0000000124 – ANTAGONISM

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Using antagonism to reveal the mechanisms of T Cell antigen discrimination

Consortium :

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1. EXCELLENCE

1.1. Pre-proposal's context, positioning and objective(s)

T cells can discriminate antigens of different affinities with remarkable efficiency. Within minutes if not seconds, they can respond to just a few antigens of high affinity while remaining irresponsive when presented with large quantities of weaker affinity antigens [1]. How T cells manage to achieve such performances has been the focus of intense efforts. However, our understanding of the molecular mechanisms mediating antigen discrimination is still incomplete. Modeling efforts have provided insights on possible signal transduction mechanisms that reproduce different features of ligand discrimination. The kinetic proof reading (KPR) model, initially introduced as a way to reduce errors during transcription and translation [2], was later proposed as a possible mechanism to achieve ligand discrimination in T cell receptor (TCR) signaling [3]. Many variants of the KPR model have since been proposed to reproduce with even more fidelity the responses of T cells to large ranges of antigen quantities and affinities [4]. Among the variations around the KPR scheme, the addition of a *proximal negative feedback* was proposed as a way to increase specificity without compromising the speed and sensitivity of the response [5], [6]. This model can reproduce most known features of TCR antigen discrimination and is in particular able to account for a special feature of TCR antigen discrimination, namely *antagonism*. Antagonism is defined as the dampening of T cell responses in the presence of *weak* affinity ligands. It reflects the fact that single TCRs do not signal in a purely independent manner. Rather, TCRs engaged with weak affinity ligands have the capacity to dampen the signaling of TCRs engaged with high affinity ligands. This antagonism due to abundant weak affinity ligands has consequences in different contexts of T cell activation, and in particular in CAR T cells where CAR signaling can be effectively dampened by endogenous TCRs engaged with weak affinity ligands, thereby limiting the efficiency of CAR therapy [7]. Production of potent TCR antagonists can also be used by pathogens or tumor cells to escape immune surveillance. Hence it is of major fundamental and clinical importance to understand how antagonism operate and to determine under which circumstances weak affinity ligands can dampen the response to high affinity ligands.

Our preliminary results show that antagonism can be observed when both high and weak affinity ligands are presented to a given T cell by the *same* antigen presenting cell (APC) but *not* when ligands are presented by different APCs in a parallel or successive presentation scheme. Our hypothesis is that antagonism operate *very locally during antigen recognition early steps*. One objective of the present project is therefore to determine on which scales, both spatial and temporal, antagonism can be observed and is affecting T cell efficiency of activation. The second objective is to determine how antagonism is implemented at the molecular level and reveal the molecular mechanisms responsible for the antagonistic effects due to weak affinity ligands.

The present project proposes to use phospho-proteomics, gene editing by CRISPR-Cas9, microscopy and innovative T cell stimulation by biophysical methods based assays to meet these different objectives. This project is at the crossroads of immunology, biophysics and systems biology and promises to offer new perspectives on T cell antigen discrimination through the prism of antagonism.

Task 1 : Probing the spatio-temporal scale of antagonism

Our preliminary results show that antagonism can be observed using irradiated splenocytes as antigen presenting cells. In this setting, antigen presenting cells bearing two different antigens are co-cultured with T cells for several hours to several days. This type of stimulation allows the quantification of T cell responses in antagonistic conditions across long time scales. For instance, one can use it to quantify T cell proliferation, the abundance of activation markers, the production of cytokines and determine how the presence of a weaker affinity ligand modulate T cell responses to a higher affinity ligand by varying the relative proportion of the two. In this setting, our preliminary results indicate that weaker affinity ligands can dampen the response to higher affinity ligands only when both ligands are presented on the surface of the same cell, and not when they are presented by different sets of cells that the T cell may encounter sequentially. This observation suggest that antagonism can only be observed when the distance between TCRs engaged with different ligands is sufficiently small. Although this type of T cell

stimulation present numerous advantages, it does not allow to control precisely the spatial organization of ligands presented to T cells, and hence cannot be used to further determine the spatial scale on which antagonistic effects can be observed.

We propose to use engineered substrates to precisely control the spatial organization of ligands presented to T cells. We will use nano- / micro-patterning techniques available in LAI, in collaboration with the CINAM (K. Sengupta), to control the distance between weak and high affinity ligands [8], [9], [10]. A pattern of circular patches of weak affinity ligands will be produced e.g. using a PDMS stamp. The inter-patch space will then be filled with high affinity ligands. By changing the patch radius (200nm-sev. µm) and the inter-patch distance (500nm-sev.µm) we will be able to control both ligand density and mean distance between ligands of different affinities. T cells will be deposited on this patterned substrate and we will monitor their response to TCR ligands by measuring the T cell-substrate contact area using RICM and the T cell intracellular calcium flux using fluorescence microscopy. This reductionist approach will therefore allow us to determine on which spatial scale antagonism operate. This setting will also offer the possibility to probe how different parameters known to regulate T cell activation such as substrate stiffness, the presence of T cell adhesion ligands (ICAM1) or co-stimulatory ligands (CD80, CD86) can also modulate antagonism. Using soft substrates may also pave the way to record exerted forces on the substrate using traction force microscopy [11].

To consolidate the results obtained using patterned substrates in more physiological settings, we propose to use supported lipid bilayer (SLB) substrates carrying two types of freely diffusing fluorescently tagged pMHC ligands, at various relative densities, and to monitor productive TCR signalling responses through the formation of LAT condensates as pioneered by the international secondment JT Groves [12], [13], [14]. To do so we will use T cells expressing a fusion LAT-GFP protein. We will use surface-based interference microscopy and fluorescence microscopy (RICM+TIRF, with L. Limozin, LAI) to track single pMHC-TCR binding events together with the global cell spreading state and to determine whether binding subsequently produce productive TCR signaling by forming a LAT condensate. Although the distance between ligands is not controlled in this setting, we will monitor their spatial localization and temporal duration using time lapse microscopy [13]. This system will therefore be ideally poised to reveal the spatial and temporal scales with which binding events with weaker affinity ligands can modulate the response to higher affinity ligands. As for patterned substrate, lipid bilayers can also be prepared with different relative ligand densities, different compositions and we shall test whether the presence of adhesion and co-stimulatory molecules affect antagonism in this setting.

These different assays will provide a unique way to characterize how T cells respond to a mixture of ligands of different affinities under well controlled conditions. T cells subsets exhibit differences in the abundances of TCR signaling machinery components and in sensitivities to antigen stimulation according to their subset (CD4 or CD8) or activation status (naive, memory, effector). We intend to use the previously described assays to examine how antagonism could be, or not, conserved among the different alpha-beta T cells subpopulations.

Task 2 : Defining the molecular mediators of antagonism using phospho-proteomics

The molecular mechanisms responsible for the antagonism mediated by weak affinity ligands is still incompletely understood. Previous studies indicated that an early negative feedback during proximal TCR signaling could be mediated by the recruitment of the phosphatase PTPN6 (SHP-1) and its dephosphorylation of proximal components of the TCR signaling pathway including LCK and ZAP70, thereby preventing the propagation of TCR signals [15], [16].

A recent phosphoproteomics study from our group at CIML showed that, under conditions of similar TCR occupancy, weak affinity ligands could trigger the phosphorylation of a group of proteins to the same extent as high affinity ligands [17]. These results suggested that the negative action of weak affinity ligands could be due to the action of a negative feedback module extending beyond the mere contribution of the phosphatase PTPN6. For instance, we could identify CD6 as a candidate for the

mediation of the negative feedback enforcing early antigen discrimination. Indeed, CD6 was found in the group of equally phosphorylated by weak and high affinity ligands and its absence increased the response to weak affinity ligands while having only a moderate impact on high affinity ligands. To consolidate these results, we propose to use antagonism to as a way to provide direct evidence of the participation of a given candidate protein in the early *negative* feedback mediating antigen discrimination. Indeed, a reduction of the intensity of the negative feedback should result in a decreased antagonistic effect. If that was indeed the case, antagonism should be *less* pronounced in the *absence* of CD6.

To refine our understanding of antigen discrimination using antagonism, we will quantify how weak affinity ligands reduce the early phosphorylation of proteins triggered by high affinity ligands. We will use mass spectrometry (MS)-based phospho-proteomics methods to identify antagonistic effects at the proteome scale. T cell stimulation with APCs is not easily compatible with MS-based proteomics assays for different reasons. First it involves two different cell types making it challenging to attribute phosphorylation events to T cells rather than to APCs. Second, synchronization of TCR engagement is hard to achieve using APCs. These issues were solved in our previous study by the use of soluble pMHC tetramers bearing TCR ligands of different affinities. We therefore intend to stimulate T cells with a mixture of tetramers with pMHC ligands of different affinities. Our preliminary results, show that this type of stimulation indeed results in antagonistic effects. Combined with phospho-proteomics methods, this type of assay will allow us to identify phosphorylation events impacted by the presence of weak affinity ligands during the first minutes following TCR stimulation.

Once candidate mediators of antagonism are obtained from the analysis of the phospho-proteomics tetramer experiment, we will confirm their role by testing whether their absence modulate antagonism. We will use CRISPR Cas9 gene editing methods to inactivate the corresponding genes in primary T cells. Antagonism effects will then be quantified in these edited cells using the different assays presented above (APCs, tetramers, patterned substrates, Supported Lipid Bilayers).

1.2. Interdisciplanary and intersectoral dimension of the project

Dr G. Voisinne is an expert in integrative biology of T cell activation, signaling pathways analysis and functional dissection of T cells. In recent years, in close collaboration with Anne Gonzalez de Peredo (IPBS, Toulouse), he has been using MS-based proteomics methods to delineate molecular mechanisms underlying T cell activation at the system-level [17], [18], [19].

Dr PH Puech has brought numerous contributions to the field of mechano-transduction phenomena in T cells over the last 15 years. He is a specialist in atomic force microscopy (and pioneer in Single Cell Force Spectroscopy), micropipettes and optical tweezers. He recently set-up traction force microscopy and the use of soft substrates as APC surrogates for T cells. He works closely with L. Limozin (an expert in cell imaging, surface microscopies, image analysis, supported bilayers, working at LAI) and K. Sengupta (an expert in soft and patterned substrates, working in CINAM).

While MS-based methods, mastered by G. Voisinne, offer a deep characterization of the molecular events triggered following T cell engagement, they are obtained through the synchronized stimulation of a large number of cells to provide enough protein material for MS analysis. However, this type of T cell stimulation do not permit to control or monitor how TCRs are engaged on a fine spatial and temporal scale.
On the other hand, biophysical assays developed by Dr PH Puech allow to control and monitor how TCRs are engaged with high resolution in space and time. Hence the expertise of both supervisors are highly complementary to study antagonism across different scales.

The intersectoral partner is the doctor Nassima Chouaki-Benmansour (MD, PharmD, PhD) who works at the Institute Paoli Calmettes (IPC) in Marseille. The IPC is an important regional hospital for cancer

treatment where immunotherapies are used and where T cell based clinical trials are conducted. The fellow will hence have a unique opportunity to witness how strategies to harness T cell responses to fight cancer are deployed in the clinic.

2. IMPACT

2.1. Expected impact of the project on the candidate's career

In this project, the fellow will receive training in many different techniques and methodologies that should be great assets for a future career inside or outside academia.

Indeed, the fellow will learn to master soft lithography techniques, advanced microscopy techniques (Fluorescence, TIRF, RICM), biophysical techniques (atomic force microscopy eg. to caracterize substrates), in vitro culture of primary cells and cell lines, flow cytometry, biochemistry, CRISPR-Cas9 gene edition methods which are all widely used in diverse fields of academic research, in particular in immunology, and are high in-demand skills in biotechnology companies. The fellow will also follow a specific training necessary to perform animal experimentation in accordance with the national legislation which could be a valuable asset as it could be a prerequisite to apply for jobs in companies conducting animal studies.

The fellow will also be trained to analyze complex data (images, phospho-proteomics data) with open source software and programming languages (Fiji, R, Python) with the respect of best practices for reproducible and open science, and will learn to test and validate research hypotheses on a solid statistical foundation.

The fellow will also have the opportunity to supervise master students and develop his project management skills. At the end of his PhD, the fellow will have learned to conduct robust and reproducible research and will have a solid basis for a successful career in science.

2.2. Expected impact for the thematic axis

T cells are crucial actors of adaptive immunity and play important roles in protection against pathogens and anti-tumor responses. As the functions of T cells are largely determined by the recognition of antigens by the TCR, this project should have an important impact in health and disease.

More specifically, the project will improve our understanding of the molecular mechanisms responsible for the high specificity and sensitivity with which T cells discriminate antigens. Building on this knowledge, new strategies could be designed to increase T cell responses against pathogens or tumor cells, or inversely to reduce recognition of self antigens to attenuate detrimental auto-immune responses.

Antibodies binding to surface proteins mediating antagonistic effects could be used to modify affinity thresholds for productive T cell responses, providing potential new candidate immune checkpoint inhibitors for immunotherapies. These thresholds could also be altered using chemical inhibition of intracellular proteins involved in antigen discrimination.

In CAR-T cells therapies, T cells are modified to express a chimeric antigen receptor recognizing a tumor antigen with high specificity. However, these cells also express an endogenous TCR that might recognize weaker affinity tumor antigens and antagonize CAR signaling. In that context, our improved understanding of antagonism will help reducing antagonistic effects by targeting its molecular mediators and improve CAR T cell responses. Similarly, in tumor infiltrating lymphocytes (TIL) based therapies where patient T cells recognizing tumor antigens are expanded in vitro before being re-injected in the same patient to amplify the immune response against tumor cells, strategies modifying antigen recognition thresholds could produce even more effective T cell responses.

2.3. Dissemination, exploitation and communication activities planned

We will encourage the fellow to participate in the “Fête de la science” festival which promotes encounters between researchers and the public. It will a great opportunity to communicate about how we carry out fundamental research and what benefits it brings to society. Because the project addresses the fundamental question of self / non-self recognition (antigen discrimination) which is a process at the heart of health and disease and because it will deliver appealing images (patterned substrates) and movies (fluorescent ligands diffusing on supported lipid bilayers), we anticipate that its presentation will encounter a great success with a wide audience. Aside, the fellow will be encouraged to participate in “Tous Chercheurs Luminy” (https://www.touschercheurs.fr/), which aims to educate and involve different audiences (schools, patient associations, professionals, the general public) in scientific research in order to promote understanding of how science is conducted in research laboratories. This can be counted in the frame of the training hours that a PhD candidate have to pursue during the duration of his/her PhD.

Scientific results will be disseminated through publications in peer-reviewed journals and participation in scientific conferences. We will base as much as possible our technical designs, programming and data analysis tools on open source softwares and languages to share them with the largest scientific community possible, for example by using dedicated data and software forges (GitHub, GitLab, Zenodo). We will contact the CNRS communication service to relay results accepted for publication to media and social networks. Dissemination of results will also be done through the websites and social network accounts of both LAI and CIML.

To complement the above dissemination strategy, experimental and theoretical outcomes of the work will also be used as up-to-date teaching material for graduates and master students.

3. IMPLEMENTATION

3.1. Work plan

The fellow will be welcomed as a full unit member in both the CIML and the LAI during the entire duration of the project. The fellow will meet with supervisors on a regular basis and at least twice a month with both supervisors present. That will ensure that both supervisors are aware of the advancement of the project and regularly update and adjust the working plan according to the results and potential difficulties encountered. The fellow will have the opportunity to attend regular lab meetings in each lab to make sure that he is involved in scientific discussions and can benefit from help and tips from labmates and researchers on both sides. The fellow will benefit from the CIML PhD program that fosters discussions and collaborations between PhD students. The fellow’s time will be shared between the two institutes depending on the project’s advancement.

During the first months, the fellow will learn the basics of T cell biology at the CIML and will have access to CIML facilities and receive the mandatory training to become a certified user of these facilities. He will be trained to dissect mice and collect immunologiocal tissues in order to recover primary T lymphocytes. He will also learn how to purify CD8 and CD4 T cells, culture primary T cells and T cell lines in vitro, activate T cells and monitor their activation status using flow cytometry and biochemical assays. Within a few months, the fellow should be able to master the experimental techniques necessary to probe antagonism using APCs and tetramers. In LAI, he will be trained on the shared cell culture platform and will receive the set of trainings required to work there as defined in the lab internal rules (safety, informatics).

For the use of patterned substrates, the fellow will benefit from the close collaboration of P-H Puech with Kheya Sengupta at CINAM to master the design of patterns and soft lithography techniques. Functionalization of patterned substrates with streptavidin makes this system compatible with any biotinylated molecule. As we will use biotinylated pMHC ligands, we are confident that the creation of patterned substrate with TCR ligands of different affinities will be achieved seamlessly. For image

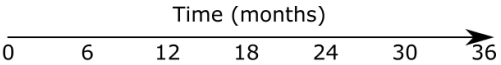
acquisition and analysis, the fellow will received training on microscopy and be tutored for the use of in-house Fiji scripts and open source software (in particular CellDetective, https://github.com/celldetective/, developped and tested at LAI by L. Limozin and his students), to quantify cell shapes and spreading.

For his work with supported lipid bilayers (SLB) and quantitative interference microscopy (IRM/RICM), the fellow will benefit from the support of recognized experts in the field. In LAI, the fellow will have support from L. Limozin who has developed specific microscopy techniques and image analysis routines to analyze the activation of T cells on these substrates. The fellow will also receive help during his visits to the lab of JT Groves. This international secondment lab has pioneered the use of SLB to track single binding events and the subsequent formation of LAT condensates. The fellow will therefore receive first-hand training to master this cutting edge technique.

In CIML, the fellow will receive extensive training to master gene edition by CRISPR-Cas9 in primary T cells. The host team has validated the efficiency of this technique for tens of targets initially identified in a previous study. However, in case where the process of CRSIPR-Cas9 gene edition is not compatible with antagonism assays (because T cells are not naive anymore, but have to be activated before transfection), we will seek to obtain mice, either from academic partners or from private companies, where the target gene is constitutively deleted in mature T lymphocytes.

To study antagonism in CD8 T cells, we will use OT-1 cells for which a collection of antigenic peptides with different affinities are available. The lab also already has stocks of biotinylated peptide MHC ligands of different affinities for the OT-1 TCR that will be used to produce tetramers, patterned substrates and supported lipid bilayer assays. As for CD4 T cells, we will use OTII cells where peptides and biotinylated pMHC monomers are also available.

We provide below a tentative Gantt chart giving a prospective overview of how the student’s time will be spent during the project.



Task 1 Characterize the spatial and temporal scales of antagonism	Set up patterned substrates with antigens of different affinities to determine the spatial scale at which antagonism operate.	
	Set up supported lipid bilayers bearing fluorescently tagged antigens of different affinities to reveal spatio-temporal dependencies between binding events.	
	Validate the role of a set of proteins as mediators of antagonism using patterned substrates and supported lipid bilayers	
Task 2 Define the molecular mediators of antagonism	Master basic T cell biology techniques. Reproduce antagonism results using antigen presenting cells.	
	Use tetramers to probe antagonistic effects on short time scales. Identify candidate mediators of antagonism using phospho-proteomics analysis.	
	Validate the role of a set of proteins as mediators of antagonism using antigen presenting cells and tetramers.	
Secondments and thesis defense	Visit to the international secondment Jay T. Grooves (3 weeks each)	
	Visit to the intersectoral secondment Nassima Chouaki Benmansour (1 week each)	
	Writing of thesis manuscript and preparation of thesis defense.	

4. ETHICS SELF-ASSESSMENT

Animals

Mice have developmental and immune response programs very similar to those of humans. In addition, the molecular pathways controlling T cell activation are very well conserved between murine and human T cells. Murine T cells therefore constitute a model of choice to study the functions of T lymphocytes. Importantly, the use of mouse T cells has led to major discoveries for human health. In particular, the immune checkpoints CTLA4 and PD1 whose discovery has radically change cancer treatment where first discovered in murine T cell studies.

The project will use OT-I and OT-II TCR transgenic mice from which we will harvest splenocytes and purify primary CD8 and CD4 lymphocytes respectively. The project will also use C57BL/6 mice that will be sacrificed to collect splenocytes serving as antigen presenting cells. No experimental procedure will be conducted on these mice.

T cells from these OT-I and OT-II mice all carry the same TCR recognizing a specific epitope from chicken ovalbumin. These models are widely used to study TCR signaling because TCR ligands of different affinities are commercially available.

When possible, we will replace mouse primary cells with cells from the Jurkat T cell line that has been derived from a human lymphoma and that will be made to express the murine OT-I or OT-II TCR. These

cell lines could be used to validate our different T cell stimulation assays. Even if they are considered as recapitulating key points of T cell activation, these cells can not entirely replace the use of primary T cells to study TCR antigen discrimination because their proteome and basal metabolic state largely differ from that of primary naive T cells,

The different mouse strains will be bred under specific pathogen free conditions in the CIML animal facility according to the French legislation. We will strictly adapt the number of breeding cages that will be set up and maintained to our experimental needs in order to minimize the generation of mice. Animals will benefit from husbandry conditions with complex social and physical environments, allowing to reduce stress and subsequent experimental variability which permits to reach statistically sound conclusions with smaller group sizes.

No overt phenotype that affects the health of the mice or results in suffering has been reported at steady-state for the strains of mice which we intend to use. Mice will be humanely killed via CO2 inhalation and secondary cervical dislocation. We estimate that approximately 200 mice will be used throughout the duration of the project.

Non EU countries

The student will visit the lab of the international secondment JT Groves in the USA. This will allow the fellow to receive precious training in a cutting edge technique that has been pioneered by the lab. Of course, her/his visit will be subjected to international regulations, such as involving the application to needed documentation and to the Inserm/CNRS/AMU FSD procedures at the date of the visit.

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