Différenciation et physiologie des lymphocytes T

Rapport Hcéres

agence d'évaluation de la recherche
et de l'enseignement supérieur

Section des Unités de recherche

Rapport d'évaluation

Unité de recherche :

Différentiation Thymique

et Physiologie des Lymphocytes T

de l'Université Paris 5

Mars 2009

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Le Président de l'AERES	Section des unités de recherche
Jean-François Dhainaut	*Le Directeur* Pierre Glorieux

mars 2009

Rapport d'évaluation

L'Unité de recherche :

Nom de l'unité : Différentiation Thymique et Physiologie des Lymphocytes T

Label demandé : UMR_S INSERM

N° si renouvellement : U591

Nom du directeur : Mme Benedita ROCHA

Université ou école principale :

Université Paris 5

Autres établissements et organismes de rattachement :

INSERM

Date de la visite :

11 Février 2009

Membres du comité d'experts

Président :

Mme Marie MALISSEN, Université Aix-Marseille 2, France

Experts :

M. Hans Reimer RODEWALD, University Hospital Ulm, Germany (Absent excusé)

M. Freddy RADTKE, Ecole Polytechnique Fédérale de Lausanne, Switzerland

M. Olivier LANTZ, Institut Curie, Paris, France

Expert(s) représentant des comités d'évaluation des personnels (CNU, CoNRS, CSS INSERM, représentant INRA, INRIA, IRD...) :

M. Grégoire LAUVAU, représentant des CSS INSERM

No CNU representative was available on the day of the visit

Observateurs

Délégué scientifique de l'AERES :

M. Marc BONNEVILLE

Représentant de l'université ou école, établissement principal :

Mme Marie-Claude LABASTIE, Université Paris 5

M. Bruno VARET, Université Paris 5

Représentante des organismes tutelles de l'unité :

Mme Christine TUFFEREAU, INSERM

Rapport d'évaluation

1 • Présentation succincte de l'unité

- Effectif : 20 personnes dont
  - 3 chercheurs
  - 3 postdoctorants
  - 5 doctorants, tous financés
  - 3 ingénieurs
  - 2 techniciens et administratifs
- Nombre de HDR : 3, tous encadrant des thèses
- Nombre de thèses soutenues depuis 4 ans : 11
- Nombre de publiants : 3 sur 3

2 • Preparation and execution of the visit

Prior to the site visit, the committee members received a detailed report prepared by the person that intends to direct the unit.

The committee first met in the morning of February 11 prior to the site visit. The scientific delegate of the AERES explained the AERES evaluation. The experts had a first short closed-door session to prepare the review. The review then took place, starting with a presentation by the present director of the unit. Then, each group leader presented her work.

In the afternoon, a visit of the laboratory was organized and the committee met privately to discuss the review. During this private session, the committee received separately engineers, technicians and administrative staff, then postdoctoral fellows and students. The committee discussed the perspectives of development of the corresponding campus with University representatives. Before the final discussion with the unit director, the committee had a short discussion with the group leaders.

The committee would have appreciated to have the unit director devoting more time to present the whole unit, the personnel, the general organization and the mode of access to various platform and facilities. This would have permitted to reach a global and integrated view of the evolution planned for the whole Unit. The presentation was almost exclusively oriented towards the actual results and future plans of her own team. Future plans for the evolution of the unit on the Necker campus were only briefly discussed in private session that occurred later in the visit.

3 • Overall appreciation of the activity of the research unit, of its links with local, national and international partners

The Unit is dedicated to T cell physiology and develops two major axes that correspond to the differentiation of hematopoietic precursors in mature T cells and to the study of T cell behavior, mainly CD8 cells, during an immune response. The strong scientific interactions that exist between the three teams are readily examplified by collaborative experimental approaches and an extensive exchange of expertises. The choice to share all financial support and most of the technical help in this small size unit is very appropriate and facilitate

management duties. The lack of administrative personal in the present unit constitutes a tremendous burden for the actual director who is faced with heavy administrative charges. A temporary solution needs to be found rapidly in the near future and a permanent solution had to be worked out for the long term (see below). In addition, the unit would really benefit from the addition of a new research team that could complement and increase their domain of expertise. However, the ongoing renovation plans of the Necker Hospital prevent such recruitment. More specifically, the Necker Hospital site has to undergo major changes with the construction of two new buildings and with major renovations occurring in other buildings. Future organization and localization of some research teams are still under debate. One of the projects is the creation of an Institute with 3 departments: Genetic diseases, Immunology/Hematology and Cell Biology. Incorporating U591 to such an institute with shared administrative and scientific platforms would certainly constitute a great opportunity for U591. Owing to the evolution of Biology, it sounds really difficult nowadays to stay competitive without access to state of the art scientific platforms.

The project of an Institute is still under study and would certainly not be finalized before a couple of years. In the meantime renovations will proceed in the existing building where Unit591 is hosted. Actually, there is no simple solution that would satisfy everyone and U591 teams would probably have to move twice. The current project is hopefully to keep every one the Necker campus.

The committee is aware of the complexity of the current situation on Hospital Necker campus and wants to stress that despite such important difficulties U591 personals have nevertheless progressed in their work in a very positive way.

4 • Specific appreciation project by project

Team 1

The leader of the team, who is also the director of the INSERM Unit, is an INSERM Directeur de Recherche Classe Exceptionelle (DREC) and has an internationally recognized stature in the field of in vivo CD8 T cell differentiation into effector and memory cells, witnessed both by the impressive list of attented conference as invited speaker as well as by the numbers of invited reviews and comments in highly cited journals (Current Opinion Immunology, Immunological reviews, Nature Immunology, Science…). By developing a highly original and powerful quantitative PCR-based assay at the single cell level (multiplex PCR), this team has made novel and conceptually important discoveries highlighting the cell heterogeneity of each activated and memory CD8 T cell and revealing distinct effector types on the different phases of the immune response. In addition, building up on its long standing experience defining the intrinsic properties of memory CD8 T cells, this team has recently discovered that memory cells are indeed in a unique G0/G1 preactivated state allowing their rapid division following antigen-driven activation. These studies have led to several publications, some of which in very high profiles journals such as Nature Immunology and The Journal of Experimental Medecine. The research projects continue to study CD8 T cell memory with new, fruitful and interesting hypotheses. However, the committee felt that although multiplex PCR technology is a very powerful technique that is unique to the unit, to rely heavily on it for most of the projects might narrow the type of questions addressed. The hypothesis of a failure of DNA repair to explain the CD8 death during primary reponses is interesting but no preliminary data were provided to support this hypothesis. To engage into extensive breeding with mouse strains deficient in DNA repair enzymes is risky in such settings. On the other hand, the committee positively noticed the willingness of the team to study models relevant to pathology (thymus graft) or to vaccines using mice infected with different pathogens.

The second long-standing research interest of team 1 is the area of T cell development. Specifically, the team has addressed the developmental origin of intestinal T cell populations. Through a series of complex thymus and bone marrow grafting experiments, evidence could be obtained to suggest that at least some of these T cell populations arise extra-thymically. Moreover, in a recent publication in Nat. Immunol., the PIs of teams 1 and 2 proposed an entirely new function of the thymus, i.e. the export of T cell progenitors from the thymus to the periphery. According to the data generated by this team, pro T cells can exit the thymus before completing their intra-thymic maturation. Next, these exported Kit+ pro T cells could migrate to the gut where they continue and complete their development. This concept is certainly very original, and the team appropriately plans to follow up on their observations. Their unpublished data along this line look very promising. It would,

however, be important to demonstrate T cell progenitor export under more physiological conditions than by thymus grafting. This is certainly one of the ongoing projects and the future plans of the team. One aspect that one might wonder about is whether the development of such minute numbers of progenitors can in the future be studied primarily by cellular means, or whether alternative approches such as genetic fate mapping would provide further or additional insight. Collectively, the questions worked on by the team are highly relevant and original in T cell development, and the team has a prominent role in the field.

Nom de l'équipe : T Cell physiology

Note de l'équipe	Qualité scientifique et production	Rayonnement et attractivité, intégration dans l'environnement	Stratégie, gouvernance et vie du laboratoire	Appréciation du projet
A+	A+	A+	NN	A+

Team 2

The major scientific focus of the PI from team 2 in the time period of 2003-2008 was T cell lineage commitment of hematopoietic progenitors. The predominant view was and is that hematopoietic bone marrow progenitors constantly seed the thymus, where they get instructed towards the T cell lineage and further mature into fully functional T cells. While most research groups focused on sorting different BM subpopulations for assessing their T cell potential, the team leader analyzed colony-forming units in the spleen. These spleen colonies form within 12-13 days after BM transplantation. Each colony is derived from a single hematopoietic stem cell which gives rise to progenitor cells with myeloid and lymphoid potential. This group was able to show that these colonies consisted not only of uncommitted progenitor cells, but indeed contained pre-T cells suggesting that the first organ where T cell lineage commitment occurs after BM transplantation is the spleen and not the thymus as previously assumed. Using various transplantation models (for example grafting nude BM into CD3KO hosts) she made sure that the formation of pre-T cells in the spleen is indeed thymus independent. Moreover, she showed that the splenic pre-T cells are also present in unmanipulated mice. Their numbers are enriched in mice with a non-functional thymus suggesting the presence of a negative feedback mechanism between the thymus and the ability of the spleen to host or generate pre-T cells. Furthermore she adopted an elegant quantitative multiplex single cell RT-PCR from the team 1 to perform gene expression analysis of various potential pre-T cells and/or thymus seeding cells. In summary the scientific contributions of team 2 were important during this time period as it showed that other organs than the thymus can generate pre-T cells which are fully competent to develop into normal functional T cells.

The future research plan of this team represents a logic continuation of what has been established in previous years. It aims at defining the cellular and molecular networks of T cell commitment using a combination of in vivo and in vitro approaches combined with an extended version of the quantitative multiplex single cell RT-PCR. The same methodology will also be used to investigate the gene expression pattern of early T cell progenitors derived from various gene targeted (Gata3 ko, IL-7Ra ko) or reporter mice (Pu-1-EGFP). Although this is a logic continuation of the previous work it lacks a little bit of novelty. For example gene expression profiling using quantitative RT-PCR of early thymic T cell progenitors has already been performed on a population level (Taghon et al. Immunity 2006). It would be worthwhile to seek advise from and to collaborate with bioinformaticians to try to integrate the data from the single cell PCR into a systems biology approach to identify potential hubs and regulatory gene networks. In summary the planned research appears to be good and solid, but lacks a little bit of novelty. There is no new opening into another or related field

Nom de l'équipe : Hemopoietic differentiation and T Cell commitment

Note de l'équipe	Qualité scientifique et production	Rayonnement et attractivité, intégration dans l'environnement	Stratégie, gouvernance et vie du laboratoire	Appréciation du projet
A	A	A	NN	A

Team 3

This team is directed by a young INSERM Chargé de Recherche 1 with HDR who was trained during her PhD by the group leader of team 1. Building up on the key finding of her PhD work (Science 2002), and after a postdoctoral training at the NIH (Bethesda, USA), the group leader continues to work on the role of CD4 T cells help on CD8 T cells differentiation via the CD40/CD40L interactions. Several papers have been published over the past years as senior author in specialized journals of the field (Journal of Immunology) and some competitive support from the ANR Jeunes Chercheurs has been obtained, witnessing the activity of this emerging team. While it is clear that this research project is under the direct and independent supervision of the team leader, the committee expresses several concerns on the scientific project. The current experimental system used by the team remains quite far from biologically relevant situations (high frequency of monoclonal T cells transferred into monoclonal T cell bearing hosts) and the specific aim related to intravital imaging is both highly competitive and technically challenging. There is also no real conceptual or technical novelty in the different projects. On a whole, the proposed projects are either directly the continuation of the previous ones with the same potential source of artefacts or are based on new technologies (intra-vital microscopy) for which no preliminary data were provided. Altogether, this situation may limit the generalization and the impact of the findings found in the experimental models studied. Another issue is the theoretical and technical proximity with the team 1 group leader. Such proximity seems to prevent team 3 leader to gain sufficient recognition and build a group of sufficient size. To solve this problem, either the head of the Unit needs to increase the support (which is already substantial) to this young team leader to allow him to develop more, or the team 3 leader should seek an independent development in another laboratory.

Nom de l'équipe : The role of CD4 help in CD8 responses

Note de l'équipe	Qualité scientifique et production	Rayonnement et attractivité, intégration dans l'environnement	Stratégie, gouvernance et vie du laboratoire	Appréciation du projet
B	B	B	NN	B

5 • Appreciation of resources and of the life of the research unit

The principles of management of the unit and the people need to be more clearly defined. A laboratory council, with representative of technicians, engeneers, students, post-docs, and researchers would help in term of management and would prevent conflicts to occur. This council should meet on a regular basis and all aspects of laboratory life discussed. English as well as non-English speakers should be able to express themselves during these regular official meetings.

The committee appreciated the quality of the pre-doc and post-doc training that involves lab meetings and journal clubs as well as strong interactions with the team leaders.

The Direction should also encourage and facilitate training of all the personal from technician to researchers.

6 • Recommendations and advice

— Points forts :

This unit is a place where immunological research is conducted with high international standard. Research publications from the unit appear with admirable frequency in the most sought-after journals and U591 scientists belong among the group of internationally renowned scientists.

— Points à améliorer :

Communication need to be improved in the unit between the group leaders but also towards all the personal.

We encouraged strongly U591 to rapidly attract new members despite the complex renovation plans afflicting the Necker campus.

— Recommandations :

The Director needs some dedicated help in terms of administrative duties, it could be a temporary help knowing that the organization may change dramatically in few years if a proper Institute is created. A consensus needs to be found rapidly concerning the first move and the renovation of the actual building but also the future plan on the whole campus. A clearer agenda will allow U591 to adequately plan its appropriate evolution through the hiring of new researchers.

Note de l'unité	Qualité scientifique et production	Rayonnement et attractivité, intégration dans l'environnement	Stratégie, gouvernance et vie du laboratoire	Appréciation du projet
A	A+	A+	C	A

UNIVERSITÉ
PARIS DESCARTES

Le Président
Axel KAHN

Paris, le 14 avril 2009

DRED 09/n° 160

Monsieur Pierre GLORIEUX
Directeur de la section des unités de l'AERES
20 rue Vivienne
75002 PARIS

Monsieur le Directeur,

Je vous remercie pour l'envoi du rapport du comité de visite concernant l'unité « **UMR-S 591 Différenciation et physiologie des lymphocytes T**» rattaché à mon établissement.

L'Université Paris Descartes est consciente de la situation difficile des équipes concernées par les restructurations sur le site Necker-Enfants Malades et s'efforcera d'en atténuer les conséquences autant que possible. L'accroissement de l'aide administrative souhaitée par le Comité de visite de l'AERES sera discuté avec l'INSERM, cotutelle de cette UMR.

Je vous prie de croire, Monsieur le Directeur, à l'expression de ma meilleure considération.

Le Président de l'Université

Axel Kahn

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Différenciation et Physiologie des lymphocytes T
Directrice : Dr Benedita ROCHA

Faculté de Médecine René Descartes Paris 5
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Dear Sirs:

Please find enclosed my comments to the committee visit (your reference # EVAL-0751721N-S210015199-UR-RPRELIM) to the Unit INSERM 591. I did not find any spelling mistakes, etc that would justify a separate file listing them. However, there was a serious misunderstanding by the committee with respect to our work, and I include two other files explaining the methodology we used.

Yours sincerely,

B. Rocha, M.D., Ph.D.

DRCE, CNRS

We receive the comments of the visit of the AERES to our Unit. In several aspects we do not agree with the Committee:

Research projects:

We deeply regret that none of the objections to our research projects now mentioned in the committee report was raised during the visit to the Unit, the place and moment when they should have been discussed at length.

1) It appears the committee did not fully understood the novelty and possible impact of single-cell quantitative gene expression profiling, as we developed in the laboratory and are applying to both the study of hematopoietic differentiation and T cell differentiation during the immune response. In the comments to team 2 it says this approach is not original to study hematopoiesis and justifies this statement by saying that arrays of T cell progenitors in the thymus have already been performed.

   Since single-cell multi-parameter quantitative analysis is not performed elsewhere, we have all reasons to believe that the approach is totally original. In the context the objection was written, the meaning of the sentence appears rather to indicate that single-cell analysis is not further required nor gives important new information once arrays are performed.

   In three of our recent publications we demonstrated that could not be further from the truth. To clarify this point we add in annex a section of the chapter "Gene expression during CD8

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differentiation" we published recently, that compares in detail both methods and shows the important new information contributed by clonal analysis does. Thus, we do believe that our method is optimal to define cell-to-cell heterogeneity and to identify new subtypes within previously known cell populations and thus will be of major importance to further our knowledge on hemopoietic differentiation. Our article in J. Exp. Med. on clonal analysis of CD8 responses demonstrates the importance of the method since we were able to identify three CD8 effector populations two of them unknown previously. Our preliminary results confirm this notion since we were able to identify new TN subtypes and a new pathway of differentiation in the thymus that previous arrays failed to reveal. We also do not plan to base the studies of either immune responses or hemopoietic differentiation exclusively in single-cell analysis as the committee suggests but rather use these data as complementary to several other complementary approaches.

Note: about the reference quoted by the Committee (Taghon, Immunity 2006). This paper analyzed the quantitative expression of several genes in bulk thymic progenitors. This approach is far from our project both by the technique used and the populations studied. However, we have used their results to select potentially interesting genes to elaborate the multiplex for our analysis on single cells (precursors from the bone marrow and the thymus).

2) The committee also raises several points to Team 3 activity that we find totally unjustified. Thus it is stated that the thematic of this team (CD4 help to CD8 responses) is very near that of Team 1 (comparison of CD8 T cell differentiation after different infections)- this problem is considered so important as damaging the recognition of Dr. Tanchot's research!

a) Dr. Tanchot is studying CD4/CD8 interactions by recommendation of our previous visit committee. Prof. Gleishenhaus (the actual president of AERES) was then quite clear that she should drop her very interesting project on T cell tolerance to concentrate exclusively in CD4/CD8 studies. They did not have any problems of "proximity with Team1".

b) The study of T cell immune responses is a major field of immunology. It is has multiple aspects that must all be explored. It is totally unreasonable to say that all scientists working on T cell responses study the same thing. Dr Tanchot investigates CD40 signal transduction and its role in memory generation. Team 1 studies infection- a very different aspect of immune responses.

c) The international community does recognize Dr. Tanchot' research: She was invited speaker in two European Congresses of Immunology (Athens and Berlin), to give seminars and to participate as invited speaker in meetings in Europe and USA.

2.2. The committee also criticizes the experimental model used referring to "the un-physiologic use of high density naïve cell transfers" a notion now popular. However, we found major flaws in the Ms. highlighting this notion. We have a paper that was revised positively that confirms the flaws we saw in other Ms. and shows that high dose transfers only accelerate antigen elimination and thus response kinetics but have no other effect on CD8 differentiation. Moreover in this paper we also show that the evaluation of normal cells early in the response has a major flaw since T cells then up to 80% down-regulate their TCR and cannot be

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recognize by tetramer binding. A paper was published last month showing that high dose transfers have NO effect on the number and quality of memory cells. So we believe that "the abnormality" of high dose transfers will be a major red hearing that will eventually be recognized as such.
The committee also raises objections on possible competitiveness of the project with respect to visualization of CD4/CD8/APC interactions. We realize imaging is difficult but we believe to be highly competitive in that endeavour. The experimental system developed has unique competitive advantages. It is the only one that allows the simultaneous visualization of all components of the immune response: Team 3 has both green-CD4 and red-CD8 T cells recognizing the same male antigen and can also visualize all male cells in vivo by a different Ly phenotype. Since this system has also the unique characteristic of the total absence of cross presentation the antigen specific CD4 and CD8/male expressing APC trio association can be directly evaluated-to our knowledge no other system allows that, what gives us an important competitive advantage. Another important competitive advantage is the capacity to study the interactions of CD8 with APC in the total absence of antigen-specific CD4 help. Finally, it is the ONLY experimental system where antigen-specific T cells can be studied in the absence of other T cells. This is possible because anti-HY CD8 T cells do not undergo homeostatic proliferation after transfer to empty hosts. We can study T/APC location in CD3e deficient mice that have conserved organ architecture- the absence of endogenous populations will facilitate greatly trio visualization. Finally we also have the great facility of the Necker environment. Frederic Geissman (with the IFR and our participation) had equiped extensively our "imaging platform" to allow intravital microscopy studies, that is not used now. Now that he left to the UK this equipment is now totally available to us. We thus dispose of large slots of time for imaging and in that aspect we are probably the Unit with best conditions in France. Again we deeply regret that none of these objections was raised during the visit since they could then be easily addressed.

Visit organization.
We though that the most important aspect of our visit was the discussion of the results and research projects but the time period indicated by AERES for that part was very insufficient. To overcome this problem we decided to reduce the scheduled period attributed to the general Unit presentation, then mention just briefly the scientific domains of each team, the complementarity of research domains and the problems we had in our research (animal house, administrative support). We did not mention platforms since we have no problems with those. The Necker' CHU problem is not under our control and it is yet not settled if /when we will move out. This problem is around for the last twelve years and we will not move out for the next two years. We left to each team the presentation of its members.

Unit organization
In relation to the Unit, we also have so far preferred to organize regular informal meetings (generally after the lab meeting) to discuss the current plans and problems rather than leave them to accumulate for rarer " lab councils". This is feasible since our Unit is small and we all

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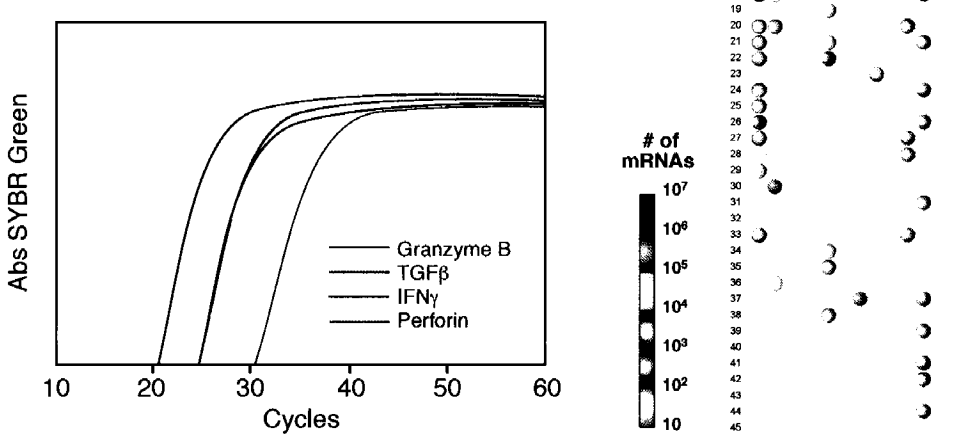
meet during the lab meetings. We also find a good strategy to solve problems when they do appear rather than letting them accumulate. We fail to understand the comments of the committee concerning the need to encourage and facilitate training of the personnel-since this is the firm policy of the Unit. This comment is totally unjustified. All demands by the personnel to attend training courses were encouraged and accepted. Besides, we organized ourselves training of personnel in new techniques like confocal microscopy, multicolour cytofluorometry and cell sorting.

Finally, we have several regrets concerning our visit. We regret that so few international experts were solicited to review our work. In the absence of Prof. Rodewal that could not be present during the visit the committee had a single international reviewer. This is not an improvement over the previous visits by the INSERM commissions where two foreign reviewers were always present. We were also very disappointed with the scientific discussion. We were looking forward to a very lively, productive and constructive discussion that would contribute with new perspectives, clues and ideas for our research, but the discussion was restricted to what appeared to us as the clarification of experimental details. We also regret that the committee did not forward their conclusions to the Unit personnel at the end of the visit, as it is usually done. The totality of the Unit waited for this till late in the afternoon, and was sadly disappointed when the committee just left.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10⁷
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10³
10²
10

Fig 1

III. A. Methodologies to evaluate gene expression by CD8 T cells.

Gene expression may be evaluated by two approach' types that give complementary but not overlapping information. In one type ("population studies") the total mRNA pool from each cell set is isolated. Then, the different mRNAs recovered from different cell sets are compared, to determine which genes are differentially expressed. The alternative approach scores transcription by individual cells ("single-cell studies"). Each of these methods has limitations of two types. The first is related to each technique's limitations that may yet be reduced with time. Importantly, there are other fundamental differences in the type of information that each method can provide, what is frequently overlooked and may lead to data over-interpretation and misconceptions in the field.

Population studies. The most currently used strategy for "population studies" compares gene expression in two or more different populations by the use of gene expression arrays. This method has as major advantages that of being quite easy to perform and of covering a vast set of genes. However, arrays were not set up to study CD8 T cells in particular, and thus probes with the capacity to detect genes that are "CD8 specific" may be underrepresented or not present at all, while positive signals may correspond to expressed sequence tag (EST) with yet unknown roles. "CD8 specific" cDNAs may be not very abundant either because transcription rates are low or/and because not all cells from that population transcribe that particular gene. Finally, "CD8 specific" cDNAs are put in contact with multiple probes, may have yet unknown cross-hybridization patterns, and less-specific, low affinity binding of cDNA to the multiple probes present in the array may "consume" less abundant cDNAs and "dump" specific signals. By these limitations, DNA arrays may fail to detect differentially expressed genes with an important role in CD8 differentiation. These inconveniences may be overcome by the more fastidious and technically demanding subtraction library technology that is yet the most accurate way to identify differentially expressed genes. The major advantage of subtraction libraries versus array studies is demonstrated in the identification of *Tbx21*. That TF that has a fundamental role in CD8 differentiation was identified in subtraction libraries [67]. In contrast, array studies comparing naïve and effector CD8 cells did not show a differential expression of these genes [68], although *Tbx21*and *Eomes* are not expressed at all in naïve cells and are expressed by virtually all CD8 effectors.

Of major interest, comparison of various CD8 populations may reveal differentially expressed genes that were not previously identified as having any role in CD8 differentiation and thus become ideal candidates for further studies. Therefore, population studies are ideally approaches to "fish genes" with previous unknown functions.

Single-cell analysis is at the opposite end of population studies [69]. It is laborious and can only address the expression of known genes. The number of genes that can be studied simultaneously in each individual cell is actually restricted to about twenty. However, **it gives fundamental information on how known genes are expressed within a population** that cannot be obtained by array studies. It

determines the frequency of cells expressing each gene. It studies gene association. These two types of information are fundamental to understand the role of each gene within that cell set, to predict cell function and to determine a possible heterogeneity and sub-division of cells types. In contrast, in population studies the mRNA are pooled from many individual cells and thus evaluate the population's mRNA expression averages, what is frequently insufficient and may even be misleading. Fig.1 exemplifies potential misconceptions by comparing the same population (CD8 T cells recovered at 4 days after priming) studied simultaneously at a "population" or at "single-cell" level.

The importance of frequency estimates. Individual genes are transcribed at very different levels, which are "characteristic" of each gene. Transcription can range from $>10^7$ mRNAs/cell (*Gzmb, Gzma*) to 10^3 mRNAs/cell (*Tgfb1*, *Prf1*) [6]. Consequently, a single cell expressing *Gzmb* at 10^6 mRNAs/cell present at 1/1,000 frequency may give the same signal as 100% of the cells expressing *Tgfb1* at 10^3 mRNAs/cell, i.e., **in "population" readouts a rare non-representative event at 10^{-3} frequency and a major property shared by all T cells may score similarly.** This major bias is evident in Fig.1. *Gzmb* was the most abundant gene expressed by the CD8 population, but single-cell analysis revealed that such signal was due to very rare cells expressing *Gzmb* at $>10^6$ copies/cell. In contrast, *Tgfb1* signal was much weaker but our single-cell analysis revealed that this gene was expressed by more than 70% of CD8s- at about 10^3 copies/cell. Single-cell read-outs are thus fundamental to determine the frequency of cells expressing each gene, i.e., how important is that property within that cell set.

Predicting functional behavior. The other major limitation of population studies is their inability to evaluate if different genes are co-expressed by the same cell or by different individual cells. This may be of importance if cell function requires the simultaneous expression of several molecules, as exemplified in Fig.1. In order to kill target cells efficiently each CD8 lymphocyte must co-express perforin and granzymes. CD8s studied at a population level appeared cytotoxic because both genes were detected but single-cell studies revealed that *Prf1* and *Gzmb* were usually expressed in different cells. These results suggested these cells were not cytotoxic, which we did confirm by *in vivo* functional tests. TGF-β may be anti-inflammatory by blocking T cell proliferation or pro-inflammatory by mobilizing and activating APCs. Response to TGF-β requires the co-expression of two receptors by the same cell, one capturing the ligand and other responsible for signal transmission. Studies at population level detected the expression of *Tgfb1* and of both receptors, suggesting an anti-proliferation role, but single-cell studies revealed that T cells cannot respond to the TGF-β they produce, because individual cells did not co-express the *Tgfbr1* and *Tgfbr2* [6]. This finding led us to envisage that cells expressing *Tgfb1* had a pro-inflammatory role of this cell set that we confirmed by *in vivo* functional studies.

Screening for new subpopulations. Single-cell analysis allows the comparison of the co-expression of twenty different genes simultaneously. Thus it allows addressing how different effector molecules and different receptors described to be involved in CD8 responses all associate between themselves. It

allows investigating if the CD8 population we are studying is homogeneous or constituted by different cell sub-types and in the later case, what are the receptors/effector molecules that define each sub-type. It must be noted that no other method allows the simultaneous comparison of twenty different parameters neither has the same sensitivity level. Flow cytometry has progressed but is yet very below 20 parameters evaluation, and available conjugates frequently impose important restrictions to association studies. Moreover, in many circumstances single-cell co-expression of different genes cannot be evaluated at protein level since Abs recognizing native proteins in the mouse are not yet available (as in the case of perforin), or importantly, protein expression levels are so low they do not allow precise discrimination between positive/intermediate/ negative cell types.

Thus, genetic arrays and single-cell analysis appear to have different complementary scopes. Genetic arrays are fundamental to identify potentially important genes that are differentially expressed in two different cell sets. Single-cell analysis, by evaluating different genes expression frequencies and on their co-expression by the same cell gives important information on the importance of each gene within a cell set; indicates potentially different T cell properties; may identify different T cell subpopulations.

Fig.1 Fig. 1. Population versus single-cell studies. Results compare gene expression levels of the same CD8 cell set that was isolated and studied simultaneously as a population (left) or as single cells (right). In single cell studies each row shows the same individual cell that is numbered. Each column shows a different gene representing the number of mRNA molecules/cell according to a colour scale. Empty symbols represent cells that do not express that gene (<2mRNA/cell) and grey symbols positive cells where gene expression levels were not quantified.