INDO SWISS JOINT RESEARCH PROGRAMME (ISJRP)

JOINT RESEARCH PROJECT

FINAL REPORT

Submitted by the Swiss Principal Investigator(s)

Part 1 - General Information

<table>
<thead>
<tr>
<th>Project Title:</th>
<th>Membrane properties affect a trans-membrane receptor’s signaling</th>
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<td>Project Start:</td>
<td>01.05.2009</td>
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<td>Project Duration:</td>
<td>36 months</td>
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Part 2 - Scientific Information

A) SYNTHESIS OF THE PROJECT

In this project, we studied the influence of the local physico-chemical properties of the cell membrane on the modulation of signal transduction by membrane receptors. More specifically, our project focused on CD40 receptor and the way its signals are modulated by the local properties of the cell membrane. Our working hypothesis was that CD40 located in rafts trigger different signalling pathways than the same receptor in non-raft regions. In addition we wanted to clarify a) the physico-chemical properties of the membrane surrounding the CD40 receptors, b) the affinity of the receptor to its ligands c) the influence of the surrounding membrane on the ligand affinity and the relative proportion of receptors expressed in raft vs. non raft regions and d) the overall mechanical properties of the macrophages.
B) RESULTS

*Exploration of the interaction forces between randomly selected peptides and CD40 receptor*

This part of the project was published in [1]

*Atomic Force microscopy.*

The aim of this part of the project was to determine if different types of CD40 receptor ligands induce different cellular responses in macrophages. The first step in such an investigation consists in isolating the peptides that interact in vitro with the CD40 receptor and to select those that bind the best to the receptor. This part of the project relies on the AFM capability to measure interaction forces between a protein (or any other chemical species) present onto the tip and another one (CD40 receptor) attached to the substrate (mica or cellular membrane). For these measurements the AFM cantilever is approached to the surface permitting to the protein present onto its tip to bind to the protein attached to the mica. When the tip is retracted the recently formed binding breaks and the deformation of the cantilever deflection during this event is used to calculate the interaction force between the two proteins. To carry on these experiments we deposited sequentially different peptides onto the AFM tip and probed their interaction force with CD40 receptors that were previously cross linked to a mica surface. The measurement was accomplished by recording consecutive force-distance curves in force volume files. By analyzing these files with OpenFovea (post processing software developed during this project), we could determine the affinity of every tested peptide for CD40 receptors. Figure 1 depicts the histograms of the interaction forces obtained after such a processing. On these histograms one can see for example that peptide nb 18 (pep18 on Figure 1) interacts stronger with CD40 receptor than peptides 5 and 1, whereas peptide 4 interacts in a similar way as the CD40 ligand.

![Figure 1](image-url)

*Figure 1*

Interaction forces between CD40 ligand and CD40 receptor (blue) compared to randomly selected peptides and CD40 receptor (red). Different patterns of interactions and different interaction forces can be distinguished on these figures depending on the tested peptide.
Interestingly, peptide7-CD40 interaction showed two means, representing a possible co-operative interaction (Figure 2). Interestingly, peptide7-CD40 interaction showed two means, representing a possible co-operative interaction (Fig. 4)

**Figure 2**: AFM force histograms

**Figure 3**: Model of CD40-peptide binding domain.

**Figure 4**: Surface plasmon resonance sensograms.

*Surface plasmon resonance*

The CD40-binding peptides- peptide 7 and peptide 19 were run on a GLM chip-immobilized CD40 in a SPR to obtain the sensograms (Fig. 4) and to evaluate their binding constants (ka, kd and Kd) using Langmuir 1:1 binding analyses. The CD40 was also immobilized on a medium density GLC chip and CD154 was flown over it to obtain the sensogram (Fig. 4).
The residuals obtained from the sensogram were well within 10% of the RUmax obtained. The binding constants (Table-1) were calculated using the Proteon-manager software.

<table>
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<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Repeats</th>
<th>$K_1$</th>
<th>$K_2$</th>
<th>$K_3$</th>
<th>Interaction force</th>
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<tr>
<td>P1</td>
<td>WIIWLPVPVPRT</td>
<td>3</td>
<td>$9.9 \times 10^4$</td>
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<td>$3.34 \times 10^6$</td>
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<td>WIIWPNPDWYLLK</td>
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<td>$5.06 \times 10^6$</td>
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<td>WIIWQPLQYPAN</td>
<td>7</td>
<td>$3.0 \times 10^6$</td>
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<td>HIIWAPWHAYT</td>
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<td>HQWYFYTFKFLNL</td>
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<td>$2.43 \times 10^6$</td>
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<td>CD154</td>
<td>-</td>
<td>-</td>
<td>$1.03 \times 10^9$</td>
<td>$1.07 \times 10^9$</td>
<td>$1.02 \times 10^9$</td>
<td>-</td>
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</tbody>
</table>

**Table 1**

**Materials and Methods**

The CD40-peptide interaction was mapped following the established protocol [1]. Briefly, freshly cleaved mica was silanized with 0.05 % of APTES (99% 3-aminopropyl triethoxysilane, Sigma-Aldrich, St. Louis, MO) diluted on water. After incubation for 1 min, APTES was removed and the mica was stored in a dry chamber. Typically, 10 μL of CD40 (50 μg/ml) diluted in phosphate buffer saline (PBS) were deposited on the APTES-treated mica. After incubation for 15 min, the mica surface was washed with ultrapure water. All chemical modification and incubations were performed at room temperature. For force spectroscopy experiments, silicon-nitride cantilever tips (DNP, Veeco, Santa Barbara, CA) were cleaned in a ultraviolet (UV) cleaner, functionalized with 1 % glutaraldehyde (Sigma-Aldrich, St. Louis, MO) and were then bound to the peptides (20 nmol). Force-volume curves were measured in PBS at room temperature using a PicoForce atomic force microscope with a Nanoscope IV controller (Veeco, Plainview, NY). All measurements were performed using a DNP cantilevers whose spring constant was calculated to be around 60 pN/nm, automatically calibrated using the thermal tune method. Peptide-protein complexes were generated by depositing CD40 on the APTES-treated mica, rinsing and after depositing peptides to a final concentration of 20 nmol of peptide. Protein-peptide interactions were examined at ambient temperature in a fluid cell with an O-ring (provider) and ambient vibrations were neutralized as the AFM scanner was placed on a VT-50 stabilizer. The AFM was operated in force volume mode at a scanning frequency of 1 Hz. Force volume data were analyzed and statistically processed using a homemade software (OpenFovea – developed under Python).

Identification of the peptides that bind to CD40: In order to identify the peptide ligands that bind to CD40, we first cloned the extracellular domain of human CD40. The CD40 protein was expressed and characterized (Fig. 5a). Similarly, for comparison of the CD40 binding of the peptides with the recombinant CD40-ligand, we cloned, expressed and characterized CD40-L (Fig. 5b). The peptide phage library was screened on the extracellular domain of CD40 protein to identify the peptide-phages that bind to CD40 (Fig. 5c). The bound phages were assayed by ELISA for CD40 bound to these phages. Those with less than 0.06 OD were considered to be non-significant and were eliminated from the study. Thus, only the peptides with significant interaction with CD40 are shown here (Fig. 5d).
Identification of the CD40-binding peptides that induce counteractive effects

The CD40-binding phages were sequenced and translated to amino acid sequences (Table-1). The peptides were synthesized and examined for their leishmanicidal effects in macrophages. The macrophage colony stimulating factor (M-CSF)-induced, IFN-γ matured macrophages were first phenotyped by high levels of CD206 and CD11b expressions (Fig. 6a). The cells were found to be more than 95% pure macrophages at the time of use in culture. These macrophages were infected with Leishmania donovani promastigotes at a 1:10 macrophage: parasite ratio and treated with four different doses of the recombinant CD40-L or the indicated peptides as described in materials and methods. CD40-L reduced parasite load and so did peptide 19 in these macrophages, but the reduction in parasite load was significantly greater in CD40-L treated macrophages (Fig. 6b). In contrast, peptide 7 significantly increased the parasite load in these macrophages (Fig. 6b) perhaps due to its ability to induce higher IL-10 production from macrophages. Because the cytokines play significant roles in regulating immune responses to various antigens, we assessed the induction of IL-12 from these macrophages. It was observed that the induction of IL-12 was comparable for both CD40-L and peptide 19 whereas peptide 7 induced significantly higher IL-10 (Fig. 6b inset). Thus, we identify the peptide 7 and peptide 19 as the CD40-binding ligands that can evoke contrasting functions.
**Exploration of the mechanical properties of macrophages**

In this part of the project published in [2], we investigated the mechanical properties of macrophages at different scales.

*Investigation of raft CD40 localisation*

The localisation of CD40 in rafts was investigated using a method developed in a previous study [3]. In this project, we compared the local mechanical properties of the cell membrane where CD40L protein localizes with the stiffness of the surrounding membrane. MeCD treatment was used as a control to disrupt rafts by extracting the cholesterol. In a previous work, we demonstrated that rafts are 30% stiffer than their surrounding membrane (i.e. a positive relative stiffness of 30%).

As shown in figure 7, the relative stiffness of the membrane domain where CD40L is expressed posses a relative stiffness value of about zero indicating that they localized outside lipid rafts. The result is similar as computed at 62, 125 and 187 nm distance from the spots where CD40L was detected.

*Investigation of cellular stiffness*
The mechanical properties at micrometre scale were investigated using the stiffness tomography technique developed in our laboratory [4]. Using this method, we highlighted column like structures which size was compatible with phagosomes (Figure 8). The effect of the actin cytoskeleton digestion was also investigated using the stiffness tomography technique. The figure R3 shows the effect of cytochalasin B as measured on the first 50nm underneath the cell membrane. The result from this part of the project were published in [2].

Figure 8
Stiffness tomography slices of fixed (A-B) and living (C-D) macrophages. These slices show stiff structures with dimensions compatible with phagosomes.

Figure 9
Effect of cytochalasin B in macrophage stiffness
Effect of cytochalasin B in the stiffness of the first 50nm of macrophages (from surface to 50nm depth). The histogram shows a clear decrease in the measured stiffness after the actin cytoskeleton digestion.
**Development of a novel fluid cell to image living macrophages**

The component of the AFM that holds the sample and the surrounding imaging medium is usually referred to as the fluid cell, the design of which determines the nature of the experiments that can be performed with the microscope. Consequently, AFM manufacturers offer a variety of fluid cells to cover the broadest possible spectrum of biological experiments. Large fluid cells are usually appropriated for the imaging of samples in Petri dishes (e.g. cells in culture). In permitting a great freedom of movement of the AFM tip, they facilitate the choice of a suitable target from amongst many cells. However, the exchange of fluid during the course of an experiment can be an expensive business if the active agent is pricey. To overcome this problem, manufacturers have developed closed, small-volume fluid cells. The disadvantages of these small-volume chambers are a reduced freedom of movement of the AFM tip and a restriction in the size of the sample or its substrate. Another drawback of these devices is the impossibility of eliminating trapped air bubbles which can ruin an experiment. In addition, depending on the location of the inflow and the outflow tubes, shear forces can detach the sample from its substrate during the fluid-exchange procedure. The secure attachment of a biological specimen to a hard substrate is indeed a universal problem and an ongoing challenge in AFM imaging. In the frame of this project we developed a novel fluid cell which optimizes the volume and the freedom of movement whilst reducing the applied shear forces. Furthermore, since our fluid cell has an open construction, air bubbles cannot become trapped.

![Figure 10. Scheme of a cross-section of the fluid cell. 1: AFM piezo scanner, tip and tip-holding device; 2: objective of the inverted optical microscope; 3: PDMS ring containing the embedded inflow and outflow tubes; 4: PDMS substrate-holder, substrate and sample; 5: inflow tube; 6: outflow tube; 7: Petri dish. The imaging medium has been colored pale blue](image)

In order to verify the fluid flow profile in the fluid cell during liquid exchange we conducted finite-element modelling. The numerical simulations confirmed that, during the fluid-exchange process, the fluid velocity is indeed very low around the AFM tip, which is consequently subjected to negligible shear stress. The simulations were further completed by experiments involving macrophages, bacteria and plant cells. We noticed that even high flow rates (1-2 ml/min) could not detach the cells of their substrate.
**Development of OpenFovea, a data processing software**

This part of the project was published in [5]. A particular effort has been dedicated during this project to the development of software to process AFM data sets. If most of the commercially available AFMs can sequentially record force distance curves i.e. force volume files, few of them are equipped with appropriate software to process this type of data. Since the present project essentially relies on recording force-volume images, in our case a classical, manual, data processing approach was not an option, regarding the tremendous amount of data we have to analyse. The software recognizes bond breaking events on force distance curves. It also can perform stiffness tomography analysis of the sample, which is a new AFM imaging mode developed in our laboratory, and which permits to distinguish structures located underneath the cell membrane.

The output generated by OpenFovea (Figure 12) permits to have a fast feedback on the experiments success, and can be used without modifications for publications.

**OpenFovea characteristics :**

- Compatible with majority of AFM (Bruker, JPK, Asylum)
- Computes stiffness from deflection distance curves.
- The point of contact detection in the force-distance curve permits to handle data with different kind of noises. It can be parameter by : 1) the dependency of the noise 2) the possibility to change the size of the fit segments 3) the possibility to limit the curve portion allowed to be fitted.
- Stiffness can be computed using the Hertz (sphere) or Sneddon (cone) model, and can use different algorithms (raw fit, linearised fit or extrema slope)
- Detects unbinding events from deflection distance curves.
- Worm Like Chain model and Free Joint Chain model allows to characterize and better filter false positive unbinding events.
- Resulting data can be displayed as histograms (Figure 12 h-k) and maps (Figure 12 a-h).
• Histograms can be Gauss fitted with an automatic detection of the peaks. This allows to have a precise and direct quantification of the results.
• Data can be Masked according to: 1) the topography (raw and corrected), 2) the indentation depth, 3) the noise, 4) the event presence, 5) all event characteristics. This allows to exclude data that are not relevant or too noisy.
• The software is compatible with windows and MacOSX.
• Data can be grouped, for example relatively to the injection of chemical, in order to be compared (Figure 12 k)

Figure 12
Illustration of OpenFovea post-processing outputs. (a) Topography image of living THP-1 macrophage. (b) Stiffness of the layer located between 0 and 50 nm depth. Blue : compliant, red : stiff. (c-d) Stiffness tomography mode. (e) y- and z- slices (top to bottom) and (d) 3D representation. (e-f) Mapping of the distribution of detected unbinding events, depicted as grey areas, in function of (e) the number of unbinding events per pixel, (f) the force of the unbinding events per pixel and (g) the distance of the unbinding event. (h) Unbinding events force histogram. (i) Event force versus loading rate scatter plot. (j) Histogram illustrating differences in the stiffness of areas in which an unbinding event was (blue) or was not (red) detected. (k) Histogram illustrating the stiffness distribution of 2 different cells which were probed with two force-volume scans each. Horizontal scale bars are 1 µm.

Bibliography


C) **LIST OF PUBLICATIONS**

**Publications in reviewed journals**


Radotić, K, Roduit C, Simonović J., Hornitschek P., Fankhauser C., Mutavdžić, D., Steinbach G., Dietler G. and Kasas S. AFM stiffness tomography on living *Arabidopsis thaliana* cells reveals the mechanical properties of surface and deep cell wall layers during growth, Accepted for publication in *Biophys. J.*

**Poster presentations**


C. Roduit, G. Dietler, B. Saha and S. Kasas AFM studies of macrophages, Red Island (HR), 12.05. 2010 Poster.


C. Roduit, G. Dietler, B. Saha and S. Kasas, AFM studies of macrophages, Linz (AT), 04.02.2011, Poster.