and FN#15) bound to the cells in both conditions (cells treated with Binding Buffer or with cell medium), though in the condition of cell medium the MFI is higher and FN#15 has an MFI higher than FN#14. This could mean that FN#15 has bound in more quantity than FN#14.

Concerning to the aptamer FN#20, it bound to both cell types (THP1 and J774A1) and in both conditions (Binding Buffer and cell medium). Like in all the results, the cells treated with cell medium have a higher MFI. And in the case of J774A1 cells, the MFI is higher, in both conditions, than in the case of the THP1 cells.

The ratio of binding in both cell types show in a clearer way that almost all the aptamers have bound to the cells more than the control **(figure 6)**. However, the statistical analysis will confirm it.

8.3 Statistical analysis of the results of the aptamers as potential candidates for immunotherapies test

In **figure 6,** a patron can be observed in the mean graphics (on the right-hand side). In both cell lines (THP1 and J774A1) when the cells are treated with the corresponding cell medium, the MFI value is higher than when they are treated with Binding Buffer (p value<0,0001) **(Table 1 and 2)**. This means that more aptamer and control sequence has bound to the cells. The reason why this has happened might be because the cell medium conditions mimics the conditions in which the cells are normally and these conditions are much more like the natural environment of the cells than the ones that can be found in the Binding Buffer. Consequently, the cells are more comfortable in the cell medium environment and all their functions are more active than in the Binding Buffer situation. For instance, phagocytosis function could be more active in the cell medium and the cells could phagocyte the aptamer, meaning that they would emit fluorescence and there would be no way of knowing if the aptamer is bound to the surface of the cell or phagocyted by the cell. This problem should be studied carefully and solved because then, it cannot be determined which is the optimum solution (cell medium or Binding Buffer) to treat the cells, as what is wanted, is the aptamer bound to the surface of the cell instead of being phagocyted.

Condition	Mean	St.Dev	Median
Binding Buffer	28,85	24,54	28,05
Cell medium	41,29	33,35	40,55

Table 1. *THP1 cells fluorescence of all FN# treatments together for the two conditions* (p<0,001).

Condition	Mean	St.Dev	Median
Binding Buffer	69,36	93,04	39,45
Cell medium	154,35	202,99	99,25

Table 2. *J774A1 cells fluorescence of all FN# treatments together for the two conditions* (p<0,0001).

As it was explained previously, there is a significant difference within the FN# treatments, in both cell lines, THP1 and J774A1 cells ($p<0,0001$). THP1 cells treated with cell medium have higher MFI values than cells treated with Binding Buffer, in general. However, the interaction of the condition and the treatment is significant ($p<0,015$). This is mainly because the values in the untreated group are similar in both conditions, which is not the case in the other groups (CTRL and FN#). Analysing the values of THP1 cells it is obvious that FN#9 and FN#12 show the highest values **(Table 3)**.

On the other hand, J774A1 cells, the interaction of treatment and condition is not significant ($p>0,05$). In all groups there is the same trend, the cell medium has higher values than the Binding Buffer ($p<0,0001$) **(Table 4)**.

In **figure 6** it is also important to remark that the CTRL MFI value is the mean of all the control MFI of all experiments, meaning that the SD might be bigger and the comparison of the individual graphics with the mean graphics may have differences concerning to the CTRL values.

Treatment	Condition	Mean	St.Dev	Median
Untreated	Binding Buffer	2,04	0,09	2,06
	Cell medium	2,07	0,10	2,10
CTRL	Binding Buffer	31,14	19,53	22,35
	Cell medium	49,63	24,45	38,70
FN#9	Binding Buffer	70,95	26,47	71,35
	Cell medium	81,68	24,67	82,00
FN#12	Binding Buffer	51,75	16,79	53,10
	Cell medium	96,10	13,34	94,30
FN#14	Binding Buffer	33,28	3,44	32,75
	Cell medium	38,13	6,86	38,90
FN#15	Binding Buffer	40,90	9,91	44,50
	Cell medium	43,50	4,65	41,55
FN#20	Binding Buffer	33,15	2,08	32,70
	Cell medium	55,18	4,10	55,30

Table 3. *THP1 cells fluorescence of all FN# treatments for two conditions:* Binding Buffer *and Cell medium* (Interaction, $p<0,015$).

Treatment	Condition	Mean	St.Dev	Median
Untreated	Binding Buffer	1,64	0,29	1,58
	Cell medium	3,53	2,24	2,81
CTRL	Binding Buffer	90,50	134,76	31,75
	Cell medium	151,86	169,88	82,50
FN#9	Binding Buffer	99,08	69,32	97,20
	Cell medium	435,95	393,98	409,25
FN#12	Binding Buffer	169,18	124,47	155,40
	Cell medium	374,00	252,31	337,50
FN#14	Binding Buffer	41,18	6,31	40,55
	Cell medium	106,28	9,85	104,00
FN#15	Binding Buffer	80,28	7,69	80,45
	Cell medium	136,75	12,31	136,00
FN#20	Binding Buffer	96,88	16,62	100,65
	Cell medium	178,75	17,35	180,50

Table 4. *J774A1 cells fluorescence of all FN# treatments for two conditions:* Binding Buffer *and Cell medium* (Treatment p<0,0001; Interaction p>0,05).

In **Table 5**, there are the means of each treatment without having into account the two conditions and there are 3 homogeneous subsets (p<0,05). In the first group, the MFI mean of the untreated cells can be found, which is the lower MFI mean given that these cells had no fluorophore. The second group corresponds to FN#14, CTRL, FN#15 and FN#20, in ascending order. Thus, there are no significant differences between the CTRL and the FN#14, FN#15 and FN#20 aptamers, so they have not bound to THP1 cells. The third group, belongs to FN#12 and FN#9, which have no significance differences between each other but they have it with the CTRL. Henceforth, aptamers FN#12 and FN#9 have bound to THP1 cells.

Treatment	N	Homogeneous Subsets		
		1	2	3
Untreated	28	2,0564		
FN#14	8		35,7	
CTRL	28		40,3821	
FN#15'	8		42,2	
FN#20'	8		44,1625	
FN#12	8			73,925
FN#9	8			76,3125

Table 5. *HSD Tukey (Post Hoc).* Fluorescence THP1 ranked by mean.

In **Table 6,** because of the high variability of this parameter, non-parametric tests (Mann Whitney) was used for this study, so, the homogeneous subsets (p<0,05) are ordered by the values of their medians. The median of the different treatments (FN#) is not having into account the two conditions and there are 3 homogeneous subsets. The first group corresponds to the untreated cells' MFI median, which is the lower MFI median given that these cells had no fluorophore. The second group belongs to the CTRL, FN#14, FN#15 in ascending order. Thus, there are no significant differences between the CTRL and the FN#14, FN#15 and FN#9 aptamers, so they have not bound to THP1 cells. The last group corresponds to FN#15, FN#9, FN#20 and FN#12, in ascending order. These aptamers have no significance differences between each other but they have it with the CTRL, not having into account FN#15 and FN#9 aptamers. Henceforth, aptamers FN#20 and FN#12 have bound to J774A1 cells.

Treatment	N	Homogeneous Subsets		
		1	2	3
Untreated	24	1,93		
CTRL	24		65,75	
FN#14	8		73,15	
FN#15	8		105,80	105,80
FN#9	8		125,75	125,75
FN#20	8			136,00
FN#12	8			206,50

Table 6. *Mann Whitney Test.* Fluorescence J774A1 ranked by median (p<0,0001).

To sum up, aptamers FN#12 and FN#9 have bound to THP1 cells and aptamers FN#20 and FN#12 have bound to J774A1 cells. Henceforth, these aptamers would be the candidates to continue this study for future medical applications in immunotherapies.

9. Conclusions

The testing of FN#9, FN#12, FN#14, FN#15 and FN#20 aptamers, two of these aptamers, FN#9 and FN#12, were satisfactory bound to the THP1 cells and FN#20 and FN#12 bound to J774A1 cells. Thus, these aptamers would be the subject of study to use them as immunotherapy tools. Especially, FN#12 because it bound to both cell types, to the human monocytes (THP1 cells) and the murine macrophages (J774A1 cells). So, in the future, the adaptation of the animal experimentation to the phase 1 of the clinical trial, in humans, would be easier.

The immunotherapies could be, for instance, in the treatment of cancer that was mentioned in the *4. Introduction*, by linking a non-immunogenic tumour antigen to one of these aptamers which would recognise an APC and that would activate the cells involved in the activation of the immune system.

The aptamers bound in both conditions (Cell medium and Binding Buffer). However, in all cases, cell medium condition showed MFI values higher than in the Binding Buffer condition. As it was explained before, this could be because the cells might have the phagocytosis function more active in the cell medium than in the Binding Buffer because of the similarity of the cell medium environment with the natural environment of the cells. Nevertheless, this theory should be confirmed since it is desirable the aptamer binding to the cell surface in order to promote APC endocytosis, instead of being phagocyted. In the future applications, when the aptamer will be conjugated with an antigenic peptide, it will present the antigen to the cell by binding to the cell surface, but, if the aptamer is phagocyted, the cell might not recognize the antigen and degrade it instead.

So, if the reason why the cell medium seems to be working better than the Binding Buffer is this one, then it does not mean that the cell medium is more optimum than the Binding Buffer. On the contrary, it would be worse because the aptamer would be phagocyted and degraded instead of bounding to the cell surface.

Concerning the results of the validation of the D#7 aptamer were not the expected results as they were not coherent. Thus, the validation of D#7 aptamer was incomplete and should be improved and repeated changing steps in the protocol, like the time of incubation for the DCs so they can upregulate or downregulate the cell receptors after their activation with LPS or MHC-I peptide, and working more accurately during the procedure.

Overall, the validation of D#7 aptamer should be done again and aptamers FN#9, FN#12 and FN#20 should be studied adjusting the protocol in order to optimise the conditions and the treatment of the cells for future applications in immunotherapies.

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11. Annex

11.1 Annex 1: abbreviations

- DC: Dendritic Cell
- SELEX: Systematic Evolution of Ligands by EXponential enrichment
- LPS: Lipopolysaccharides
- MHC: Major Histocompatibility Complex
- APC: Antigen-Presenting Cell
- Ag: Antigen
- BCR: B-cell Receptor
- TCR: T Cell Receptor
- Tc: Cytotoxic T cell
- Th: T helper
- Treg: T regulator
- IL: Interleukin
- IFN-γ: Interferon Gamma
- IL-xR: Interleukin x Receptor
- TAA: Tumour Associated Antigens
- BMDCs: Bone Marrow Dendritic Cells
- PCR: Polymerase Chain Reaction
- CTRL: Control sequence
- MFI: Mean Fluorescence Intensity
- GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor

11.2 Annex 2: BMDC Culture protocol

Part 1 - 1st Day

Note: every time that something is mixed (dilutions, etc.) → do up and down before using it.

Note: check every antibody twice (one Ab/week will be analysed as you perform two experiments per week).

1. Place the DC culture medium (DC-medium) in the warm bath for 30 minutes at 37ºC.

DC-medium: IMDM, 10 % heat inactivated FCS, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml, streptomycin, 2.5 % R1/J558 supernatant w/ GM-CSF.

2. Pipette 5mL of DC culture into a 50mL falcon (50F).
3. Defreeze the BMDCs (they are in the -18ºC freezer) by holding them into the warm bath.
4. Pour the BMDCs into the 50F.

   Note: the cells are in their sixth day of differentiation into dendritic cells.
5. Centrifuge the 50F for 5 minutes at 25ºC at 200g.
6. Remove the supernatant (suck it).
7. Add 15mL of DC culture (avoid bubbles).
8. Collect all the liquid and put it into a Petri dish (distribute the volume equally by drawing an 8 with the Petri dish).

   Note: you use this Petri dishes and not the special cell culture flask because the plastic is treated in order to avoid contaminations and this plastic activates de DCs (you don't want that) and the Petri dishes used for microbiology are not.
9. Incubate at 37ºC for 48 hours.

Part 2 - 3nd Day (48 hours later)

1. Place the DC analysis medium in the warm bath for 30 minutes at 37ºC.

DC analysis-medium: IMDM, 10 % heat inactivated FCS, 50 µM β-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml, streptomycin, 2.5 % R1/J558 supernatant.

2. Take the dPBS 1X pH 7.2 gibco® out of the fridge (leave it at room temperature).
3. Have a look at the incubated Petri dish (it has been incubated for 48 hours). There should be, no contamination, and cells in suspension and adhered to the Petri dish with star shape).
4. Remove the supernatant (~15mL) and put it into the 50F.
5. Add 3mL of EDTA at (0,5M, you have to dilute it into 2mM EDTA in dPBS) to the Petri dish (prepare it freshly every time).
   Note: cells cannot be put into water because they would die by explosion (osmosis, the water enters into the cells and make them explode).
6. Incubate the Petri dish for 5 minutes at 37ºC.
7. Have a look on the cells in the microscope. If they are still fixed to the Petri dish:
   a. Do up and down, remove the liquid and put it into de 50F.
   b. Add 5ml of dPBS, do up and down and put it into the 50F.
8. Now you can throw the Petri dish away.
9. Centrifuge the 50F for 5 minutes at 25ºC at 200g.
10. Remove the supernatant.
11. Add 10mL of DC analysis (the last 5 up and down times, let the liquid flow gently against the wall of the falcon tube so you acquire individual cells).
12. Label the 24 well plate (name, date and cell type).
13. Add 900µL of dPBS in an Eppendorf.
14. Add 100µL from the 50F to the Eppendorf.
15. Prepare the Neubauer chamber
16. Place 10µL from the Eppendorf in the Neubauer chamber (do it in both sites).
17. Count the cells and calculate.

$$\frac{nº\ of\ cells}{4} \cdot 10^4 \cdot dilution\ factor$$

18. Add x mL of DC analysis to the 50F until it reaches the desired volume.

Example: I have 10mL in the 50F and I want 20mL because I will add 1mL of solution per well and I will use 18 wells, so I need to add 10mL.

Note: remember to calculate the final nº of cells/mL (when the x mL has already been added).

$2 \cdot 10^5$cells/well are needed. Always count 2 wells more (pipette error).

It is better to collect the number of cells that you want per well ($2 \cdot 10^5$cells/well) and then add the necessary DC analysis.

19. Put 1mL/well and incubate the plaque for 45 minutes at 37ºC.

    Note: never less than 45 minutes but if it's a bit more it's okay.

20. Remove the supernatant.
21. Add 200µL of the sample per well.

	Dilutions		**Samples ***		**µL/well**
12 wells (in total) *	Stock solution	dPBS	From the dilution	DC analysis	
X2 MHC-I peptide Stock solution: 2,2 mM Desired concentration: 1 nM/well	1 µL	10mL	2,045 µL	447,95 µL	200
X2 LPS Stock solution: 1mg/mL Dilute it into 1ng/µL and then into 1:400 per well	1 µL	999 µL	1,1 µL	438,9 µL	200
x2 Aptamer Stock solution: 100 µM Desired concentration: 250 nM/well	X	X	1,1 µL	438,9 µL	200
x2 Control sequence Stock solution: 100 µM Desired concentration: 250 nM/well	X	X	1,1 µL	438,9 µL	200
X2 Untreated +Ab	X	X	X	200 µL	200
x2 Untreated	X	X	X	200 µL	200

*If the optimum time of incubation is being, 18 wells must be used. If the optimum time of incubation is already known, 12 wells are needed.

Note: be careful with the volume, recalculate it if you need more.

22. Incubate for 10 minutes at 37ºC.
23. Remove the supernatant.
24. Add 1mL of DC analysis/well.
25. Incubate for 4 hours at 37ºC.

    Note: 4 hours exactly.

ALWAYS CHECK THE BUFFERS AND THE MEDIA (SEE IF THERE IS SOMETHING GROWING IN THERE)

26. Scratch the cells from the wells.
27. Add 1mL of Wash Buffer to FACS tubes.
28. Transfer the cells into the FACS tube.
29. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
30. Remove the supernatant.
31. Add 100µL of blocking solution to the cells.

    Note: Add the blocking buffer (which contains FCS) to block all the non-specific bindings, as the FCS has lots of proteins and stuff. THE BLOCKING BUFFER HAS ALWAYS TO BE KEPT AT 4ºC.
32. Incubate 15 minutes at 4ºC.
33. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
34. Remove the supernatant.
35. Add 50µL of the antibody solution (1:200) to LPS, MHC-I and UN +Ab (it's in FACS buffer).

    Note: We use 1:200 dilution of the Ab because someone else did it and it works.
36. Incubate for 20 minutes at 4ºC.
37. Add 1mL of FACS buffer (without the antibody).
38. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
39. Remove the supernatant.
40. Stain the cells into 1:1000 of the secondary antibody (same that you did with the primary Ab, you do a 1:100 dilution and then multiply for the volume that you want).
41. Incubate for 20 minutes at 4ºC.
42. Add 1mL of FACS buffer.
43. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
44. Remove the supernatant.
45. Add 100µL of FACS buffer.
46. FACS measurement.

Material, reagents, media and buffers

- DC analysis
- 5mL and 10mL pipette
- 50mL falcon
- FACS tubes
- EDTA (Ethylenediaminetetraacetic acid)
- dPBS (Dulbecco's Phosphate-Buffered Saline)
- 24 well plate
- Eppendorf
- Ethanol

Primary antibodies

- Anti-CD40 antibody
- Anti-CD86 antibody
- Anti-MHC-II antibody

Secondary antibodies

- Anti-Armenian hamster IGG antibody and its conjugated with the fluorophore PE. (It binds to the anti CD80 antibody).
- Anti-mouse IGG antibody and it's conjugated with Alexa-Fluor 647 and it binds to anti-MHC-I antibody.

Indirect staining

- Ab Anti-MHC-I (it was isolated from a mouse) you need a secondary Ab (anti-mouse Ab).
- Ab Anti-CD80 (it was isolated from an Armenian hamster) you need a secondary Ab (anti-Armenian hamster Ab).

Note: The Ab are in the fridge.

If the antibody is not working, meaning that you don't see any upregulation, first check all the calculations and if everything is good, increase the time of incubation (instead of 4 hours try Over Night).

Check if the parameters of FACS are correct (if the light is not out of range).

Fluorophores:

- Measured with PE Channel:
  - eFluor PE for anti-CD40 antibody.
  - eFluor 450 for anti-CD80 antibody.
- Measured with APC Chanel:
  - Alexa-Fluor 647 for anti-CD86 antibody.
  - A647N for the Ctrl and the aptamer (D#7) sequence.
  - Alexa-Fluor 647N for anti-MHC-II antibody.
  - Alexa-Fluor 647N for anti-MHC-I antibody

FACS buffer	• In dPBS
	  ○ 0.1% BSA
	  ○ 0.005% NaN_3
	• Ab diluted in FACS buffer 1:200 (only when it's necessary) *
		• 100µL of BSA
		• 5µL of NaN_3
		• 99.9mL of dPBS
		• For 6 samples: 3.5µL of Ab + 346.5µL of FACS buffer.

Blocking buffer	• dPBS with 10% FCS (Fetal Calf Serum)	• 10mL of FCS in 90mL of dPBS
Wash buffer	• dPBS in 1mM Magnesium Chloride	• Solution A: 1.0165g of magnesium chloride in 50mL of distillate water • Add 500µL of solution A to 49.5mL of dPBS

*Final dilution: 1:200, so count the 50µL that remain in the FACS tube after centrifugation and the 50µL that have to be added, having a total of 100µL. So, first a dilution 1:100 must be done (1µL of Ab + 99µL of FACS buffer). Then, when the 50µL are added to the FACS tube there will be a final dilution of 1:200.

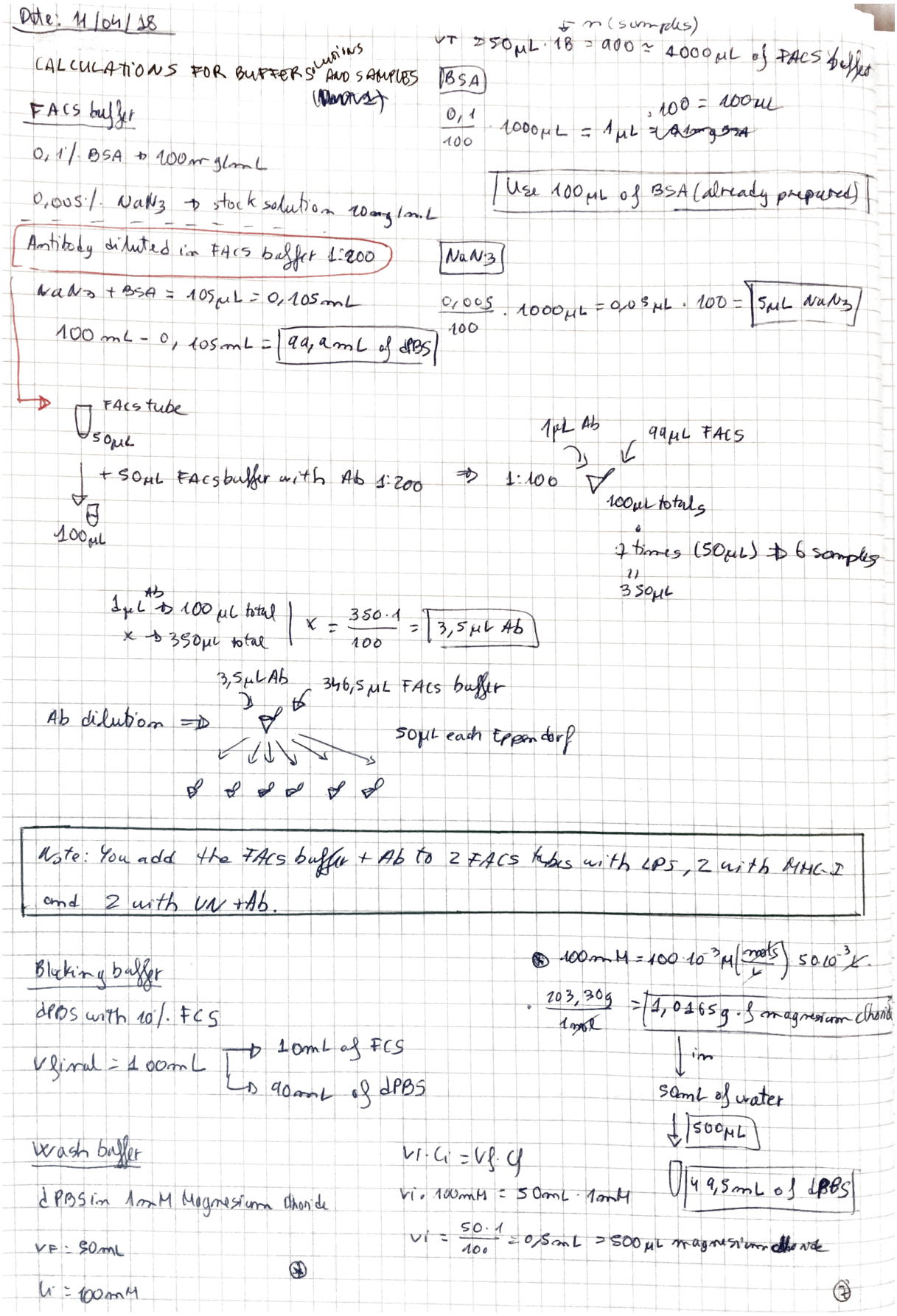

Figure 7. *Calculations for buffers and solutions.*

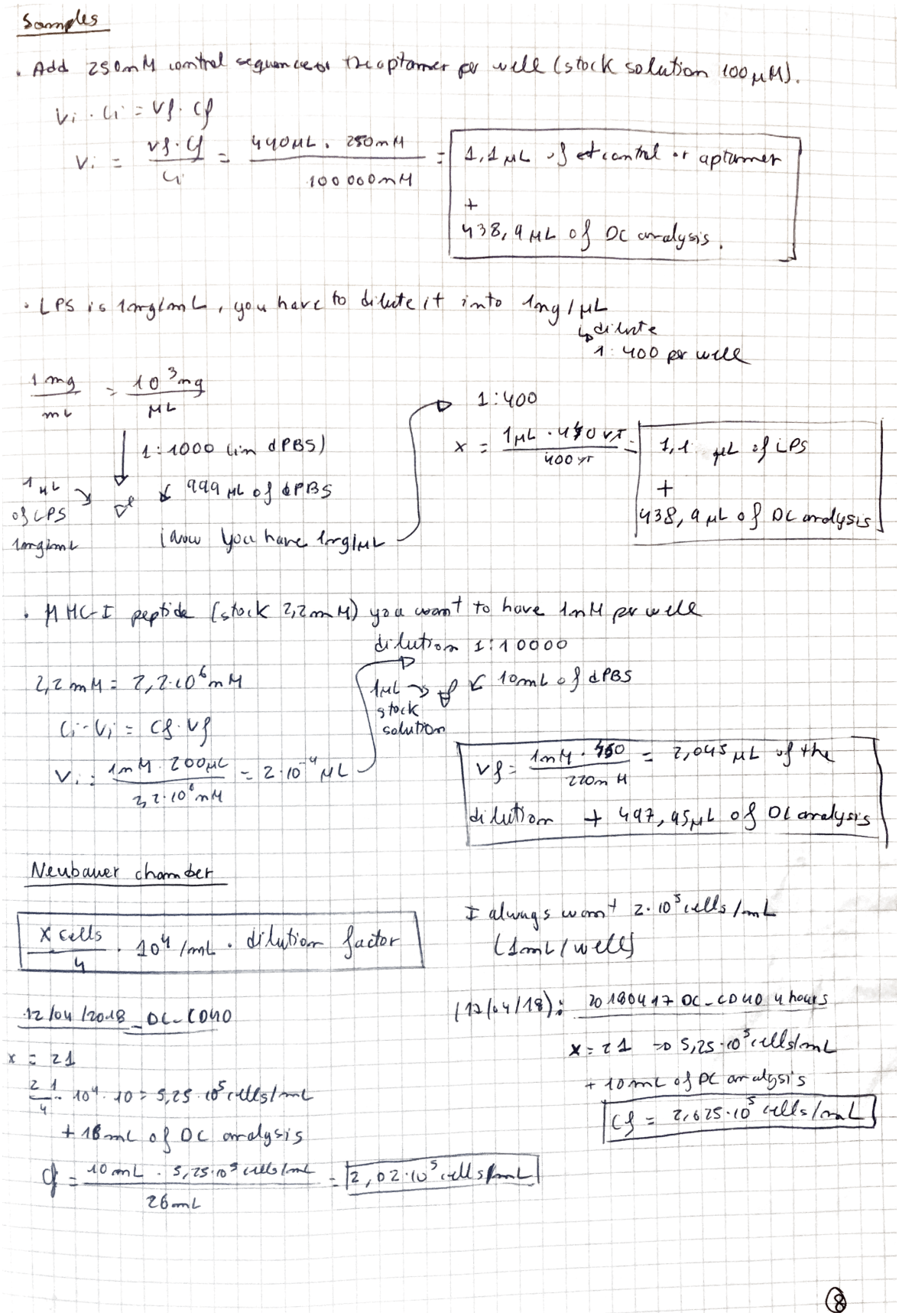

Figure 8. *Calculations for the samples.*

11.3 Annex 3: flow cytometry protocol

Switching On (30 minutes before using it)

Note: reserve the time for using the FACS (sign in a calendar).

Note: the first and the last person who uses the FACS must annotate their name and the time when they switch it on or off, respectively.

1. Switch the FACS on (green button).
2. Switch the computer on. (password written in the first page of the register book).
3. Check if there are bubbles in the tubes, if yes, remove them by letting the liquid flow a bit (clean everything).
4. Check the if the WASTE bottle is full (if it weights a lot it's full), if it is full, click "cytometer → cleaning mode → bubble filter purge and degas flow cell. Throw the content down the sink and add a trickle of Kohrsolin.
5. Click on the program "DIVA" → username: Silvana, password: silvana
6. Click cytometer → fluidics start up → confirm (OK).
7. Refill the other tanks if necessary.

In use

1. Click cytometer → cleaning modes → SIT Flush
2. Click to the folder "Claudia", on the experiment and add a new tube.
3. Before the measurement vortex the sample.
4. Place the sample gently in the sensor.
   Note: Check if the parameters of FACS are correct (if the light is not out of range).
5. Click "Acquire Data". When red things in the graphics are visible, click "Record Data".
6. Remove the FACS tube immediately. VERY IMPORTANT.
7. Click "Next Tube". The FACS tube can be renamed.
8. Save the data. Click "File" → export → FCS Files → FCS 2.0 → OK → Claudia

Wash

1. Click "shard view" → washing → tubes → add a new tube (click on the drawing) and rename it with the date and the name of the user.
2. Follow the instructions written in the FACS.

- Put FACS Rinse in a FACS tube and press only “Acquire Data” for 5 minutes.
- Put distillate water in a FACS tube and press only “Acquire Data” for 6 minutes. Record the last minute (clicking “Record Data”).

3. Check the WASTE, empty it if it’s necessary.
4. Click “File” → Log Out → Log Out Only

Switch off

1. Click “cytometer” → fluidics shutting down → OK

Note: When the FACS is measuring, it is using the FACS Flow. When it is switched off, this flow is substituted by the Shut Down.

2. Switch off the FACS (green button) and the PC.

ANALYSING THE RESULTS

Note: Work in the MAC PC.

1. Click “Data” → Clàudia
2. Click “FlowJo” → File → New Workspace → drag the results of the pen drive → save as → data → Clàudia → *Name it as the experiment (Ex: 120418_DC_CD40).*
3. Double click to the UN sample, now you can gate the important part.
    - The colours represent the number of cells that are in the same state, meaning that are in the same position in the graphic; forward scatter (x, represents the size of the cells) and complexity or granulator scatter (represents the complexity of the cells). The order is red → yellow → green → blue
    - The red and yellow part are the desired ones, so circle that contains these must be drown.
    - You can discriminate between two populations.
        - The one in the left are the DCs.
        - The one in the right are macrophages and other cell types.
    - When the cells are gated, name them “cells” and apply it to ALL the samples.

    Note: make sure that this circle covers the red and yellow zone IN ALL THE SAMPLES.

Note: SHIFT means that the population is moving to the right, to the left, up or down.

4. Click "Layout" (the fifth button). There are two types of fluorophore:
   Note: the gated region is the one that should be used, but if it doesn't make any sense, use all the sample.
   - **APTAMER fluorophore**: XAxis-APC-A and YAixis Histogram (Ctrl, UN and the aptamer (D7).
     - Click in the legend → Click Σ → Mean → Parameter →APC-A
       Note: With the mean, fluorescence value can be seen.
   - **ANTIBODY fluorophore**: XAxis-PE-A and YAixis Dot Plot (if you don't see it right, select the Histogram) (UN, UN +Ab, LPS and MHC-I).
     Note: CAREFUL! The fluorophore changes depending on the antibody that's being used.
   - First, drag the original results (not gated) of the UN and put the duplicates overlapped.
5. Save (all in the same page) → Print → Save as PDF → save it in the MAC and in the pen drive.

<u>Channels</u>

- APC: in this channel, the Alexa-647 (A647N) fluorophore can be observed (anti-MHC-II, anti-MHC-II, anti-CD80 antibodies and the aptamer and control sequence have this fluorophore).
- PE: in this channel the PE fluorophore can be observed (anti-CD40 and anti-CD86 antibodies have this fluorophore).

Note: ALWAYS have a look on the parameters (look if they are out of range or not).

11.4 Annex 4: THP1 and J774A1 cell culture protocol

Cultivation of the cell lines

J774A1-Passage on Monday, Wednesday and Friday

- Cell medium: DMEM + 10% FCS.
  1. Add 14mL of DMEM + 10% FCS medium in the flask.
  2. Add 1mL of the cells that are in the previous flask (before doing it, observe it in the microscope and do up and down).

THP1-Passage on Tuesday and Friday

- Cell medium: RPMI + 10% FCS.
  3. Add 8mL of DMEM + 10% FCS medium in the flask.
  4. Add 2mL of the cells that are in the previous flask (before doing it, observe it in the microscope and do up and down).

The aim of the experiment is to find new aptamer candidates for being used as part of an immunotherapy in the future.

Cell types:

- THP1 (suspension human cells, monocytes), $4 \cdot 10^5$ cells/FACS tubes are required.
- J774A1 (adherent mice cells, macrophages), seed them on a well plate one day before using them (duplication tax: 24 hours, so $2 \cdot 10^5$ cells/well must be seeded).

Buffers:

Binding Buffer (incubating buffer) PREPARE FRESHLY! (5ml/day)	• dPBS • 1mM Magnesium Chloride • 1mM calcium chloride (desired stock solution: 100mM in water) • 0,01mg/mL BSA • 0,01mg/mL salmon sperm (it is a competitor* and it is already prepared, it is 10mg/mL)	• 4,9mL of dPBS • 50µL of $MgCl_2$ • 50µL of $CaCl_2$ • 0,5 µL of BSA • 5 µL of salmon sperm
Wash buffer PREPARE FRESHLY! (210ml/day)	• dPBS • 1mM Magnesium Chloride • 1mM calcium chloride	• 205,8mL of dPBS • 2,1mL of $MgCl_2$ • 2,1mL of $CaCl_2$

Note: keep the magnesium chloride, the calcium chloride and the dPBS at room temperature (the stock solution).

**that means that it serves for non-specific bindings.*

Aptamer candidates with Atto647N fluorophore:

1. FN#9
2. FN#12
3. FN#14
4. FN#15
5. FN#20

All the aptamers must be used at 250nM and dilute it into 200µL of Binding Buffer or cell medium (it is unknown in which one, Binding Buffer or cell medium, is going to work or not).

Part 1 – 1st Day-Preparation of J774A1 cells

Preparation of J774A1 cells				
Tuesday, 22	Wednesday, 23	Thursday, 24	Monday, 28	Tuesday, 29

Note: every time that you mix something (dilutions, etc.) → DO UP AND DOWN before using it.

1. Detached the cells (doing up and down). KEEP 1ML TO CONTINUE THE CELL LINE.
2. Count the number of cells with the Neubauer chamber (10µL) and multiply it until the desired amount, which is $2{\cdot}10^5$ cells/well · 17 wells = $34{\cdot}10^5$ cells
3. Calculate how many ml are required. $\frac{x}{4} \cdot 10^4 \cdot dilution\ factor\ (if\ it's\ necessary) = number\ of\ \frac{cells}{mL} = Ci$ and then (Vi·Ci=Vf·Ci)

   Note: the volume needed in order to have $2{\cdot}10^5$ cells/well or mL will be known here (y)
4. Rest: 17ml – y = z ml of fresh medium.
5. Put it into a falcon tube (z ml of cell medium + y ml of the medium with the cells).
6. Pipette 1mL per well (17 wells).
7. Incubate at 37ºC for 24 hours.

Part 2 – 2nd Day

	Days for J774A1 cells				
	Wednesday, 23	Thursday, 24	Friday, 25	Tuesday, 29	Wednesday, 30
Aptamers	FN#9 and FN#12	FN#9 and FN#12	FN#14 and FN#15	FN#14 and FN#15	FN#20 x2
	Days for THP1 cells				
Aptamers	Tuesday, 22	Wednesday, 23	Thursday, 24	Friday, 25	Monday, 28
	FN#9 and FN#12	FN#9 and FN#12	FN#14 and FN#15	FN#14 and FN#15	FN#20 x2

Note: Before doing the incubation, cells must be washed (the THP1 are washed directly in the FACS tubes and the J77A41 in the well plate). This is done because the cell medium must be totally removed before working with the cells. It would only be needed while the cells are treated with the Binding Buffer *but it is done in both cases (with the* Binding Buffer *and the cell medium) to have the same conditions.*

Preparation of THP1 cells

1. Do up and down. KEEP 2ML TO CONTINUE THE CELL LINE.
2. Count the number of cells with the Neubauer chamber (10µL) and multiply it until the desired amount, which is $4 \cdot 10^5$ cells/well · 17 FACS tubes = $68 \cdot 10^5$ cells
3. Calculate how many ml are required to have these cells. $\frac{x}{4} \cdot 10^4 \cdot dilution\ factor = number\ of\ \frac{cells}{mL} = Ci$ and then (Vi·Ci=Vf·Ci)

   Note: the volume needed in order to have $4 \cdot 10^5$ cells/well or mL will be known here (y)
4. Put the y ml into a falcon tube + z mL of cell medium until 17mL (in total).
5. Put 1mL of it in each FACS tube (16 in total).

Aptamer staining

1. Add 1mL of wash buffer to the FACS tubes (THP1) or to the wells (J774A1).
2. J774A1 cells: just mix the wash buffer with the cells (move the plate gently) and remove it very carefully.
3. THP1 cells: Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
4. Remove the supernatant
5. Add 200µL of the aptamer

	Samples * x4			µL/well
16 wells or FACS (in total) *	From the stock solution	Binding Buffer or cell medium*	Total	
X8 Aptamer Stock solution: 100 µM Desired concentration: 250 nM/well	1,1 µL	438,9 µL	440 µL	200
X4 Control sequence Stock solution: 100 µM Desired concentration: 250 nM/well	1,1 µL	438,9 µL	440 µL	200
X4 Untreated	X	200 µL	200 µL	200

***For each cell line, do duplicates for the cell medium and for the Binding Buffer.

*Cell medium:

- THP1 cells: RPMI 1640 + 10% FCS
- J774A1 cells: DMEM + 10% FCS

1. Incubate for 30 minutes at 37ºC.

   Note: there are no 4 hours-incubation this time.
2. J774A1 cells: Scratch the cells and transfer them to the FACS tubes.
3. (In both cell types) Put 200µL (what was in the wells) in 2mL of wash buffer. In the case of THP1, the 200µL are already in the FACS tubes and 2mL of wash buffer are added (everything in the FACS tubes).
4. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
5. Remove the supernatant´
6. Add 1mL of wash buffer
7. Centrifuge the FACS tubes for 5 minutes at 25ºC at 200g.
8. Remove the supernatant
9. Add 100µL of wash buffer
10. FACS measurement

Note: it would be interesting to find an aptamer with affinity for one of the cell lines.

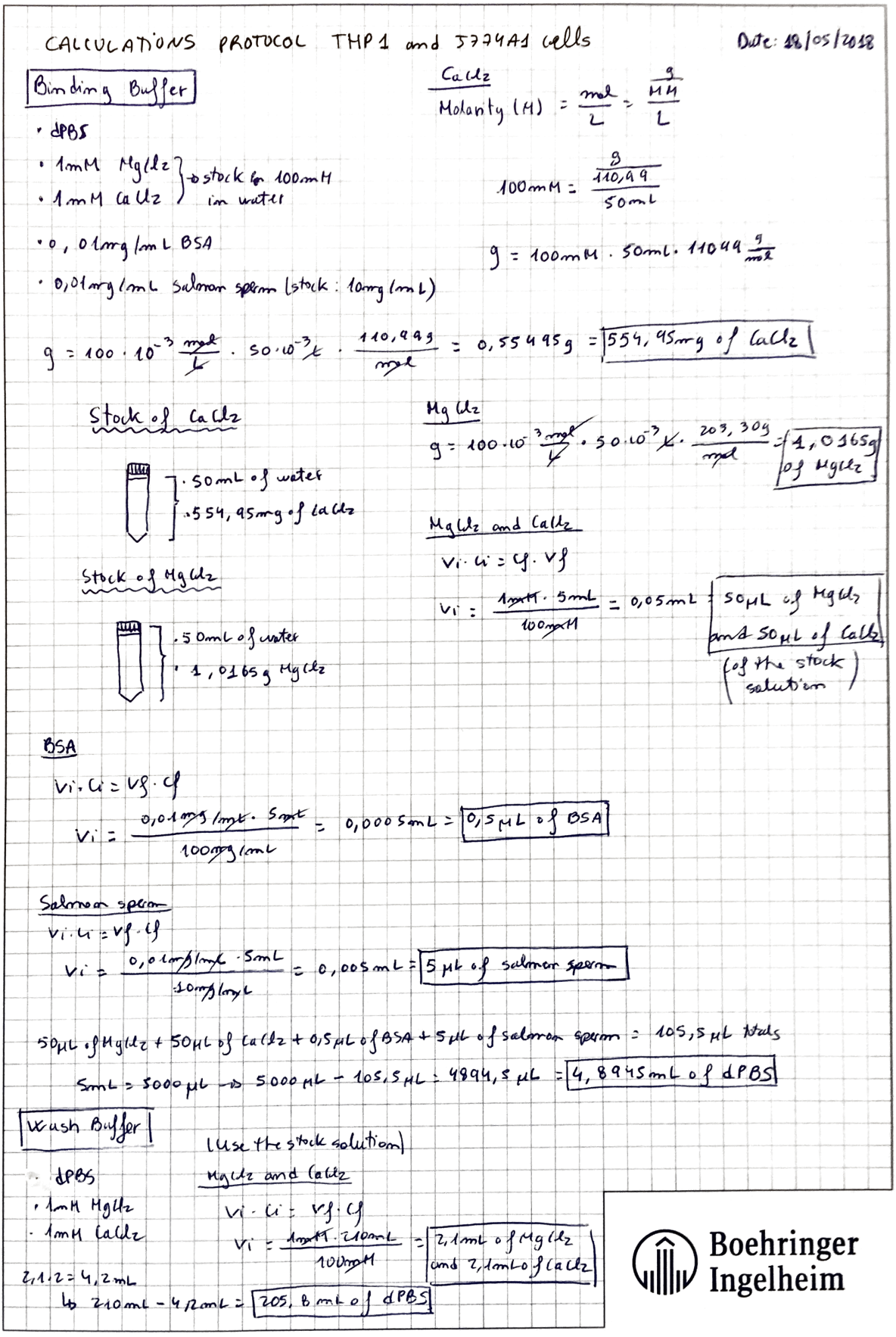

Figure 9. *Calculations for buffers.*

12. Self-evaluation

Before doing the internship, I was not expecting to work in only one field, I thought that I would learn different techniques in the laboratory. Despite not having worked in too many different fields, I performed several repetitions of cell culture experiments and flow cytometry measurement. Thus, I was able to go deeper in what I worked with. Moreover, the cell culture laboratory was in a radioactive area so I also learned the safety instructions for the radioactive laboratory and how to work properly in there.

I would have liked to repeat some of the experiments that I performed as the results were not good enough. Unfortunately, I did not have time to do it and for personal reasons I could not extend the Learning Agreement. Nevertheless, I appreciate every day that I spend in the laboratory and I did not only learn laboratory techniques but also the reality of the labour market.

In the future, I would like to do a longer internship and plan my schedule more accurately in order to optimise the time and be able to perform more repetitions if it is necessary.