API - Agents pathogènes et inflammation

Rapport Hcéres

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

Section des Unités de recherche

AERES report on the research unit

Agents pathogènes et inflammation

From the

University of Franche-Comté

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AERES report on the research unit

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Le Président de l'AERES

Didier Houssin

Section des unités de recherche

Le Directeur

Pierre Glorieux

November 2010

Research Unit

Name of the research unit: Agents pathogènes et inflammation

Requested label : EA

N° in the case of renewal: EA 4266

Name of the director: M. Georges HERBEIN

Members of the review committee

Committee chairman:

M. Olivier LANTZ, Institut Curie, Paris

Other committee members:

Mrs Nathalie CHAPUT, Institut de Cancérologie Gustave Roussy, Villejuif

Mrs Sophie DE BENTZMANN, Université Aix-Marseille 2, Marseille

M. Peter STAEHELI, University of Freiburg, Germany

M. Michael SCHINDLER, Heinrich Pette Institute, Germany

M. Bruno POZZETTO, Université St. Etienne, St. Etienne (membre du CNU)

Observers

AERES scientific advisor:

Mrs Ana-Maria LENNON

University, School and Research Organization representatives

Report

1 • Introduction

- **Date and execution of the visit :**

The visit took place on November, 4, 2010 from 11 AM to 7 PM. After a closed door meeting between the comittee members, G. HERBEIN, the head of the unit, presented the general strategy of the unit as well as the main results. After a poster sessions, the 3 different group leaders presented their past, present and future projects. The committee then met with the ITA, students and staff scientists before outlining the main conclusions during a closed door meeting of the AERES committee.

- **History and geographical localization of the research unit, and brief presentation of its field and scientific activities:**

The unit headed by G. HERBEIN and affiliated to the University of Franche-Comté encompasses a virology and a bacteriology group. The virology group is headed by G. HERBEIN and is located in the former hospital Saint Jacques in the center of Besançon. Affiliated individuals to this group are involved in liver transplantation and the study of rheumatoid arthritis. The second group is headed by P. PLESIAT and studies the virulence factors of Pseudomonas aeruginosa and its resistance to antibiotics. This second group is located in the new hospital in the outskirts of Besançon. The project plans to adjoin 2 other groups from the University of Bourgogne located in the Dijon teaching hospital. These 2 groups are headed by P. POTHIER and C. NEUWIRTH. C. NEUWIRTH will join P. PLESIAT's group whereas P. Pothier who studies the epidemiology and virulence of selected gastroenteritis and respiratory viruses will constitute a third team in the future unit.

- **Management team :**

The unit is headed by G. HERBEIN and the 2 other teams will be headed by P. PLESIAT and P. POTHIER, respectively.

- **Staff members (on the basis of the application file submitted to the AERES):**

	Past	Future
N1: Number of researchers with teaching duties (Form 2.1 of the application file)	12	24
N2: Number of full time researchers from research organizations (Form 2.3 of the application file)		
N3: Number of other researchers including postdoctoral fellows (Form 2.2 and 2.4 of the application file)	3	12
N4: Number of engineers, technicians and administrative staff with a tenured position (Form 2.5 of the application file)	2	7
N5: Number engineers, technicians and administrative staff without a tenured position (Form 2.6 of the application file)	1	3
N6: Number of Ph.D. students (Form 2.7 of the application file)	4	7
N7: Number of staff members with a HDR or a similar grade	8	10

2 • Overall appreciation on the research unit

- **Summary:**

The publication record is good, with an abundant production including several papers in the best journals of the speciality during the last 4 years. Two leaders in this group are national reference center for pseudomonas and enteric viruses, respectively. The future projects were clearly described by the bacteriology group and the scientific strategy was found appropriate and of good quality by the committee. For the virology groups, the committee had a lot of difficulties to correctly assess the projects in the absence of real description either in the written document or during the oral presentation. Furthermore, the presentation document was mainly redacted in French, making it difficult for the international members of the committee to make a proper evaluation. Some of the projects are clearly sound and feasible whereas others are based on strong hypothesis which would need to be better demonstrated before going further.

The fusion between the Besançon and Dijon groups is clearly synergistic for the bacteriology groups with complementary expertise and interests; the collaboration is already set up and the official fusion between the 2 locations will be easy. These points are much less clear for the 2 virology groups because the real way the fusion will be implemented was not provided to the committee neither in the written document nor in the oral presentation. The clinical studies done in the context of liver pathology and transplantation as well as the rheumatoid arthritis works are thematically divergent from both the virology and bacteriology topics.

The students are well mentored and the technical staff is well managed. The technical ressources either internal or external to the unit look appropriate to carry out the proposed work.

- **Strenghts and opportunities:**

Good scientific production, good student mentoring. National reference centers. The bacteriology group is well organized.

- **Weaknesses and threats:**

The organisation is not well thought. Some projects would need to stand on stronger demonstration of the hypothesis. The leadership of the unit head is not clear. How the interactions between the Dijon and Besançon groups will be organized is not clear.

- **Recommendations to the head of the research unit:**

Some of the virology projects should be better justified and documented. The organization of the unit should be rethought with better interactions between the 2 virology sites. The liver and rheumatology projects may benefit to become affiliated to another research unit as these topics are quite different from the infectious disease themes of the other members.

- **Production results:**

A1: Number of permanent researchers with teaching duties (recorded in N1) who are active in research	12
A2: Number of permanent researchers without teaching duties (recorded in N2) who are active in research	
A3: Ratio of members who are active in research among staff members [(A1 + A2)/(N1 + N2)]	100%
A4: Number of HDR granted during the past 4 years	2
A5: Number of PhD granted during the past 4 years	4
A6: Other relevant item in the field *(i.e. number of first and/or last authors original publications in peer review journals)*	94

3 • Specific comments

- **Appreciation on the results (relevance and originality of the research, quality and impact of the results):**

Two of the teams are national reference centers allowing the groups to get adequate funding. Participation to European projects.

- **Appreciation on the strategy, management and life of the research unit:**

How the virology sites of Besançon and Dijon will interact and work together is not clear. How the different teams (bacteriology and virology) will interact is not clearly stated.

All the permanent scientists have important teaching work load. They are all implicated in national and international collaborations.

- **Appreciation on the project :**

This point is discussed in the report that assesses each team.

4 • Appreciation project by project

- **Title of the team and name of the team (project leader):**
- **Chronic viral infections and inflammation (G. HERBEIN).**
- **Staff members:**

	Past	Future
N1: Number of researchers with teaching duties (Form 2.1 of the application file)	9	5
N2: Number of full time researchers from research organizations (Form 2.3 of the application file)		
N3: Number of other researchers including postdoctoral fellows (Form 2.2 and 2.4 of the application file)	3	3
N4: Number of engineers, technicians and administrative staff with a tenured position (Form 2.5 of the application file)	2	1
N5: Number of engineers, technicians and administrative staff without a tenured position (Form 2.6 of the application file)	2	1
N6: Number of Ph.D. students (Form 2.7 of the application file)	5	3
N7: Number of staff members with a HDR or a similar grade	8	3

- **Appreciation on the results:**
  - Relevance and originality of the research, quality and impact of the results:

The team of G.HERBEIN studies HIV and HCMV replication in the context of infected macrophages. Macrophages are targeted by HIV in vivo and they are key cells in HCMV pathogenesis. They can function as viral reservoirs and transmit

the virus via the mucosa and the blood brain barrier. Thus, the topic of research that is focused on in the team is highly relevant and interesting. More specifically, research in the group aims to elucidate mechanisms of NF-kappaB activation by viral isolates or specific viral proteins. In the context of HIV-1, they follow up on the interesting hypothesis that the viral accessory proteins Nef and Vpr activate NF-kappaB in infected cells and non-infected bystander cells to permit viral replication. Ultimatively, these mechanisms might contribute to the high levels of generalized immune activation in HIV-infected patients, which correlate with high viral loads and AIDS progression.

The frequency of publication in the activity period is high. In regard to the speciality, the quality of publications is good but not outstanding, having published most studies in journals ranging between IF 4 and 6 (Retrovirology, Journal of Immunology, JBC).

The team has a number of fruitful and longlasting national and international collaborations that are documented well by the bibliography.

- **Appreciation on the impact, the attractiveness of the team and of the quality of its links with international, national and local partners:**

The department is visible and recognized internationally and has attracted PhD students and a PostDoc from abroad. G.HERBEIN has an impressive record of acquired extramural funding (e.g. 6th EU FrameworkProgramm and a number of national and international grants) demonstrating his outstanding ability to successfully apply for competitive funding.

As already stated above there is an international network of foreign collaborators. Unfortunately, there was no presentation of potential joint projects of the Herbein and Pothiers groups. This makes it difficult or impossible for the evaluation panel to assess if there are synergies between both groups.

- **Appreciation on the strategy, management and life of the team:**

There is a weekly team meeting and despite the organizational involvement of G.HERBEIN in his overall management duties the team seems well organized including lively scientific discussion.

- **Appreciation on the project:**

The written documents and the information provided during the site visit envisage the follow up on three projects for which already a substantial amount of preliminary data has been collected. In more detail, G.HERBEIN and his team aim to (i) elucidate the impact of HIV-1 Nef protein interacting with HCV core in the context of HIV and HCV coinfected macrophages and the consequences on viral replication of both viruses; (ii) assess the role of Nef in activation of Akt and the subsequent pathways, which might prime infected or non-infected T-cells for efficient HIV replication;(iii) follow up on the hypothesis that HCMV might be an oncogenic virus or influence oncogenesis. These are strong hypothesis which are based on an impressive amount of preliminary data. Unfortunately, there was no specific strategy presented on how these project will be pursued in the next four years and no detailed research plan was presented to the evaluation committee. This is disappointing, especially since we are convinced that the projects might have important implications and potentially are of hight impact. However, a number of important questions concerning these hypotheses should be answered in the upcoming years. The PI might have a detailed research plan at hand, but this plan could not be evaluated properly due to the lack of a detailed project description. For example, the hypothesis that HIV proteins get secreted and prime adjacent cells for replication is provocative. It might have an enormous impact on immune activation and viral replication. However, since formal proof that this really happens in vivo is still lacking, this point should have been addressed. Another exciting area of research the team wants to follow up on is the issue of HIV and HCV coinfection of macrophages. Questions arising are for example if macrophages of HIV- and HCV-coninfected patients are more permissive for infection due to higher NFkappa-B activation. Or if coinfected macrophages can be isolated from patients. Furthermore, it was not clear to the committee how the hypothesis that HCMV might modulate oncogenesis or might be involved in oncogenesis arose in first place and which experiments are proposed in the future to answer this question.

In sum, the proposed projects are of high relevance and might have great impact. Nevertheless, final evaluation is difficult due to the lack of a detailed project description.

- **Conclusion :**

  - Summary:

G.HERBEIN and his team have a strong track record in the past which is well documented by the amount and quality of publications as well as the amount of extramural funding. The preliminary data of the presented projects is solid and most of the hypotheses are strong. The members of the team were motivated and discussed lively. Unfortunately, there is a shortcoming in the detailed description of the planned projects and the proposed interaction with the team of P. POTHIER.

  - Strengths and opportunities:

The virology group of G.HERBEIN has three pathogens in hand that all target the macrophage. In fact, HCMV and HCV are most important opportunistic infections in the context of HIV/AIDS. Studying all three in the in vivo primary target macrophage is of high relevance and impact, and it might greatly stimulate an overall underdeveloped research topic. We see this strength as an opportunity and chance to start research in the exciting field of viral coinfections.

  - Weaknesses and threats:

The specific research plan for the projects of the team G. HERBEIN did not contain enough detailed information. Further, the proposed collaboration with the team of P. POTHIER was not outlined well enough to permit a complete assessment of possible synergistic effects.

  - Recommendations:

Document with more evidences the hypotheses funding some of the projects.

Precise the collaboration and the complementarity with P. Pothier's group.

Focus the projects of the clinical groups in order to better fit to the main projects of the team.

- **Title of the team and name of the team (project leader):**
- **Bacteria and chronic inflammatory diseases (P. PLESIAT)**
- **Staff members:**

	Past	Future
N1: Number of researchers with teaching duties (Form 2.1 of the application file)	4	6
N2: Number of full time researchers from research organizations (Form 2.3 of the application file)	0	0
N3: Number of other researchers including postdoctoral fellows (Form 2.2 and 2.4 of the application file)	0	4
N4: Number of engineers, technicians and administrative staff with a tenured position (Form 2.5 of the application file)	1	1
N5: Number of engineers, technicians and administrative staff without a tenured position (Form 2.6 of the application file)		1
N6: Number of Ph.D. students (Form 2.7 of the application file)	2	4
N7: Number of staff members with a HDR or a similar grade	3	4

- **Appreciation on the results:**

  - Relevance and originality of the research, quality and impact of the results:

The research activity of PLESIAT's team concerns the study of resistance mechanisms in a gram negative bacterium, Pseudomonas aeruginosa, that is a threatening infectious agent responsible for nosocomial infections and chronic infections in cystic fibrosis patients. The team has mainly concentrated its activity onto the resistance mechanisms of efflux pumps of the RND family (MexXYOprM and MexABOprM). The very exciting point is that the research work of this team is based on clinical observations of multiresistance phenotypes of clinical isolates, collected by the team through its national reference center activity, that are further dissected from the molecular point of view. This represents a very productive transversal research, relevant towards clinical situations. Another aspect has been more recently developed regarding new enzymatic resistance mechanisms, in particular those due to the emergence of ▫-lactamases and carbapenemases.

  - Number and quality of the publications, scientific communications, thesis and other outputs:

During the activity period, the team has published very regularly in good journals of the speciality (IF 5). From this research activity aiming at understanding and anticipating resistance mechanisms in a bacterium for which toto resistant strains exist in parallel to the crual lack of new molecules, the team contributes to the development of inhibitors of efflux mechanisms and managing epidemiological survey.

  - Quality and stability of partnerships:

Besides it own activity research, the team has generated a CNRS national network gathering twelve teams dedicated to fundamental research on P. aeruginosa for exchanging genetic, structural, phylogenetic and virulence skills, that Pr Plésiat is coordinating. In addition, P. PLESIAT is the leader of the French reference Center for P. aeruginosa, highlighting his strong connection with clinical aspects of P. aeruginosa.

- **Appreciation on the impact, the attractiveness of the team and of the quality of its links with international, national and local partners:**

Participation to international or national scientific networks, existence of stable collaborations with foreign partners.

The leader has networked long-term collaborations with eminent foreign scientists, these numerous collaborations being consolidated through the obtention of national and international grants. The clinical collaborative aspect through the CNR activity is outstanding.

Exchanges of young scientits from the lab and from outside laboratories belonging to the national network coordinated by P. PLESIAT have been numerous in the last two years, demonstrating the unique expertise of the examined team as well as the strong will of acquiring new skills.

- **Appreciation on the strategy, management and life of the team:**

  - Relevance of the team organization, quality of the management and of the communication policy:

Despite the heavy clinical and teaching involvment of permanent researchers, there is a very good quality of management, and of communication through one to one result discussion and team meeting every week.

  - Relevance of the initiatives aiming at the scientific animation and at the emergence of cutting edge projects:

Local scientific animation has benefited from the integration of the national network that P. PLESIAT is coordinating, allowing the members of the team to exchange on technical and scientific points. From the very local point of view, the team is also well trained with clinical aspects of bacteriology.

  - Contribution of the team members to teaching and to the structuration of the research at the local level:

Despite the heavy teaching and hospital contributions of team members, there is a high quality management at the bench; the students are well-coached and have many opportunites to present their work in national and international conferences. The team leader is also deeply involved in the Doctoral Student school at the local level.

- **Appreciation on the project:**

  - Existence, relevance and feasability of a long term (4 years) scientific project:

The four year project implies the integration of Catherine NEUWIRTH‘s group to Patrick PLESIAT‘s team. The project will characterize from the molecular point of view resistance phenotypes of P. aeruginosa and Achromobacter xylosidans clinical isolates, particularly those associated with efflux pumps of RND family, beta-lactamases and porins. From the scientific aspect, it is obvious that this is a very good and solid fusion. Catherine NEUWIRTH and her team will integrate the team to work on resistance mechanisms of an emerging CF pathogen, Achromobacter xylosidans, for which poor data is available. The expertise of C. NEUWIRTH on beta-lactamases will benefit to the preexisiting team, and the genetic and genomics skills of P. PLESIAT’s team will undoubtly benefit to C. NEUWIRTH‘s team since no genetic tool or genome sequence are available for this emerging pathogen. Based on clinical observations, the NEUWIRTH group identified recently an efflux pump homologous to the MexABOprMP efflux pump of P. aeruginosa; thus the scientific crosstalk between the two thematics is actually fruitfull and synergistic. From the oral presentation, it must be notified that the objectives are perfectly defined and realistic thanks to the expertise of members of the team and to existing collaborations.

Beside the heavy clinical coordination activity of the leader, the team has demonstrated a high quality research activity and proposes a well-structured research project for the next four years relative to antibiotic resistance mechanisms of P. aeruginosa and A. xylosidans based on a strong and well-know expertise in the field of gram-negative non fermentative bacteria.

- **Conclusion:**

  - Summary:

The project is based on strong expertise with original topics based on molecular dissection of antimicrobial resistance mechanisms observed in clinical situations of P. aeruginosa and A. xylosidans human infections that researchers thanks to their clinical positions are knowing very well. The past activities and the project are very coherent and the management is very impressive.

- Strengths and opportunities:

The association between P. PLESIAT and C. NEUWIRTH teams is clearly synergistic as the respective skills are complementary and can be applied to the specific bug studied by one team or the other. This will be very productive and has been very well defended during the visit.

- Weaknesses and threats:

How the association will be practically organized has not been clearly explained. The number of permanent scientists and of Post-doctorant fellows needs to be increased to consolidate the good quality of science they are producing.

- Recommendations:

Continue with the interesting research proposed by the team.

Develop the synergy between Plésiat and Neuwirth groups.

Precise the complementarity with the virology groups.

- **Title of the team and name of the team (project leader):**
- **Enteric and respiratory viruses. Immune response (P. POTHIER).**
- **Staff members:**

	Past	Future
N1: Number of researchers with teaching duties (Form 2.1 of the application file)	1	1
N2: Number of full time researchers from research organizations (Form 2.3 of the application file)		
N3: Number of other researchers including postdoctoral fellows (Form 2.2 and 2.4 of the application file)	1	5
N4: Number of engineers, technicians and administrative staff with a tenured position (Form 2.5 of the application file)		5
N5: Number of engineers, technicians and administrative staff without a tenured position (Form 2.6 of the application file)		1
N6: Number of Ph.D. students (Form 2.7 of the application file)		0
N7: Number of staff members with a HDR or a similar grade	1	4

- **Appreciation on the results:**

  - Relevance and originality of the research, quality and impact of the results:

The team of P. POTHIER was active in 3 fields, namely (i) epidemiological aspects of viruses infecting the gastrointestinal tract, (ii) basic reasearch aimed at understanding the interactions between noroviruses and cellular entry receptors and (iii) fundamental aspects of antiviral and vaccine strategies in mice. Epidemiological work performed in recent years is of high relevance and has high impact. It is closely related to the core expertise of the team and profits from the fact that the Pothier laboratory is the French national reference center of enteric viruses. Similarly, the norovirus receptor studies are closely related to the core expertise of the team. The recent hMPV infection studies with aged mice are of great interest as they contribute to the understanding of an important clinical problem.

  - Number and quality of the publications, scientific communications, thesis and other outputs:

Results were published in highly respected speciality journals. Overall, the publication record of the Pothier team is good but not outstanding.

  - Quality and stability of partnerships:

Several active collaborations are documented. The well established networks will certainly help to maintain high quality research.

- **Appreciation on the impact, the attractiveness of the team and of the quality of its links with international, national and local partners:**

  - Ability to recruit high levels scientists, post-docs and students, and more particularly from abroad:

The team is visible. Thus, future recruiting of highly talented students and postdocs should not be a problem.

- Ability to raise funds, to successfully apply for competitive funding, and to participate to scientific and industrial clusters:

P. POTHIER has a good record of extramural funding. He is involved in several national and international networks.

- Participation to international or national scientific networks, existence of stable collaborations with foreign partners:

Such activity is well documented.

- **Appreciation on the strategy, management and life of the team:**

  - Relevance of the team organization, quality of the management and of the communication policy:

The team seems to be organized well.

  - Contribution of the team members to teaching and to the structuration of the research at the local level:

No information provided.

- **Appreciation on the project:**

The Pothier team would like to continue all previous research activities. A first focus will remain on epidemiological studies of rotaviruses and noroviruses, and on the molecular characterization of newly discovered gastrointestinal viruses. This part of the proposal is straightforward and will almost certainly yield interesting results. It makes a lot of sense that these projects are pursued.

A second focus will be on the norovirus-host cell receptor interaction. The ambitious goals are to understand the molecular details of virus docking, internalization and uncoating, as well as getting insights into host cell signalling events during the infection process. This part of the research project is highly attractive and, if successful, will yield important information. Unfortunately, only few details of the chosen experimental approaches were revealed in the written proposal and only very few additional details were provided during the site visit. Thus, evaluating the suitability of the experimental strategy and the likelihood of success is difficult.

A third focus will be on the immune response in the intestinal mucosa after intra-rectal application of VLP-based rotavirus vaccines. Again, only few experimental details were given in the written proposal and only very little additional information was provided during the site visit, rendering a proper evaluation of the project difficult.

A final forth focus of the current research proposal will be on hMPV. P. Pothier proposes to continue studying the age-related disease aggravation in hMPV-infected mice and to initiate in vivo studies aimed at determining the efficiency of siRNA administration as antiviral strategy. The review team got convinced that the age-related disease aggravation issue is an important and underdeveloped field of research, but no thorough research plan was provided which would have shown how the problem is being addressed experimentally. The reviewers further felt that the siRNA-based antiviral approach is build on preliminary data that are not very convincing and that this part of the project should not be pursued.

- **Conclusion:**

  - Summary:

P. POTHIER and his team performed well in the past. This successful work is documented by several publications in highly respected journals and by successful acquisition of extramural funding. The proposed future work is, in most parts, well founded on solid preliminary work of the team. Unfortunately, the planned work was not described in sufficient detail.

  - Strengths and opportunities:

The epidemiological work which is partly based on truly original observations of the Pothier laboratory is of high quality. The proposed molecular work of norovirus entry, developed in collaboration with the team of J. LE PENDU in

Nantes, has a high potential provided that important technical issues can be solved. Characterizing mucosal immune reactions is of great relevance for antiviral protection. If the available model systems are properly used, they might yield important insights.

- Weaknesses and threats:

The specific research plan for some projects of the P. POTHIER team did not contain enough detailed information, which seriously complicated a fair assessment. Further, the proposed collaboration with the team of G. HERBEIN was not outlined well enough to permit a good assessment of possible synergistic effects.

- Recommendations:

Focus the research work on enteric viruses, in relation with the reference center, especially the part dealing with the entry of noroviruses and the interaction with the intestinal mucosa.

Document more precisely the different steps of the research project in order to better assess its feasability.

Explicit the complementarity with HERBEIN's team.

Intitulé UR / équipe	C1	C2	C3	C4	Note globale
AGENTS PATHOGÈNES ET INFLAMMATION (API)	**B**	**A**	**B**	**B**	**B**
CHRONIC VIRAL INFECTIONS AND INFLAMMATION [HERBEIN-HERBEIN]	B	A	Non noté	B	B
PSEUDOMONAS AERUGINOSA AND INFLAMMATION [HERBEIN-PLESIAT]	A	A	Non noté	A	A
RESPIRATORY VIRUSES, ENTERIC VIRUSES, AND THE IMMUNE DEFENSE [HERBEIN-POTHIER]	B	A	Non noté	B	B

C1 Qualité scientifique et production
C2 Rayonnement et attractivité, intégration dans l'environnement
C3 Gouvernance et vie du laboratoire
C4 Stratégie et projet scientifique

Statistiques de notes globales par domaines scientifiques

(État au 06/05/2011)

Sciences du Vivant et Environnement

Note globale	SVE1_LS1_LS2	SVE1_LS3	SVE1_LS4	SVE1_LS5	SVE1_LS6	SVE1_LS7	SVE2 _LS3 *	SVE2_LS8 *	SVE2_LS9 *	Total
A+	7	3	1	4	7	6		2		30
A	27	1	13	20	21	26	2	12	23	145
B	6	1	6	2	8	23	3	3	6	58
C	1					4				5
Non noté	1									1
Total	**42**	**5**	**20**	**26**	**36**	**59**	**5**	**17**	**29**	**239**
A+	16,7%	60,0%	5,0%	15,4%	19,4%	10,2%		11,8%		12,6%
A	64,3%	20,0%	65,0%	76,9%	58,3%	44,1%	40,0%	70,6%	79,3%	60,7%
B	14,3%	20,0%	30,0%	7,7%	22,2%	39,0%	60,0%	17,6%	20,7%	24,3%
C	2,4%					6,8%				2,1%
Non noté	2,4%									0,4%
Total	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%	100,0%

*** les résultats SVE2 ne sont pas définitifs au 06/05/2011.**

Intitulés des domaines scientifiques

Sciences du Vivant et Environnement

- **SVE1 Biologie, santé**
  - **SVE1_LS1 Biologie moléculaire, Biologie structurale, Biochimie**
  - **SVE1_LS2 Génétique, Génomique, Bioinformatique, Biologie des systèmes**
  - **SVE1_LS3 Biologie cellulaire, Biologie du développement animal**
  - **SVE1_LS4 Physiologie, Physiopathologie, Endocrinologie**
  - **SVE1_LS5 Neurosciences**
  - **SVE1_LS6 Immunologie, Infectiologie**
  - **SVE1_LS7 Recherche clinique, Santé publique**
- **SVE2 Ecologie, environnement**
  - **SVE2_LS8 Evolution, Ecologie, Biologie de l'environnement**
  - **SVE2_LS9 Sciences et technologies du vivant, Biotechnologie**
  - **SVE2_LS3 Biologie cellulaire, Biologie du développement végétal**

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PRESIDENCE

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BORDEREAU DES PIECES ADRESSEES A :

AERES
Professeur Olivier LANTZ
Président du comité d'évaluation
De l'EA 4266 API

DESIGNATION	OBSERVATIONS
Volet général : observations générales sur le rapport d'évaluation de l'EA 4266 API	
Volet corrigeant les erreurs factuelles du rapport d'évaluation	
Projet détaillé de l'équipe « Virus hématogène et Inflammation » dirigée par G. Herbein	
Projet détaillé de l'équipe « Virus entériques et respiratoires et Immunité » dirigée par Pierre Pothier	Pour attribution

Besançon, le 9 mars 2011

Le Président de l'Université,

Pour le Président et par délégation
Le Directeur Général des Services
Claude CONDÉ

Louis BÉRION

UNIVERSITÉ DE FRANCHE-COMTÉ

EA 4266 API
Agents pathogènes et inflammations

Pr. Olivier LANTZ

President of the Review Committee

AERES

Dear Professor Lantz and members of the review committee,

We would like to thank you for the AERES report on the research unit "Agents pathogènes et inflammation" EA 4266 from the University of Franche-Comté. We believe that your recommendations will be of great help for the achievement of the next research plan of the unit.

The first major concern arising from your evaluation was about the practical reorganization of the laboratories of virology and bacteriology located in Dijon and Besançon.
The head of the unit will be Georges Herbein with three teams headed by Pierre Pothier (Dijon), Patrick Plésiat (Besancon) and Georges Herbein (Besancon). The topics of the clinical groups (hepatology, rheumatology) will no more be addressed in the revised version of the project. The unit will be affiliated to the University of Franche-Comté and be part of the PRES Bourgogne-Franche-Comté. Although located on two distinct campuses, strong interactions between Dijon and Besancon researchers result from regular laboratory meetings based on a monthly schedule, the writing of common grant applications, the exchange of graduate students in the context of the newly created Ecole Doctorale Bourgogne-Franche-Comté (co-headed by Patrick Plésiat), the development of a Bourgogne-Franche-Comté biobank of microorganisms, and ultimately on shared research programs. These latter will benefit from the interaction of the virologists (Pierre Pothier, Georges Herbein) and bacteriologists (Patrick Plésiat, Catherine Neuwirth) from both sides. The Pothier's team will benefit from the

knowledge of the macrophage and of cell signalling from Herbein's team for the achievement of his project of signal transduction following infection with norovirus. The Herbein's team will benefit from the knowledge of animal model developed by Pothier's team to translate *in vivo* his research on HCMV and oncogenesis by injecting HCMV infected cells in nude mice.

The collaboration between virologists and bacteriologists will allow the study of the role of macrophages (Herbein) in the context of infection with *Pseudomonas aeruginosa* (Plésiat), especially in assessing the role of secretins. The virology group of Pierre Pothier (Dijon) has recently joined (January 2011) a national expert network led by Patrick Plésiat on the analysis of cystic fibrosis pathogens. The task of Pierre Pothier will be to identify respiratory viruses responsible for infectious exacerbations in cystic fibrosis patients. This expertise will be complementary to that of Patrick Plésiat (*Pseudomonas aeruginosa*) and Catherine Neuwirth (*Achromobacter xylosoxidans*), and will benefit of the clinical samples collected by the two bacteriology groups. In addition, group meetings between the teams of Patrick Plésiat (Besançon) and Catherine Neuwirth (Dijon) have already been scheduled on a quarterly basis. The first meeting will be held in April 2011.

Following the recommendation of the committee and in order to better clarify several aspects of the virological research projects we have joined **in the annexe** a complete description of the experimental work (summarized in the introduction section of each project) we are going to perform in the laboratories of virology of Dijon and Besançon in the next few years. In particular we provide here detailed information about research on norovirus-host cell interactions and on the intestinal immune response after immunization with rotavirus like particles in mice performed by the Pothier's group and on the pathogenesis of HIV infection and the oncomodulatory effect of HCMV performed by the Herbein's group. Synergy and know-how between Herbein and Pothier's teams are also underlined in the introductory sections of each project. Pierre Pothier's research activity on immune response is making obvious the common interest that is shared for this topic with Besançon virology laboratory. We strongly believe that cooperation between both virology laboratories will be fruitful in

many aspects, such as allowing student exchanges through a common Bourgogne-Franche-Comté doctoral school, better research funding for setting common grant proposals, benefiting from the expertise of each other to ultimately lead to synergistic successful research programs.

We would like to thank again the review committee for his advice allowing us to shape a better research project and we hope that the members of the review committee will be satisfied with our comments.

Sincerely yours,

Georges HERBEIN

Annexe : * Detailed Project Team 1 EA4266 G. Herbein
* Detailed Project Team 3 EA4266 P. Pothier

PROJECT Team 1 (Georges HERBEIN)
2012-2015

SUMMARY

Introduction

Following recommendations of the review committee, we will focus our work for the next research plan on the role of three viruses, namely HIV, HCV and HCMV, that all target the macrophage. The topics of the clinical groups (hepatology, rheumatology) will no more be addressed in the revised version of the project, thereby allowing to focus on the main expertise of our team the study of HIV pathogenesis, especially in the context of coinfections with HCV and HCMV that are most important opportunistic infections in the context of HIV/AIDS.

The annexes contain a comprehensive description of our research projects (projects 1 and 2). In the first section we will detail the experiments for investigating the role of HIV-1 Nef interacting with the cellular protein Akt (aim 1/project1). We will determine the molecular consequences of Nef-Akt binding in T-cell function in HIV pathogenesis and in the formation of viral reservoirs. Our working hypothesis of this aim is that Nef binds to Akt to enhance IL-2 production and favors the formation of reservoirs and that Akt-blocking agents such as HIV protease inhibitors (PI) disrupt its function. The team of Pierre Pothier will participate in the harvest of blood samples from PI-treated patients to complete our study. Since HCV/HIV coinfections are quite frequent, we will detail the experiments for investigating the role of Nef interacting with the HCV core protein in macrophages (aim2/projet1). Our working hypothesis of this aim is that HIV Nef binds HCV core associating TRAF2, 5 and 6 in a multiprotein complex and that this will result in enhanced HIV and HCV replication in macrophages. Since we and other have shown that HIV Nef modulates the apoptotic process, we will determine the molecular consequences of HIV Nef binding to the the translation elongation factor EF1A in macrophages (aim3/project 1). Our working hypothesis of this aim is that HIV Nef interacts with EF1A and Exportin t resulting in a cytoplasmic relocalization of the Nef/EF1A complex leading to the blockade of stress-induced apoptosis.

In the second section, we will describe the experimental plan for investigating the role of HCMV in oncogenesis. HCMV infection has recently been detected in a high percentage of human malignant glioma cells *in vivo*, but not adjacent normal brain, but also the role of HCMV as an "oncomodulator" has been suggested in colon cancer, prostate cancer, liver cancer and breast cancer. Therefore we would like to reassess the role of HCMV in two frequent human cancers, namely the liver cancer and the breast cancer. We will determine the role of HCMV in liver cancer (aim1/project2). Our working hypothesis of this aim is that HCMV triggers oncogenesis in hepatocytes and that the IL-6/STAT3 axis could be involved in this phenomenon. We will also determine the role of HCMV in breast cancer (aim2/project2). Our working hypothesis of this aim is that HCMV infects Human Mammary Epithelial Cells (HuMEC) and favors their transformation. We will detail the experiments that will be performed to test this hypothesis, including the analysis of several signaling pathways involved in breast cancer (Ras, Akt, STAT3), the measurement of HuMEC proliferation after HCMV infection and the use of soft agar colony formation assay. We will test *in vivo* the oncogenic potential of HCMV-infected HuMEC in collaboration with the team of Pierre Pothier at the University of Burgundy that has extensive expertise in small animal models.

As requested by the review committee, we provide below a detailled approach of our scientific project for the 2012-2015 period.

PROJECT 1. ROLE OF NEF IN HIV-1 PATHOGENESIS

The Nef protein of HIV-1 is an important factor in AIDS pathogenesis. In addition to downregulating CD4 and the major histocompatibility complex class I molecules from the cell surface, as well as increasing virion infectivity, Nef triggers activation of the T-cell receptor (TCR) cascade to facilitate virus spread and modulates cellular signal transduction pathways. Besides endogenous Nef protein expressed within infected cells, exogenous Nef protein is detected in the serum of the HIV-infected subjects. Exogenous Nef protein has been shown to enter the cell by absorptive endocytosis following binding to the surface of $CD4^+$ T cells, primary macrophages, and U937 promonocytic cells and to activate the signal transducer and activator of transcription 1 (STAT-1) and nuclear factor-kappa B (NF-kB) in human monocytes/macrophages.

SPECIFIC AIMS of PROJECT 1
The specific aims of our project are as follows :

1. Determine the molecular consequence of Nef-Akt binding in T cell function in HIV pathogenesis and in the formation of viral reservoirs. *Our working hypothesis of this aim is that Nef binds to Akt to enhance IL-2 production and favors the formation of reservoirs and that Akt-blocking agents such as HIV protease inhibitors (PI) disrupt its function.*

2. Determine the molecular consequence of HIV Nef/HCV core binding in macrophages and to elucidate the role of macrophages in HIV/HCV coinfection. *Our working hypothesis of this aim is that HIV Nef binds HCV core associating TRAF2, 5 and 6 in a multiprotein complex and that this will result in enhanced HIV and HCV replication in macrophages*.

3. Determine the molecular consequences of HIV Nef/EF1A binding in macrophages. *Our working hypothesis of this aim is that HIV Nef interacts with EF1A and Exportin t resulting in a cytoplasmic relocalization of the NEF/EF1A complex leading to the blockade of stress-induced apoptosis.*

1. Specific Aim 1.

Determine the molecular consequence of Nef-Akt binding in T cell function in HIV pathogenesis and in the formation of viral reservoirs. *Our working hypothesis of this aim is that Nef binds to Akt to enhance IL-2 production and favors the formation of reservoirs and that Akt-blocking agents such as HIV protease inhibitors (PI) disrupt its function.*

1.1. Background and significance

Symptoms of immune hyperactivation in HIV-1-infected individuals are noted throughout the course of infection and include spontaneous lymphocyte proliferation; expression of T cell activation antigens; lymph node hyperplasia; increased cytokine

expression; and elevated levels of neopterin, β2-microglobulin, acid-labile interferon, and IL-2 receptors. Immune hyperactivation is probably critical for the maintenance of the infectious process, as HIV-1 cannot infect resting T-cells and therefore depends on a pool of activated T cells in the host. Proposed mechanisms for this immune activation include among others the persistence of virus and viral antigens throughout the course of the disease, the presence of a superantigen encoded by HIV or another microbe, and the presence of autoimmune phenomena.
The serine/threonine kinase protein kinase B (PKB) Akt is a key regulator in the phosphoinositol-3-kinase (PI3K) signalling pathway and plays important role in many cellular processes such as cell survival, metabolism, growth, proliferation, and mobility that could be involved in HIV-1 pathogenesis. The HIV accessory protein negative factor (Nef) is one of the earliest and most abundantly expressed viral proteins. Extracellular Nef protein is also found in the serum of infected individuals and has immunomodulatory effects such as the suppression of immunoglobulin class switching in bystander B cells. Since most of the T cells present in the peripheral blood of HIV-1-infected subjects are bystander uninfected cells and since HIV-1 Nef has been reported to interfere with several signalling pathway in T cells, we considered the possibility that soluble HIV-1 proteins, such as the Nef protein, could lead to the superactivation of T cells via its potential interaction with Akt.

1.2. Preliminary studies

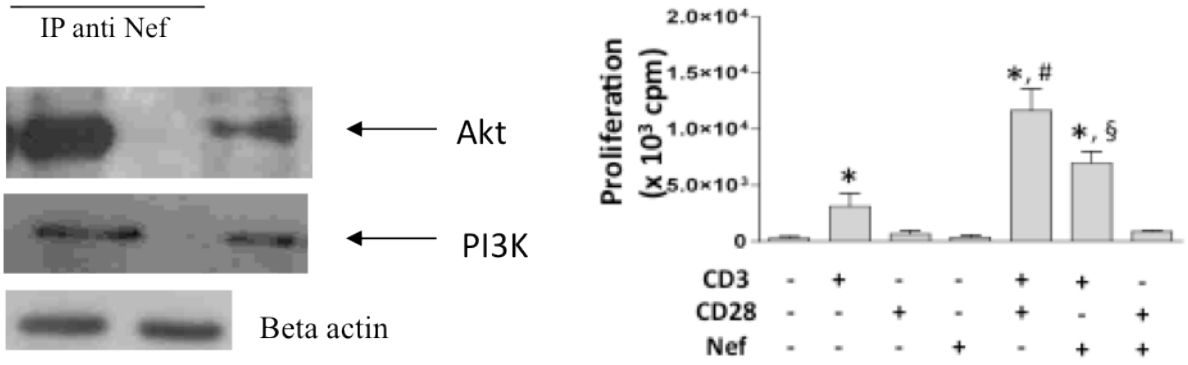
Exogenous HIV-1 Nef binds Akt and PI3K and favors IL-2 production and T-cell proliferation.

We have shown that :

1) Nef interacts with Akt and PI3K by coimmunoprecipitation

2) Akt undergoes serine-473 and threonine-308 phosphorylation following direct interaction with the HIV-1 Nef protein and translocates into the lipid rafts.

3) treatment of TCR-stimulated T-cells with exogenous Nef « mimics » the CD3/CD28 co stimulation with increased IL-2 production and T-cell proliferation.



1.3. Research design and Methods

We observed that endogenous Akt coimmunoprecipitated with HIV-1 Nef in peripheral blood lymphocytes (PBLs) treated with exogenous Nef. To further test whether this interaction is direct or indirect, we will express HIV-1 Nef as a GST fusion protein in *E. Coli* and we will test its ability to interact with Akt present in cell lysates of T cells. We will also assess the interaction of HIV-1 Nef and Akt by transient transfection of T cells with a pNef plasmid. This will indicate whether HIV-1 Nef interacts with Akt not only in

primary T cells treated with exogenous Nef, but also following endogenous expression of Nef. To determine the extremity of HIV-1 Nef that binds AKT, we will test GST-nef plasmids truncated at their C-terminal or N-terminal extremity for their binding to Akt using a pull-down assay.
We will also further test the interaction between HIV-1 Nef and PI3K, an upstream kinase involved in Akt phosphorylation. We observed that endogenous PI3K coimmunoprecipitated with HIV-1 Nef in PBLs treated with exogenous Nef. We will determine whether HIV-1 Nef bounds to PI3K by pull-down assay. To determine the extremity of HIV-1 Nef that bind PI3K, we will test GST-nef plasmids truncated at their C-terminal or N-terminal extremity for their binding to PI3K using a pull-down assay. All together our results should indicate that HIV-1 Nef binds to both Akt and PI3K, delineate the regions of Nef involved in binding to Akt and PI3K, and ultimately might suggest a critical role for Nef as an adaptator protein in the PI3K/Akt pathway.

The next series of experiments will aim to determine the intracellular signalling pathways elicited by Nef/Akt pathway in primary T cells. Because of its putative role as co-stimulator in activated T cells, exogenous Nef was tested for its ability to influence Akt activation, one of the main molecules phosphorylated following CD28 costimulation. We observed that in PBLs treated with exogenous Nef, Akt phosphorylation started as early as 1 min and peaked at 5 min post treatment. We will use an Akt inhibitor, Akt inhibitor VIII, to block the phosphorylation of Akt on Ser473 and Thr308 in PBLs treated with exogenous Nef. To determine whether the effect of HIV-1 Nef on Akt phosphorylation is dependent upon cellular PI3K activity, we will use PI3K inhibitors LY294002 and wortmannin. Since CD3/CD28 costimulation is a key regulator of T cell activation and proliferation, we will test the effect of exogenous Nef on phosphorylation of Akt in the context of TCR mediated CD28 costimulation. Our results should indicate that exogenous Nef alone or in conjunction of TCR stimulation is able to significantly activate Akt in primary T cells. We will also assess whether exogenous Nef treatment alone or combined with TCR stimulation induces the translocation of activated Akt to lipid rafts, using western-blotting in the presence or absence of methyl-beta-cyclodextrin, a compound known to disrupt lipid rafts (this will be done in collaboration with Pierre Pothier's team).

Nef by itself has been reported to activate NF-kB in T cells and TCR/CD28 costimulation activates CD28RE and NF-kB that can recruit the p300/CREB-binding protein histone acetyltransferase to the *IL-2* promoter. Therefore, we will test the effect of Nef by itself and in TCR-mediated costimulation context on CD28RE and NF-kB activation in primary T cells, using EMSA. These experiments will be performed in the presence or absence of the Akt inhibitor VIII to determine whether Akt is involved in both CD28 RE and NF-kB activation by exogenous HIV Nef. We will also perform experiments to determine whether exogenous Nef alone or in combination with TCR stimulation modulates IL-2 production and T-cell proliferation. In agreement with our preliminary data, we expect to observe that Nef in combination with TCR stimulation, but not Nef alone, stimulates IL-2 production and T-cell proliferation. To show that the Nef-mediated IL-2 production and T-cell proliferation is Akt-dependent and Nef-specific, we will use the Akt inhibitor VIII and neutralizing anti-Nef antibodies.
Since T-cell activation is critical for HIV-1 replication, we will investigate the potential biological role of exogenous Nef and Akt in PBLs infected with HIV-$1_{89.6}$ and NL4-3. We

expect to observe that exogenous Nef increases HIV-1 replication in acutely infected PBLs that could be blocked by the AKT inhibitor VIII and the PI3K inhibitor LY294002.

To further demonstrate the critical role of Nef and Akt in HIV pathogenesis, we will compare HIV-1-positive patients infected with a wild-type virus (Nef-expressing virus) naive of any treatment, patients from the Sydney Blood Bank Cohort (SBBC) infected with an attenuated nef-deleted HIV-1 (collaboration with the University of Melbourne) and healthy HIV-negative controls. In contrast to HIV-1-positive patients infected with a wild-type virus, we do not expect to detect significant Akt activation in PBLs isolated from HIV-1 patients infected with an attenuated nef-deleted HIV-1, as measured by western-blot or flow cytometric analyses. In addition, since HIV-1 protease inhibitors (PI), but not non-PI drugs (INRT, INNRT) have been shown to block Akt activation in peripheral blood of cancer patients, we will assess the potential use of PI as immunomodulatory molecules in addition to their antiviral effect in HIV infection. The team of Pierre Pothier will participate in the harvest of blood samples from PI-treated patients to complete our study. We expect to see low level of Akt activation in PBLs isolated from HIV-1 patients under HAART treated with PI, but not treated with nonPI drugs. We will also measure both the production of IL-2 and T-cell proliferation in PBLs isolated from HIV-1 patients infected with an attenuated nef-deleted HIV-1 when compared with HIV-1 patients infected with a wild-type virus. We expect to observe that the production of IL-2 and T-cell proliferation are significantly lower in PBLs isolated from HIV-1 patients under HAART treated with PI in comparison to PBLs isolated from HIV-1 patients treated with nonPI. All together we expect to show *ex vivo* that the PBLs of naive ΔNefHIV-infected patients and PI-treated patients, but not the PBLs of naive Nef+HIV+ patients and nonPI-treated patients, have decreased IL-2 production and T-cell proliferation. We are aware that the limited cell number obtained from patients infected with deltaNef HIV could make these experiments difficult. Therefore we will use assays which require only a limited number of cells to be performed: MTT assay or Ki67 measurement by FACS for the measure of T-cell proliferation, measure of Akt phosphorylation by FACS.
Although the detection of soluble Nef in the serum of HIV-1 infected subjects has been previously reported, we will measure the amount of soluble Nef present in the serum of HIV-1 patients infected with an attenuated nef-deleted HIV-1 when compared with HIV-1 patients infected with a wild-type virus (HIV Nef ELISA Kit, Immunodiagnostics).

Since T-cell proliferation and IL-2 production are critical factors for the replication of HIV-1, we hypothesize that the size of the HIV-1 cellular reservoir could be limited in naive ΔNefHIV-infected patients and PI-treated patients, and expanded in naive Nef+HIV+ patients and in nonPI-treated patients. To evaluate this hypothesis, we will purify PBMCs from blood of naive ΔNefHIV-1-positive patients, naive Nef+ patients, PI- and nonPI-treated patients and we will deplete them in CD8+ T cells before stimulation with anti-CD2 Ab + antiCD28 Ab and measurement of HIV-1 genomic RNA concentrations in culture supernatants, a marker of the size of HIV-1 reservoirs. We expect to show that the reactivation of HIV-1 from latency will be barely detectable in naive HIV-1 patients infected with an attenuated nef-deleted HIV-1 whereas this reactivation will be positive in naive Nef+HIV+ patients infected with a Nef-expressing virus. Also, we expect to show that the HIV-1 cellular reservoir is significantly decreased in HIV-1 patients treated with PI versus HIV-1-infected patients treated with nonPI. Among the cell types present in CD8+-depleted PBMCs, latently-infected resting memory CD4+ T cells that harbour integrated replication-competent viral DNA represent the primary long-lived source of HAART-persistent HIV-1. Therefore, we will purify HLA DR-CD4+ T-cells from blood samples obtained from naive and HAART-treated patients, we will stimulate them with antiCD2 Ab + antiCD28 Ab treatment and measure the HIV-1

reactivation. We expect to show that the HIV-1 cellular reservoir is significantly smaller in HIV-1-infected patients treated with PI versus HIV-1-infected patients treated with nonPI.

In conclusion, we expect to show that Akt activation of T-cells mediated by exogenous Nef is critical in HIV-1 pathogenesis, and that the immunomodulatory effects of PI which block Akt could prevent the formation of cellular reservoirs of HIV-1, especially if used early after infection.

2. Specific Aim 2

Determine the molecular consequence of HIV Nef/HCV core binding in macrophages and to elucidate the role of macrophages in HIV/HCV coinfection. *Our working hypothesis of this aim is that HIV Nef binds HCV core associating TRAF2, 5 and 6 in a multiprotein complex and that this will result in enhanced HIV and HCV replication in macrophages.*

2.1. Background and significance

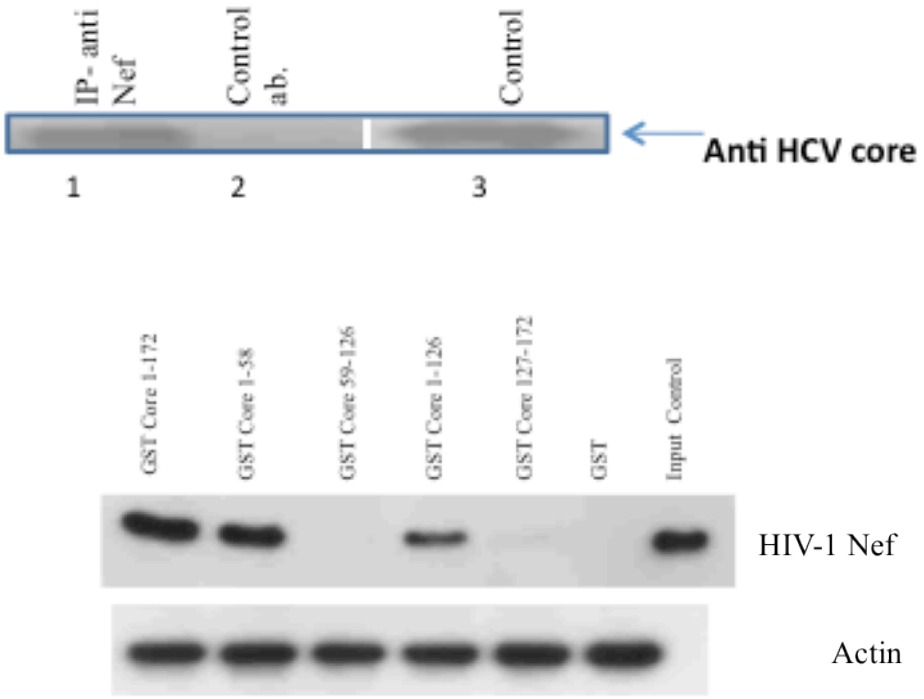
Hepatitis C virus (HCV) infection is common in HIV-1 infected patients and each of these infections may affect the other. Thus, several reports found that HIV infection accelerates the development of severe liver disease and HIV facilitates infection and replication of HCV in human macrophages. It was also reported an association between HCV coinfection and progression of HIV disease. HCV was originally thought to be a strictly hepatotropic virus, but there is mounting evidence that it can also replicate in peripheral blood mononuclear cells (PBMC) and human macrophages, particularly in patients with HIV infection. Extrahepatic replication of HCV could be facilitated by immunosuppression and HCV replication may be directly enhanced by the presence of HIV. Among HCV structural proteins, the HCV core protein has also been shown to affect various cellular signalling pathways, to activate different promoters, e.g., c-myc, c-fos, and has further been shown to modulate death ligand-mediated apoptosis. Besides endogenous HCV core protein expressed within infected hepatocytes, but also T lymphocytes and monocytes/macrophages, exogenous HCV core protein is detected in the serum of HCV infected subjects and has been reported to activate both MAPK and NF-kB signalling in several cell types. Since both HIV Nef and HCV core proteins activate NF-κB and stimulate HIV-1 replication in several myelomonocytoid cell types, we investigated their respective role in HIV-1 and HCV replication in primary macrophages.

2.2. Preliminary results

HIV Nef interacts with HCV core protein in a multiprotein complex including TRAF2, TRAF5, and TRAF6

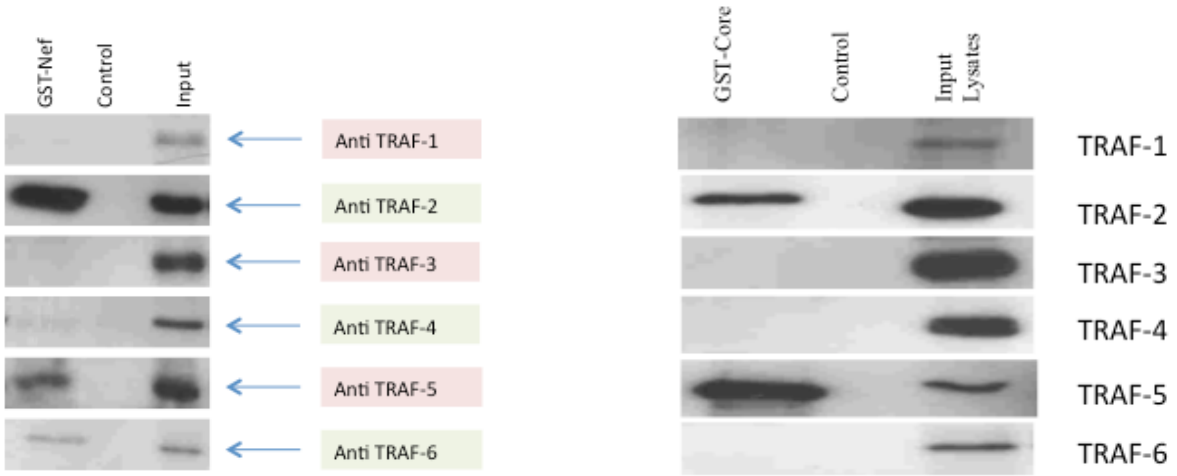
We have shown that :

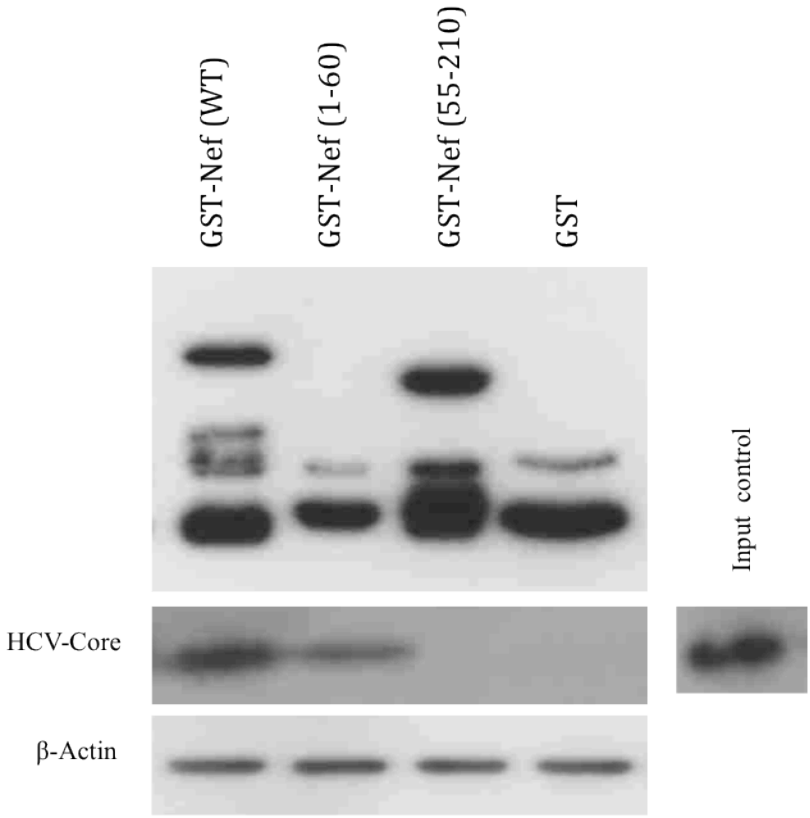
1) the N-terminal extremity of HIV-1 Nef (aa1-60) interacts with the N-terminal extremity of HCV core (aa 1-58)



2) HIV-1 Nef interacts with TRAF2, TRAF5, and TRAF6 via its C-terminal extremity (aa55-210)

3) HCV core interacts with TRAF2 and TRAF5 via its region (aa1-126)





2.3. Research design and methods

Since HIV-1 Nef and HCV Core proteins are independent activators of NF-κB in several cell types, we will investigate their possible synergistic activity in primary monocyte-

derived macrophages (MDM). We will treat MDM with either HIV-1 rNef or HCV rCore protein and measure NF-κB activation by EMSA. We expect to observe that in contrast to a treatment with either of the viral proteins, a cotreatment with HIV-1 Nef and HCV Core proteins will result in the most potent NF-κB activation in MDM. We will verify that the gel shift band is specific as shown by diminished formation of the complex by inclusion of an unlabeled NF-kB oligonucleotide but not by inclusion of a mutated NF-kB oligonucleotide. We will also identify the subunits involved in the NF-kB complexes following treatment of MDM with rNef, rCore or with the two recombinant proteins used together using Abs against different NF-kB subunits before measuring DNA-binding activity by EMSA. We will determine whether the p50/p65 canonical NF-kB pathway is activated, or whether a non-canonical NF-kB pathway plays a role. We will measure the phosphorylation and degradation of IkBα following treatment with the two viral proteins alone or together, as typically occurs during the activation of the canonical NF-kB pathway, using western-bloting. We will also measure the respective expression levels of IKKα, IKKβ and IKKγ and determine their phosphorylation levels in MDM treated with rHIV-1 Nef and rHCV core alone and together. We expect to show a synergistic effect of HIV-1 Nef and HCV core proteins on canonical NF-κB activation in MDM. To confirm our preliminary data indicating that a TRAFs complex plays a role in HIV-1 Nef and HCV core mediated activation of NF-kB, we will transfect MDM with siRNAs directed against TRAF2, TRAF5, and TRAF6, but also against TRAF1, TRAF2 and TRAF3 as negative controls, 48 hours before treatment with HIV-1 Nef and HCV core. We are aware that the transfection of primary MDM is not easy, but we already performed successfully this methodological approach in the past (TransRNAMax). We will then assess the effect of HIV-1 Nef, HCV core protein, or both proteins together on NF-κB-dependent long terminal repeat (LTR) stimulation. U937 cells will be transiently transfected with a target plasmid that contains the luciferase reporter gene under the control of the HIV-1 LTR promoter, pLTR-Luc. Twenty-four hours later, transfected cells will be treated for 24 h with HIV-1 Nef, HCV core protein, or both proteins together, harvested, and luciferase activity will be measured in cell lysates. We expect to observe that HIV-1 Nef treatment and HCV core protein treatment of transfected U937 cells increase HIV-1 LTR stimulation, but that the co-treatment of transfected U937 cells with HIV-1 Nef and HCV core proteins would result in the highest LTR stimulation. We will also verify that the LTR activation induced by HIV-1 Nef, HCV core protein, or both proteins together is not observed when a plasmid containing mutated NF-κB sites, pLTR-mut-NF-κB-Luc, will be used instead of pLTR-Luc. We expect our results to indicate that HIV-1 Nef and HCV core proteins activate synergistically the LTR *via* NF-κB stimulation in promonocytic cells U937. Since NF-κB DNA-binding sites are present in the HIV-1 LTR, we will determine the effect of HIV-1 Nef and HCV core proteins, alone and together, on provirus transcription in the promonocytic cell line U1, U937 cells that contain two integrated HIV copies per cell. We will measure the HIV-1 replication in U1 cells following treatment with HIV-1 Nef and HCV core protein, alone or together. We expect to observe that both HIV-1 Nef and HCV core protein stimulate viral replication in U1 cells as measured by p24 assay, but that the highest viral replication will be detected in culture supernatants of U1 cells co-treated with HIV-1 Nef and HCV core proteins, indicating a synergistic effect of the two proteins on HIV-1 transcription. To rule out the possibility that a TNFα inducer, such as LPS, enhanced viral replication in U1 cells, we will boil HIV-1 Nef and HCV core protein at 100°C; boiling should abolished viral replication in U1 cells stimulated with the two proteins either alone or together,

indicating that both HIV-1 Nef and HCV core proteins, but not LPS contamination, are responsible for enhanced replication.

We will also assess the effect of HIV-1 Nef and HCV core protein on HIV-1 growth following acute infection of MDM with HIV-1 $_{89.6}$ (10 ng p24/4 X 10^5 cells). We expect to see that each of the HIV-1 Nef and HCV core proteins will enhance HIV-1 replication in primary MDM versus untreated infected cells. Also when acutely infected macrophages will be treated with the two proteins together, we expect the HIV growth to be the highest, indicating a synergistic action of Nef and HCV core proteins on HIV-1 replication in primary MDM.

In addition, we will transfect MDM with a HCV infectious clone (Jc1-Luc) in the absence or presence of treatment with HIV Nef and HCV core alone or together, and measure the luciferase activity in cell lysates. We expect to observe the highest luciferase activity in transfected MDM co-treated with HIV rNef and HCV rCore.

To expand our *in vitro* studies, we will then analyzed both plasma and intracellular HIV-1 and HCV loads in PBMC, PBL and MDM isolated from the peripheral blood of 20 HIV-1/HCV coinfected patients, 20 HIV-1 infected patients, 20 HCV infected patients and 20 healthy donors using the measurement of intracellular HIV RNA and HCV RNA by RT-PCR. We have recently developed a RT-PCR assay in our laboratory that allow the accurate and reliable measurement of intracellular HIV and HCV viral loads. We expect to observe that the plasma HIV-1 load will be higher in HIV-1/HCV coinfected patients in comparison to HIV-1 infected patients. In addition, we expect to show that both intracellular HIV-1 and HCV viral loads are higher in MDM from HIV-1/HCV coinfected subjects in comparison to HIV-1 or HCV monoinfected subjects. Since NF-kB activation has been reported to stimulate the HIV-1 long terminal repeat (LTR) resulting in enhanced viral production and is also observed in HCV-infected cells, we will measured NF-kB activation in PBMC, PBLs and MDMs isolated from the peripheral blood of HIV-1/HCV coinfected patients, HIV-1 infected patients, HCV infected patients, and healthy normal donors. We expect to observe that NF-kB activation is higher in MDM and in PBMC of HIV-1/HCV coinfected subjects in comparison with HIV-1- or HCV monoinfected subjects. We will also measure the level of NF-kB activation of autologous PBLs, as an internal control. We will confirm the presence of soluble HIV Nef and HCV Core in the serum of patients by using ELISA. We are aware that the direct detection of HIV-1 and HCV Core in the MDM isolated from HIV/HCV coinfected patients will be difficult. Nevertheless by using large amount of MDMs isolated from the peripheral blood of HIV/HCV coinfected subjects (after agreement of the Ethical Committee of the University Medical Hospital of Besançon) we might be able to detect Nef/Core/TRAF2,5,6 complexes using coimmunoprecipitation assays.

3. Specific Aim 3

Determine the molecular consequences of HIV Nef/eEF1A binding in macrophages. *Our working hypothesis of this aim is that HIV Nef interacts with EF1A and Exportin t resulting in a cytoplasmic relocalization of the NEF/EF1A complex leading to the blockade of stress-induced apoptosis.*

3.1. Background and significance

Identification of new cellular factors that interact with a specific domain of HIV-1 Nef might help elucidate the function of that domain in virus replication. To this end, HIV-1 Nef was used as a bait in a far western assay screen of a mammalian cDNA library. An interaction between Nef and the translation elongation factor 1-alpha (eEF1A) was identified. This elongation factor is an essential component of the cellular translational machinery. In its GTP-bound form, eEF1A delivers aminoacyl-tRNA to ribosomes. Once associated with the ribosome, eEF1A hydrolyzes GTP, is released from the tRNA, and leaves the ribosome. Although the conventional role of eEF1A during protein synthesis is to bind and transport aminoacyl-tRNA to the A site of the ribosome in a GTP-dependent mechanism, an increasing body of evidence suggests that, in addition to its role in peptide elongation, eEF1A may have functions beyond translation. One of these proposed unconventional functions of eEF1A is its role in the regulation of dynamics of the cytoskeleton. Because eEF1A is an abundant protein in most eukaryotic cells, and it binds to actin filaments with relatively high affinity, it could be a potent regulator of the dynamics of the cytoskeleton.

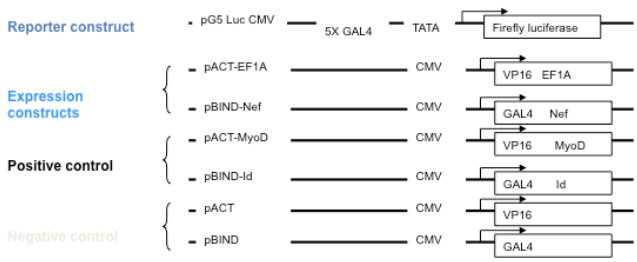
3.2. Preliminary results.

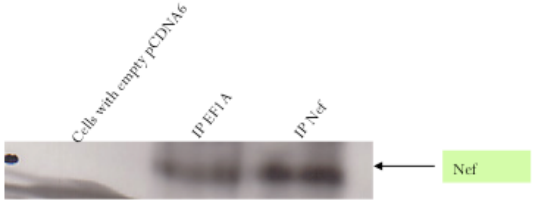
HIV-1 Nef interacts with eEF1A in primary macrophages.

We observed the direct interaction of Nef with the translation elongation factor 1 alpha (eEF1A) by coimmunoprecipitation assays, pull-down assays, and two-hybrid system analysis.

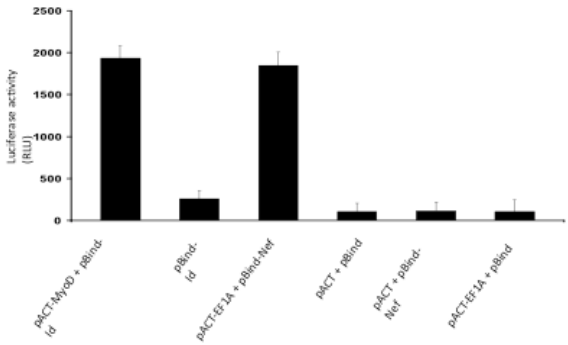
We have shown:

1) the interaction between eEF1A and Nef by coimmunoprecipitation and two-hybrid assay.



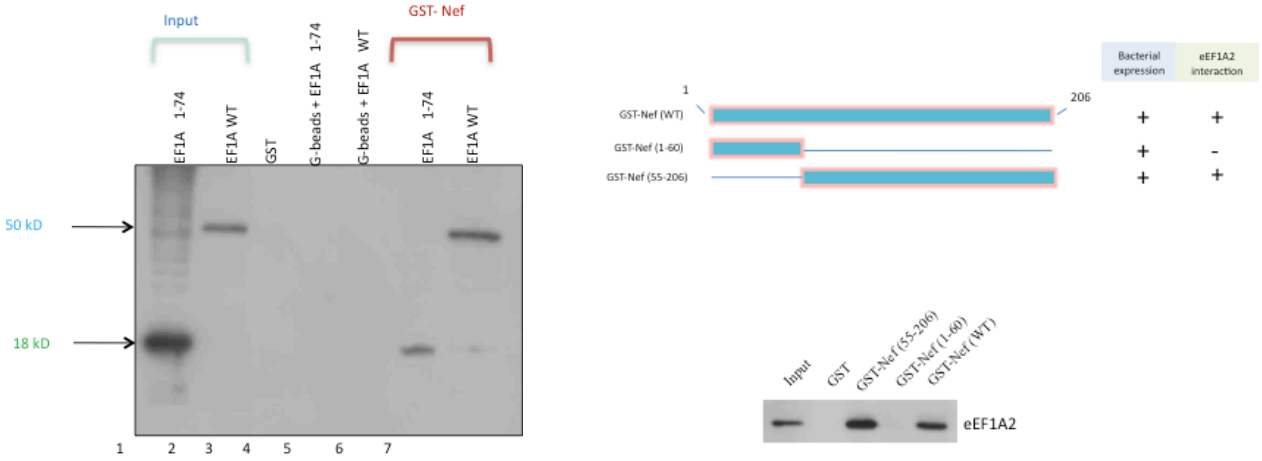


Coimmunoprecipitation of eEF1A and Nef in MDM transfected with pNef (or pcDNA6 control)



Two-hybrid assay

2) that the N-terminus of eEF1A (aa1-74) and the domain 55-206 of Nef are involved in EF1A/Nef binding



3.3. Research design and methods

eEF1A is a protein found both in the nucleus and in the cytoplasm of cells and its nucleocytoplasmic shuttling allows the transport of tRNA which is mainly processed through Exp-t. To determine whether Nef via its interaction with eEF1A could interfere with the nucleocytoplasmic repartition of eEF1A, we will treat MDMs with exogenous Nef. Nuclear and cytoplasmic extract of cells treated up to 5 hours with exogenous Nef will be prepared and the eEF1A/Nef interaction will be assessed in both cellular compartments. We expect to show that in MDMs, exogenous Nef enhances the nucleocytoplasmic shuttling of eEF1A. We will determine whether this nucleocytoplsmiac shuttling occurs in a Nef-mediated dose-dependent manner.

Since the active export of proteins from the nucleus has been reported to involve exportins, we will then test two exportins, Exp-t and Exp-1, in regard to the nucleocytoplasmic shuttling of eEF1A in the presence of Nef. We are aware that other exportins (e.g. Exp-5) could be involved in this shuttling, but Exp-1 is the exportin which is most often involved in such processes and Exp-t has been described to favor the nucleocytoplasmic transport of tRNAs which have been reported to bind to eEF1A. Using lysates of MDM, we will try to detect a direct interaction between HIV-1 Nef and Exp-t using pull-down-assays. We do not expect to detect a direct interaction between HIV-1 Nef and Exp-1 since eEF1A nuclear export has been reported to be mediated via Exp-t. To determine the region of HIV-1 Nef responsible for binding to Exp-t, the two C-terminus (GST-Nef1-60) and N-terminus (GST-Nef55-206) deleted GST-Nef mutants will be assayed for their ability to associate *in vitro* with Exp-t. Using lysates of MDM, we will also try to detect an interaction between eEF1A and Exp-t by pull-down assay and coimmunoprecipitation. To determine which portion of eEF1A is responsible for association with Exp-t, deletions mutants engineered within the cDNA encoding eEF1A will be used.

Following treatment with exogenous Nef of MDMs, we expect to detect the transient appearance of a Nef/eEF1A/Exp-t complex in the cytoplasm followed by a release of Exp-t from the complex. After the release of Exp-t from the complex, we expect the

Nef/eEF1A complex to be detected in the cytoplasm of exoNef-treated MDMs for a longer period of time. We will also transfect MDM with Exp-t siRNA 48 hours prior treatment with exogenous HIV-1 Nef. Knockdown of the Exp-t protein in MDM will be monitored by Western-blot. Band density will be quantified using ImageJ 1.40 software, and the results will be shown as relative intensities. We expect the knockdown of Exp-t to result in enhanced nuclear retention of eEF1A and Nef in MDM cultures treated with exogenous Nef. We also expect not to detect any nuclear retention of Nef and eEF1A following treatment of MDM with the Exp-1 inhibitor leptomycin B. Altogether, we expect that our results will indicate that in Nef-treated MDM, the nucleocytoplasmic transport of the Nef/eEF1A is preferentially mediated by Exp-t.

Brefeldin A (BFA) disrupts the organization of the microtubule and the actin cytoskeletons and exerts endoplasmic reticulum (ER) and Golgi stress that result in apoptosis. Interestingly eEF1A is an actin/microtubule binding protein and thereby could interfere with BFA-induced apoptosis. eEF1A has been reported to modulate apoptosis depending on the cellular compartment involved, being proapoptotic in the nucleus and antiapoptotic in the cytoplasm. Therefore, we will measured the respective amounts of cytoplasmic and nuclear eEF1A present in MDM treated with BFA or not. We expect to observe that in the absence of Nef following BFA treatment eEF1A will present mainly in the nucleus parallel to increased apoptosis of MDM. Since HIV-1 Nef binds to eEF1A and could favor its nucleocytoplasmic transport, we will assess whether a pretreatment with HIV-1 Nef inhibits BFA-induced apoptosis in MDM in a dose-dependent manner. Since Exp-t could be involved in the nucleocytoplasmic translocation of the eEF1A/Nef complex, we will assess apoptosis of MDM treated with exogenous Nef prior to BFA treatment, and transfected or not with siRNA Exp-t. We expect to show that a treatment with siRNA Exp-t inhibits the Nef-mediated blockade of apoptosis in MDM treated with BFA. Altogether, we expect our results to indicate that the nucleocytoplasmic translocation of the Nef/eEF1A complex mediated by Exp-t is involved in the inhibition of MDM apoptosis triggered by stress, such as following BFA treatment.

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PROJECT 2. ROLE OF HCMV IN ONCOGENESIS

More than 70% of the world's adult population is infected by human cytomegalovirus (HCMV), a β-herpesvirus, and once infected the host remains persistently infected for life. On initial infection or viral reactivation, HCMV can cause serious and even fatal complications in fetuses or immunocompromised individuals. HCMV infection has recently been detected in a high percentage of human malignant glioma cells *in vivo*, but not adjacent normal brain, but also the role of HCMV as an "oncomodulator" has been suggested in colon cancer, prostate cancer, liver cancer and breast cancer. Therefore we would like to reassess the role of HCMV in two frequent human cancers, namely the liver cancer and the breast cancer.

Specific aims of Project 2

The specific aims of our project are as follows :

1. Determine the role of HCMV in liver cancer. *Our working hypothesis of this aim is that HCMV triggers oncogenesis in hepatocytes.*

2. Determine the role of HCMV in breast cancer. *Our working hypothesis of this aim is that HCMV infects Human Mammary Epithelial Cells (HuMEC) and favors their transformation.*

1. Specific Aim 1

Determine the role of HCMV in liver cancer. *Our working hypothesis of this aim is that HCMV triggers oncogenesis in hepatocytes.*

1.1. Background and significance

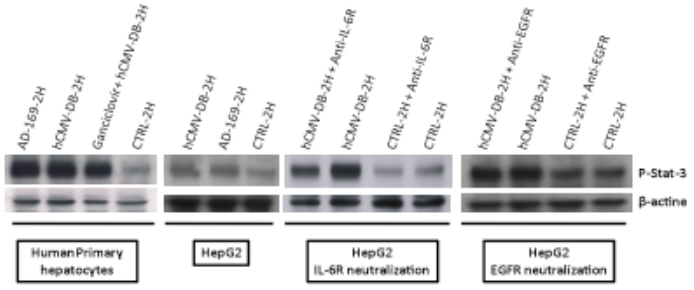
Hepatocellular carcinoma (HCC) is the common form of liver cancer and is the most strongly affected by obesity among all cancers. In addition, inflammation has been reported to play a critical role in the appearance of numerous cancers, including HCC. It has been recently reported that obesity-promoted HCC development is dependent on enhanced production of the tumor-promoting cytokines IL-6 and TNFα, which cause hepatic inflammation and activation of the oncogenic transcription factor STAT3.

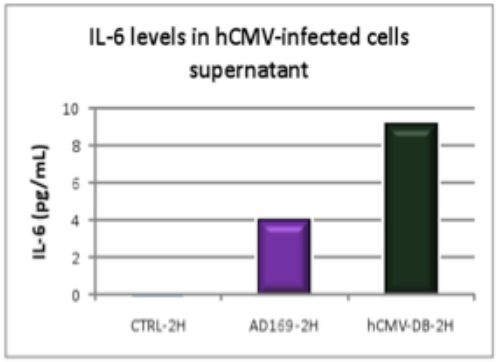
1.2. Preliminary results

HCMV triggers the production of IL-6 from HepG2, a human liver carcinoma cell line, and stimulates STAT3 phosphorylation through IL-6 receptor in primary human hepatocytes and HepG2 cells.
We have shown:

1) early phosphorylation of STAT3 (Tyr 705) in human primary hepatocytes and HepG2 cells exposed to HCMV (2 hours post-infection) using western-blotting.

2) phospho-STAT3 activation is mediated through IL-6 release and IL-6R autocrine activation by HCMV-exposed hepatocytes.





1.3. Research design and methods

We observed in our preliminary results that the production of IL-6 is increased in the supernatant of HepG2 cells exposed to HCMV parallel to the activation of STAT3. We will first confirm an increased production of IL-6 by primary human hepatocytes exposed to HCMV using measurement of IL-6 in culture supernatants by ELISA. We will confirm that several HCMV strains (laboratory isolates, clinical isolates) can enhance IL-6 production by hepatocytes and HepG2 cells. We will follow the replication of the virus not only by measurement of HCMV DNA in the culture supernatants by real-time PCR assay, but also by measuring the number of PFU/ml. This latter method should definitively allow us to demonstrate the replication of HCMV in hepatocytes. Alternatively, we might also quantify the intracellular viral load by detection of the HCMV genome by real-time PCR in cell lysates. We will also determine the presence of IE proteins and pp65 proteins by immunofluorescence in HCMV exposed hepatocytes. As a control, we will test in parallel the effect of ganciclovir on the replication HCMV in hepatocytes.

Since IL-6 binding to the IL-6 receptor is involved in STAT3 activation, we will assess the involvement of JAK1/JAK2 in STAT3 activation by using western-blot. We will also test in parallel the role of negative regulators of the STAT pathway such as SOCS proteins by using western-blot analysis. As an internal control we will measure in parallel the levels of phospho-STAT3 and of unphosphorylated STAT3 to show that the STAT3 effect results from post-translational modification of STAT3 and does not depend on STAT3 gene expression. To demonstrate that the IL-6/STAT3 axis is critical in HCMV-exposed hepatocytes we will test in parallel the expression of STAT5 and of its phosphorylated form using western-blot. In

addition to an inhibitor of the IL-6 receptor, we will use IL-6-neutralizing antibodies and short hairpin RNA targeting IL-6 to show that HCMV activates the IL-6-JAK-STAT3 signaling axis through activation of the transcription factor nuclear factor kappaB and the consequent production of IL-6. We will also test the production of other proinflammatory cytokines such as TNFα following exposure of hepatocytes to HCMV and we will assess whether the TNFα/TNF receptor axis could be also involved in hepatocyte proliferation and survival.

Since phosphoSTAT3 has been reported to target the promoters of genes involved in cell proliferation (cyclin-D1) and in cell survival (survivin), we will measure the expression of cyclin-D1 and survivin in HCMV-exposed hepatocytes and in uninfected controls. We expect to observe an increase expression of cyclin-D1 and survivin in HCMV-exposed hepatocytes versus mock controls. To further measure the effect of HCMV on hepatocyte proliferation, we will use a MTT assay, the measurement of Ki67 Ag by flow cytometry and [(3)H]thymidine incorporation. Treatment of cells with specific inhibitors of JAK and STAT3, AG490 and NSC74859 respectively, will be tested to inhibit HCMV-dependent [(3)H]thymidine incorporation and foci formation, suggesting further a key role for JAK/STAT3 axis in the HCMV-mediated proliferative phenotype. We will also assess the resistance to apoptosis of hepatocytes following HCMV exposure by using an annexin-V and propidium iodide FACS assay. We expect to see enhanced hepatocyte proliferation and/or survival following HCMV exposure. We are aware that primary human hepatocytes are very fragile cells that could survive in culture only for a limited period of time and therefore the measurement of HCMV replication and the study of signaling in these cells could be difficult. Nevertheless, our preliminary experiments indicate that we are able to growth hepatocytes for 7-10 days even after exposure to HCMV. Since the IL-6 production and STAT3 activation are very early events (in the 24-48 h post-exposure to the virus), we believe that we will be able to demonstrate the role of the IL-6/JAK/STAT3 signaling axis in this model. We expect that our results will indicate a critical role of the IL-6/JAK/STAT3 signaling axis in hepatocytes proliferation in parallel to their enhanced survival.

2. Specific Aim 2.

Determine the role of HCMV in breast cancer. *Our working hypothesis of this aim is that HCMV infects HuMEC and favors their transformation.*

2.1. Background and significance

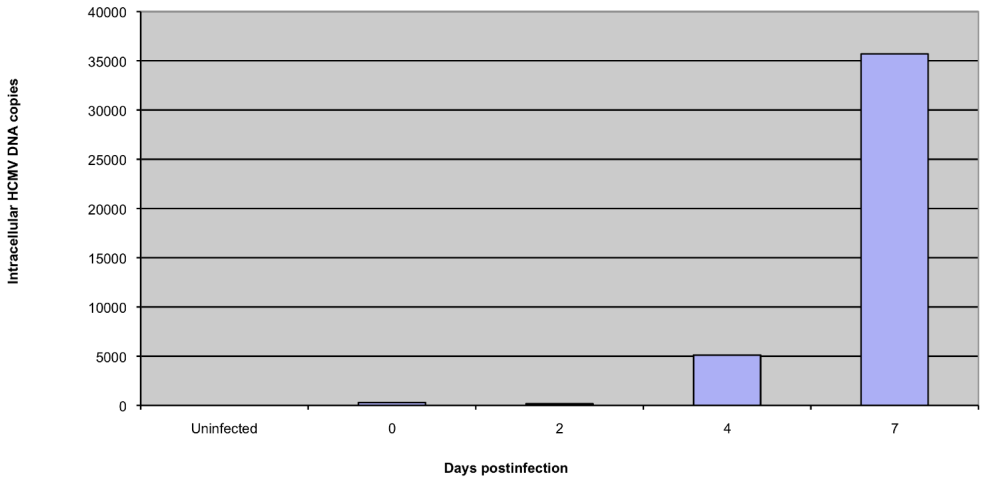
Although HCMV is generally not regarded to be an oncogenic virus, HCMV infection has been implicated in malignant diseases from different cancer entities. It has been hypothesized that breast cancer can be caused by late exposure to common viruses such as HCMV and Epstein–Barr virus (EBV). Elevation in serum CMV IgG antibody levels has been reported to precede the development of breast cancer. To address this issue we propose to test the sensitivity of human mammary epithelial cells (HuMEC) to HCMV and to determine whether HCMV-exposure of HuMEC results in the activation of signaling pathways involved in human breast cancer.

2.2. Preliminary results

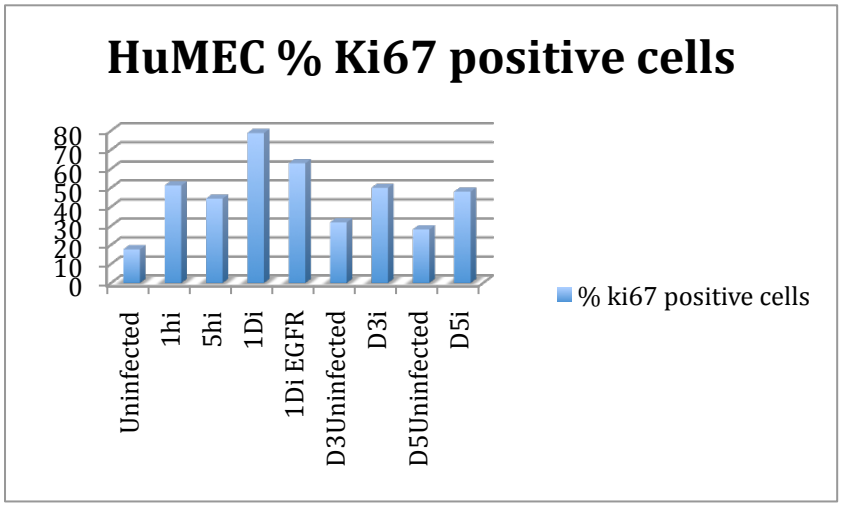
HCMV infects humain mammary epithelial cells (HuMEC) and triggers the synthesis of cyclin-D1 and phospho-STAT3.

We have shown that:

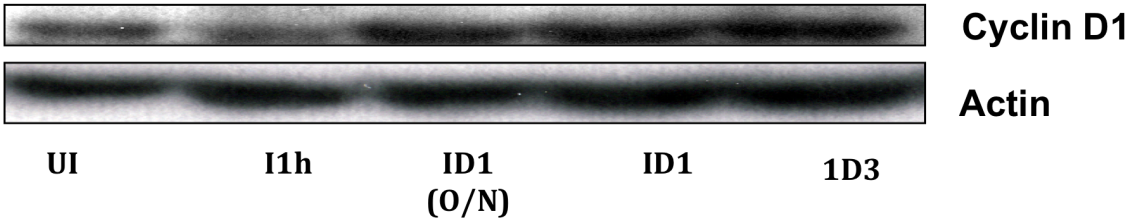
1) HuMEC are permissive to HCMV

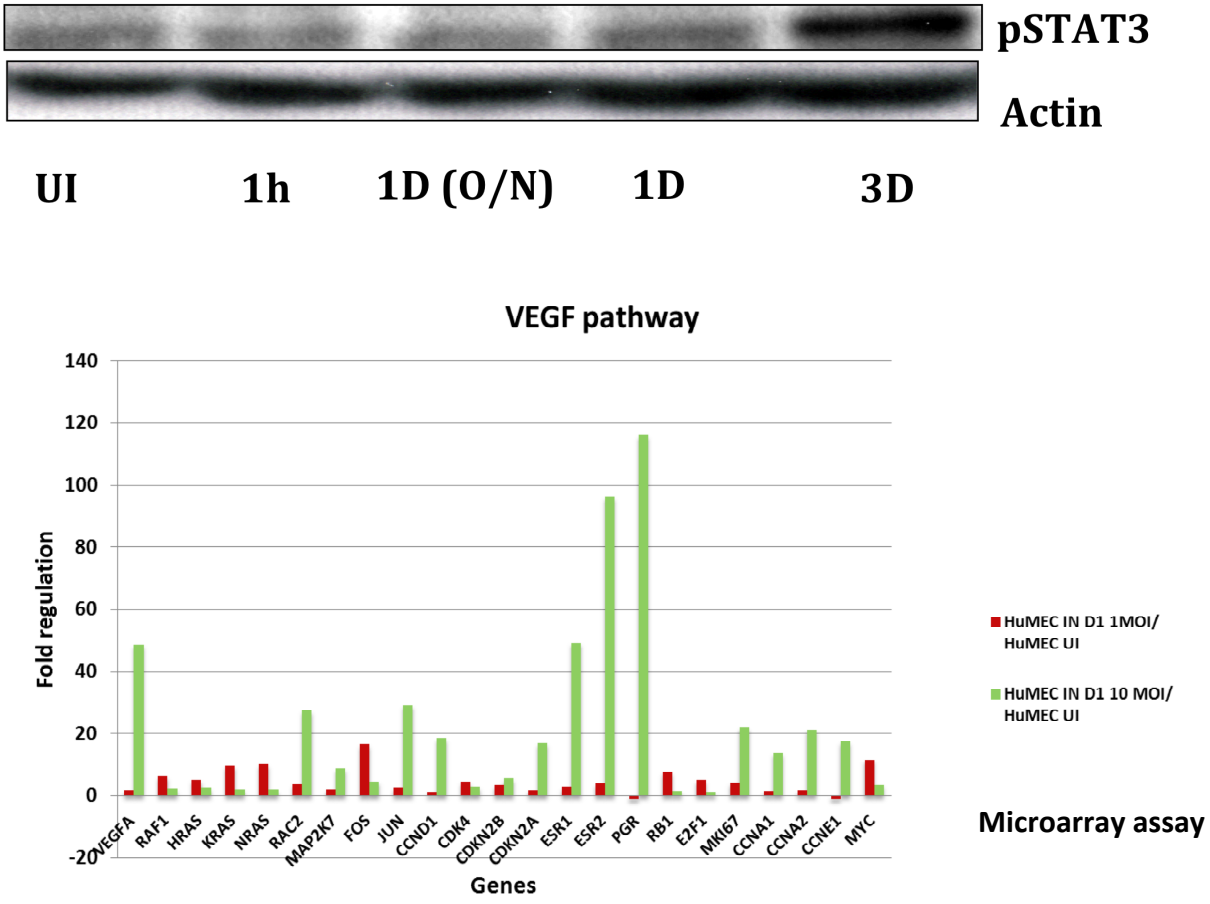


2) HCMV favors proliferation of HuMEC cells (as measured by Ki67 Ag expression)



3) HCMV strains activate several pathways involved in breast cancer : cyclin D1 and phospho-STAT3 upregulation after infection (western-blot and microarray analysis)





2.3. Research design and methods

Since epithelial cells are usually permissive for HCMV, we will further demonstrate that several strains of HCMV can replicate in HuMEC. We will therefore infect HuMEC with the HCMV strains AD169, DB, but also with the fibroblast tropic strain TB40/F and the endothelial tropic strain TB40/E. We will follow the replication of these viruses not only by measurement of HCMV DNA in the culture supernatants by real-time PCR assay, but also by measuring the number of PFU/ml. This latter method should definitively allow us to demonstrate the replication of HCMV in HuMEC. We will also quantify the intracellular viral load by detection of the HCMV genome by real-time PCR in cell lysates. We will determine the presence of IE antigens and pp65 proteins by immunofluorescence in HCMV exposed HuMEC. It would not be unexpected to detect only very few HCMV positive cells post-exposure, that could result either from the low permissivness of HuMEC cells to HCMV and/or to the fact that the proliferation of HuMEC cells could shut down the viral replication as previously reported in other cell types. We will also use GFP-labelled HCMV isolates that will further allow us to measure the replication of HCMV in HuMEC. As a control, we will test in parallel the effect of ganciclovir on the replication HCMV in HuMEC.

In efforts to delineate a potential function of HCMV in the neoplastic process, we will investigate biological properties emanating from the exposure of HuMEC to strains of HCMV. To determine whether HCMV affects signaling pathways controlling mitogenesis and oncogenesis, we will investigate in HCMV-exposed HuMEC the activity state of downstream effectors of the following pathways:

- the VEGF pathway which leads to the activation of Ras, MAPKK7, Fos/Jun, cyclin-D1, cyclin E , cyclin A and modulation of the E2F/Rb complex leading ultimately to cell proliferation.
- the EGFR/ERBB2 pathway which leads to the activation of JAK, STAT3 and via the phosphorylation of STAT3 can activate cyclin D1, and also favor cell cycle progression.
- the PI3K/Akt pathway leading to NF-kB activation and delivering anti-apoptotic signals.
- the ATM pathway which has been reported activated following HCMV infection, resulting in increased p53 expression which is blocked by the IE86 HCMV protein and ultimately could result in the decrease of the expression of p21waf, thereby favoring cell cycle progression.

More specifically, following infection of HuMEC with HCMV (AD169, DB, TB40/F, TB40/E), we will harvest the HCMV-exposed HuMEC at 1h, 5h, day 1, day 3, day 5 and day 10 to prepare: 1) total cellular protein extracts to perform western-blotting; 2) total RNA extract to perform microarrays assays. Thus, we will test the above signaling pathways at both the protein level and the mRNA level. Additional experiments could be performed using Northern blotting to detect more specifically the amount of cellular mRNA expression in HCMV exposed HuMEC. We will also test uninfected HuMEC, HuMEC exposed to heat-inactivated HCMV, HuMEC infected with HCMV in the presence of ganciclovir. The proliferative capacities of the HuMEC will be measured by detection of Ki67 Ag by flow cytometry and by a MTT test. We will also test low (0.1), intermediate (1) and high (10) MOI (multiplicity of infection) during the HCMV infection of HuMEC. High MOI might be required to express a high amount of IE proteins such as IE72 and IE86 which will block the p53 overexpression observed in HCMV infected cells as previously reported, favoring the entrance into the proliferative cell cycle. Since its has been postulated that the HCMV replication requires cell cycle arrest, we expect to observe that a higher proliferation of the HCMV-exposed HuMEC will be observed parallel to restricted viral growth, even if IE antigens are highly expressed. Thus, a delicate balance between viral replication and cellular cycling could favor the progression toward a proliferative (and potentially oncogenic) phenotype.
We expect to show that exposition of HuMEC to HCMV favor the activation of VEGF pathway, the EGRF/ERBB2 pathway, the PI3K/AKT pathway and the ATM pathway. It might be also possible that only parts of these pathways are activated, or that these cytoplasmic signalling pathways are only activated with some viral strains, or might depend of the initial viral input (low, intermediate or high MOI).

Even if we expect to detect more proliferation of HuMEC following exposure to HCMV, it does not implicate that HuMEC are transformed. To directly test the issue of oncogenesis of HuMEC following HCMV exposure, we will perform a soft agar assay for colony formation. The soft agar assay for colony formation is an anchorage independent growth assay in soft agar, which is considered the most stringent assay for detecting malignant transformation of cells. For this assay, HuMEC at day 1, day 5, day 10 and day 20 post-infection with HCMV (AD169, DB, TB40/F, TB40/E), at low, medium and high MOI, will be cultured with appropriate controls (uninfected HuMEC, HuMEC infected with heat-inactivated HCMV, HuMEC infected with HCMV in the presence of ganciclovir as negative controls ; HeLa cells and NIH3T3 cells as positive controls) in soft agar medium for 21-28 days. Following this incubation period, formed colonies will be analyzed morphologically using cell stain and we will quantify the number of colonies formed per well. We expect to observe in HuMEC

exposed to HCMV *in vitro* cellular transformation associated with certain phenotypic changes such as loss of contact inhibition and anchorage independence. The process by which these phenotypic changes occur, is assumed to be closely related to the process of *in vivo* carcinogenesis. In general there is reasonably good correlation between *in vitro* transformation and *in vivo* carcinogenesis, although the correlation varies depending on the system being studied. These systems are believed to be reasonably good predictors of *in vivo* activity, and positive results are viewed as potential indications of *in vivo* carcinogenesis.
Therefore, future studies will test *in vivo* the oncogenic potential of HCMV-infected HuMEC in collaboration with the team of Pierre Pothier at the University of Burgundy which has extensive expertise in small animal models. We will determine whether HCMV-infected HuMEC xenografted into athymic nude mice result in enhanced tumorigenicity compared to control mice xenograft with uninfected HuMEC. Even if we do not find classic transforming activity associated with HuMEC exposure to HCMV, sustained expression of IE proteins along with multiple other HCMV gene products that can inhibit cell apoptotic pathways and promote neoplastic transformation could greatly affect the oncogenic phenotype of tumor cells expressing such HCMV gene products. These findings may have relevance to the pathogenesis of breast cancer.

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SOMMAIRE

Introduction

Following recommendations of the review committee, we focused our work for the next research plan on enteric viruses, their interactions with the host and the mucosal immunity.

The annexes contain a comprehensive description of our research projects (projects 1 and 2). Epidemiology of gastroenteritis viruses is not detailed in this annex since no precision was required by the review committee. In the first section we will detail the experiments for investigating the cellular and molecular events involved in the early stages of norovirus (NoV) infection, from binding to uncoating. During the last four years we have been studying the interactions of NoV with the human blood group antigen (HBGA) carbohydrates, which participate to the docking of the virus onto the cells. In particular we have measured the affinity toward different HBGA ligands of the GII.4 variant that is largely predominant among NoVs, according to our epidemiological survey performed each year as National Reference Center for Enteric Viruses. This work has been carried out by Pierre Pothier and Gaël Belliot in the Laboratory of Virology of Dijon in collaboration with Dr. Jacques Le Pendu of the INSERM Unit 892 at the University of Nantes and Dr. Wilfrid Boireau of the FEMTO Institute at the University of Franche-Comté. Part of this study has been recently accepted for publication in the Journal of Virology (Rougemont et al. 2011 Journal of Virology, May, Vol 85(9)) and this project has been recently awarded a joint BQR grant from the Universities of Burgundy and Franche-Comté, encouraging further collaborations between the groups of the two universities. Together with our partners we would now like to explore the tropism of NoVs at molecular level (aim1/section 3). Then we will focus on the biochemical events involved in the docking and internalization of NoV viral particles (section 4). Our preliminary results strongly suggest that lipid raft domains are involved in the process (section 4.3) and we can anticipate that many cellular factors might play a role during NoV internalization. Finally, in the long term, we also propose to characterize the signaling pathways that are triggered during NoV infection (aim 3/section 5). We believe that the experience of the George Herbein's laboratory, especially on adhesion molecules (section 4.6), will be of paramount importance for the success of this project.

In the second section we will describe an experimental vaccination strategy against rotavirus (RV) infection in mice with recombinant rotavirus-like particles (VLP) administered by intrarectal (i.r.) route. This model has been set up by Pierre Pothier and Davide Agnello at the Laboratory of Virology of Dijon. We have already published that i.r. immunization with VLPs composed of viral protein (VP) 2 and VP6 (2/6-VLPs) protects adult mice against RV infection and induce a RV-specific immune response in the small intestinal mucosa. In order to understand how antigen administration by i.r. route is able to induce an immune response in the small intestine, we are evaluating the homing properties of antigen-specific lymphocytes induced by i.r. immunization in comparison with immunization by other routes. We have started our investigation by studying the chemokine and adhesion molecule receptors of 2/6-VLP-specific IgA antibody secreting cells and we are now going to extend our investigations to memory B cells (aim 1). We would also like to elucidate which are the immunological mechanisms that mediate protection against RV infection in mice immunized with 2/6-VLPs by i.r. route. The experimental strategy we describe here includes the use of genetically modified immune deficient mice together with the depletion of some lymphocyte populations with monoclonal antibodies (aim 2).

Project 1. Study of the early stages of the NoV infection, from binding to uncoating

1. Background

Human noroviruses (NoV) are one of the major causes of gastroenteritis, principally in adults (5). Thanks to improvements in surveillance systems it has been shown that type GGII.4 NoV has been the predominant genogroup for 20 years. Phylogenetic analyses, which are going into greater and greater detail, have shown that GGII.4 were subject to genetic drift and that since 2002, the dominant variant has been replaced by another variant every 2 to 3 years (7, 10). Several hypotheses have been put forward to explain these cycles, in particular host immunity, which puts pressure on NoV (3). It has also been shown that NoVs recognize the sugars that define blood group antigens, which are expressed by intestinal cells (8). Several studies have shown that susceptibility to NoV infection was also hereditary (6).
The study of human NoVs has often been hampered by the lack of a culture system, and molecular biology techniques are therefore necessary to study components of the virus such as its capsid. The discovery of the first norovirus that could be cultured, a murine NoV (MNV), opened the doors onto new fields of research into NoV (14). MNV is being used more and more as a substitute in the study of human NoV despite its immunity-related tropism rather than intestinal tropism (14). The propagation of MNV has made it possible to characterize its receptor as a member of the ganglioside family (13).

1. Main results of the precedent research plan

The study of the interaction of the human GII.4 NoVs with the Human Blood Group Antigens (HBGAs) was initiated beginning of the year 2006. In our previous studies, we investigated the binding capacity of 6 variants, representative of GGII.4 from 1987 to today, to the sugars that define blood group antigens (A, B, H and Lewis antigens). The main objective was to show whether there had been a qualitative or quantitative increase in the binding capacity of GGII.4 NoVs during their evolution. In the previous project, we, in collaboration with the Institut FEMTO, studied the binding of VLP to saliva types and synthetic sugars and showed that the genetic drift found in GGII.4 NoVs was accompanied by quantitative and qualitative modifications in binding.

2. General objectives for the next research plan

The aim of our project for the next research plan is to study in greater detail the molecular and cellular mechanisms that govern binding and internalization of NoVs until the replication of the viral genome. In order to carry out this study, we will make use of our expertise in MNV (two ANR + 1 publication) and our studies of the human NoV capsid (2 publications). The 4-year plan that we propose can be divided into three main themes.

Aims 1: Influence of the capsid on virus tropism.

Aims 2: From the docking to the internalisation of the NoV viral particle. Implication of the lipid raft in the attachment process.

Aims 3: Internalisation of the NoV.

3. Aims 1. Influence of the capsid on virus tropism

3.1. Introduction

Except for MNV, which affect macrophages and dendritic cells, other NoV groups, in particular genogroups I and II, present intestinal tropism. Our objective is to determine what parts of the capsid confer tropism to NoV. The study will consist in conducting a comparative analysis of MNV and human type GGII.4 NoVs. This study follows on from earlier work that we conducted on the role of the mutagenesis of certain essential amino acids in the binding of human NoVs to their glucidic receptor.
In the literature, it has been shown that the structure of the NoV capsid can be divided into several domains: N, S, and P. N and S make up the internal part of the capsid and P is the part oriented towards the outside. The P domain can be divided into two sub-domains, P1 and P2. The characterization of a part of the human NoV receptor showed that the P2 domain or the hypervariable domain was essential in the binding of NoV to its receptor. The information that we have on this subject for MNV is much more fragmented.

3.2. Rationale and hypothesis

We do know that MNV bind via gangliosides that present sialic acids at the end of the chain. The residues involved in MNV binding are not yet known. We can, however, predict that the equivalent of the P2 domain in the human homologue is also important. We hypothesize that the P2 domain is involved in NoV tropism.

3.3. Specific aims :

➔ To determine which amino acids are important in the binding and tropism of human and murine NoVs.
➔ To determine the affinity constant of NoV attachment to the HBGAs

3.4. Previous studies and preliminary results

3.4.1. Materials and detection tools

In our laboratory over the past few years, we have developed baculoviruses that we use to produce VLP of human and murine NoVs. The VLP can be expressed in large amounts and can be easily purified by ultracentrifugation. For the detection of GII.4 NoV, we have produced several monoclonal antibodies raised against the GII.4 capsid, some of which recognize the MNV capsid in denaturing conditions. Additionally, we have developed baculovirus VLP for MNV and obtained from the NIH capsid-specific immune serum raised against MNV viral particles. For the MNV-related study, the laboratory has developed a specific real-time RT-PCR and plaque assay titration method (1).

3.4.2. Mutagenized and chimeric VLP

The laboratory possesses several plasmid constructions corresponding to the capsid encoding ORF2. Additionally, the laboratory possesses full-length genome constructs of MNV (11) and human GII.4 NoV (MD145 isolate) (2). This library of recombinant plasmids will constitute the backbone for making new constructs.

3.4.3. SPR analysis

The SPR is a long-term collaboration with the FEMTO Institute from the University of Franche-Comté. We previously showed that VLPs from GII.4 NoV could specifically

recognize ABO antigens that were covalently attached on a gold chip. Our colleagues from FEMTO characterized the chip (manuscript in preparation). Because the intensity of the interaction is magnified by the large mass of the VLP, the dissociation constants (Kon and Koff) could not be accurately calculated since one VLP possesses 180 putative binding sites. However, a relative binding affinity, based upon visual examination of the binding curves as reported previously could be estimated.

3.5. Research design and methods

3.5.1. Mutagenesis of the VLP and domain swapping

To reach our objectives, we propose to carry out a comparative analysis of the P2 domain of human and murine NoVs. The mutagenesis of certain capsid amino acids involved in human NoV binding has allowed us to modify the binding profile of NoVs to synthetic sugars. In the next 4-year plan, we propose to replace all or part of the P2 domain in GGII.4 NoV with the P2 domain of MNV and vice-versa.

1/ The first phase will consist in characterizing the chimeric VP1 proteins and evaluating their ability to form synthetic particles.
2/ We also plan to further explore the mutagenesis of the amino acids of the P2 domain that are not directly involved in the binding of the HBGA for GII.4 NoV. This is a follow up study from our previous work where we analyzed the importance of the inserted amino acids (e.g. T395), which is a common feature of the post 2002 GII.4 strain.
3/ The mutagenized and chimeric VLPs will be screened against a broad panel of saliva and synthetic carbohydrates to determine whether the modifications brought to the VLP are lethal or induce a modification of the binding profile.
4/ We then aim to test the ability of modified VLP to bind to RAW (MNV) and Caco-2 (human NoV) cells to demonstrate the change in tropism. VLP binding assays will be developed on RAW and Caco-2 cells as described previously (8). VLPs will be detected by immunofluorescence assay. Qualitative and quantitative measurements will be performed by using confocal microscopy and flow cytometry assays, respectively. For the cytometry assays, binding assays will be conducted in the presence of VLP and non-attached cells. The VLP attached to cells will be labelled with ITCF-Mab. The magnitude of the cell binding will be expressed by the ratio labelled cell number/total cell number.

3.5.2. Anticipated results

We anticipate that the chimeric particles will no longer recognize certain antigens to the benefit of others. For example, our previous work showed that human NoV VLPs did not bind to GD1 and GM1 gangliosides, and we predict that chimeric VLPs will be able to do so. However, in the event that binding is totally abolished for chimeric VLPs, we will make smaller changes in the GII.4 VLP by substituting with a βsheet and α-helix structure from their MNV counterpart within the P2 domain. This work will be the first step in the study of tropism in noroviruses.

3.5.3. Study of the interactions between carbohydrates and NoV by SPR

For the SPR analysis, we will focus our work on mutants that presented a modified binding profile. Previous studies showed that truncated P domains could be overexpressed in bacteria and form dimers for the GII.4 NoV (12). We obtained this construct and recombinant protein for the GII.4 NoV (strain Dijon171) from our colleagues from Nantes (Jacques le Pendu, INSERM U892). Because each dimer only possesses two carbohydrate binding sites and it is

possible to control the exact number of glycoconjugates grafted onto the chip, we will be able to determine the dissociation constant for each dimer.

1/ The first step will be to produce a construct for the MNV P domain which is similar to that of the GII.4 Dijon171 strain.

2/ To express P domain dimers from MNV and GII.4 NoV in bacteria. For the second step of this work, we will express mutagenized and chimeric P domains.

3/ To analyze the attachment of the dimers by SPR. This study is ongoing for the GII.4 NoV. It will be extended to the MNV and the mutagenized constructs. The final objective will be to determine dissociation constants (K_{on} and K_{off}) for the ABO and Lewis antigens (Human GII.4 NoVs) and the gangliosides (MNV) by using dimers. This party of the project is the direct follow up of the research project that we conducted during the previous plan. For the coming project and for epidemiological purposes, we anticipate that we might predict the pathogenicity of newly emerging NoV GII.4 variants by characterizing the magnitude of the binding to HBGA.

3.6. Collaboration with the FEMTO Institute

The fine study of the interaction between the VLP and its receptor will be carried out using the SPR technique in collaboration with the FEMTO Institute based at the University of Besançon. This collaboration was initiated during the previous plan and is ongoing.

4. Aims 2. From the docking to the internalisation of the NoV viral particle. Implication of the lipid raft in the attachment process

4.1. Introduction

The second objective will be to evaluate the role of lipid rafts, specialized microdomains of the plasma membrane that are rich in cholesterol and sphingomyelin, and their potential role in NoV binding to it receptor and then internalization.

The lipid membrane of a cell is extremely dynamic and can undergo local changes in its properties. The presence of cholesterol and sphingolipids in certain places on the cell surface structures the microdomains or raft-type platforms. These structures play a role in the internalization of certain viruses (HIV, Influenza). It has been shown for MNV that the addition of high doses of methyl-β-cyclodextrin (MβCD), a cholesterol inhibitor in rafts, reduces infection (4, 13). Indirectly, these studies suggest that rafts may be involved in MNV vacuolization.

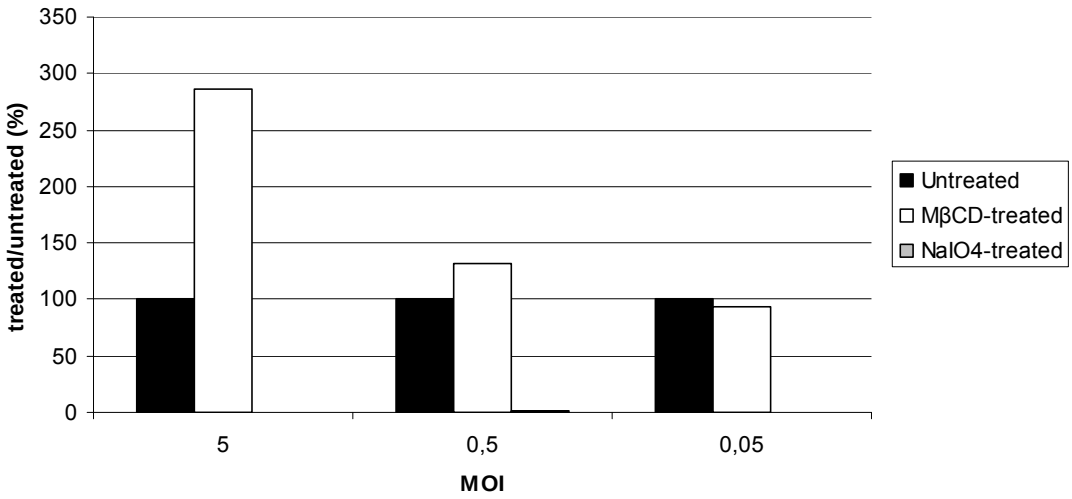
4.2. Hypothesis

The binding and internalization of MNV as well as human NoV is raft-dependent.

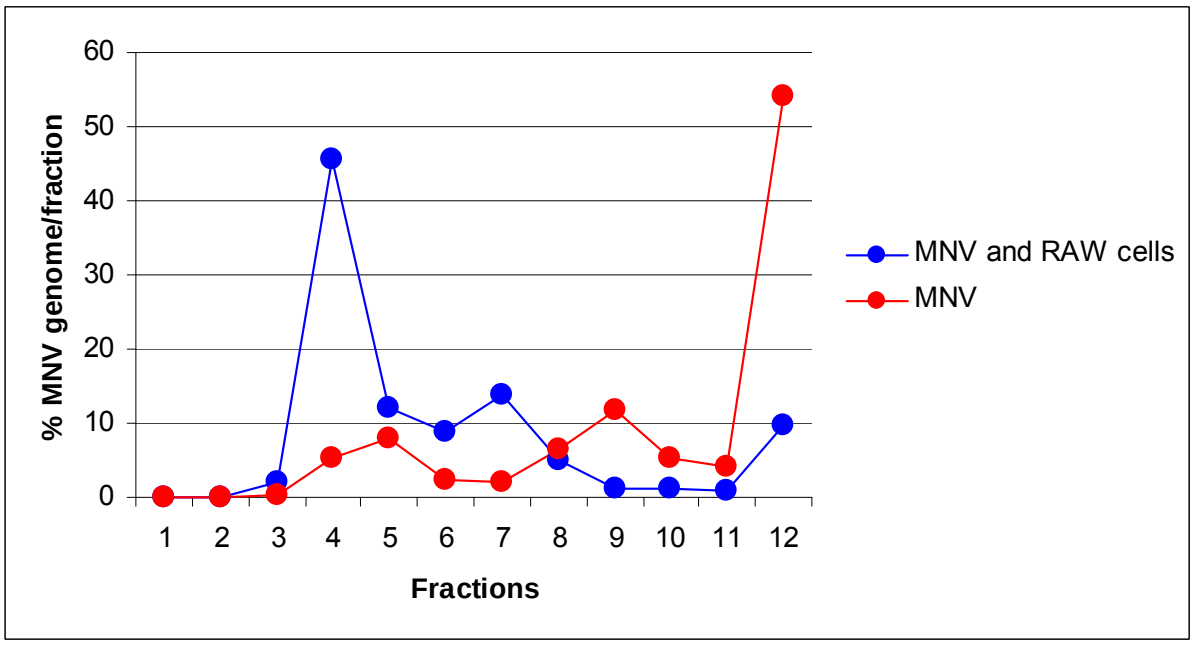
4.3. Preliminary results

Our preliminary work and the results of published studies suggest that rafts are important and this was revealed thanks to the use of MβCD. However, we found that the addition of MβCD led to an almost 3-fold increase in MNV binding. In a second experiment we found that sodium periodate totally abolished virus binding suggesting that carbohydrates were involved in virus attachment. Therefore, the disappearance of rafts does not lead to the disappearance of ganglioside receptors at the cell surface. These results, however, are based on indirect

methods and our main objective will be to find direct evidence of the association that may exist between rafts and MNV. To localize virus-receptor complexes in rafts, we will separate the rafts from other lipid components of the membrane by treating the cells with a non-ionic detergent, Triton X-100.



In a preliminary experiment, we purified lipid rafts by sucrose gradient ultracentrifugation from MNV-infected and mock-infected RAW cells (please see figure below). Freon-extracted MNV cell lysate was used as control. The viral genome and infectious particles were detected after fractionation by qRT-PCR and plaque assay, respectively. Our results clearly showed there is an association between the raft-enriched fractions (i.e. fractions 4 and 5) and the MNV. This experiment confirmed preliminary data with MβCD.



4.4. Specific aims

➔ To determine whether lipid rafts are directly associated with the attachment and the internalization of NoV.

➔ To characterize the entry of the virus. Preliminary results in the literature showed that the entry of MNV is mediated by vaccuolisation.
➔ To determine what type of molecules participate in the docking and the internalization of the virus. We hypothesise that the purification of rafts from MNV-infected cells will enable us to isolate potential receptors.

4.5. Research design and methods

The cultivatable MNV will be used as a substitute for the human NoV. For human NoVs, binding assays will be conducted using VLP and the Caco-2 cell line to determine whether lipid rafts are implicated in the event before the replication. The same approach as that for MNV will be used.
The data that we could obtain for MNV will provide the basis for the study of human NoVs. As there is no culture system for human NoVs, we will work with the capsids that we have produced and characterized at the laboratory. Earlier work has shown indirectly that the Caco-2 cell line expresses the receptor(s) of human NoVs. In the same way as for the MNV, we will determine whether or not rafts are involved in human NoV binding and internalization. The knowledge we acquired during the previous 4-year contract on blood antigens serving as receptors will be essential.

Aim 2a: This part of the study is ongoing (please see the preliminary results).
By enriching the fraction with raft, we will be able to use ELISA to determine the presence or absence of MNV, western blot to detect the capsid and RT-PCR to detect the genome. This experiment is ongoing for MNV as described above (please see paragraph 5.3). The enriched fractions in rafts will be characterized by measuring the quantity of raft-specific GM1 ganglioside marker. This standard experiment should allow us to determine whether the MNV (and later on human NoV) is directly related to the lipid raft during its attachment.

Aim 2b: These biochemical experiments will be completed by visualization of the virus on the cells treated or not with MβCD using confocal microscopy.
The second step will be to show that rafts are involved in the internalization of MNV. These studies will essentially require the use of confocal microscopy to visualize cells infected with MNV that have been treated or not with MβCD. The raft will be visualized on confocal microscopy using cholera toxin, which strongly binds to the raft-specific GM1 ganglioside. Cholera toxin will be detected by specific labelled-antibodies, available on the market. We anticipate that such experiments would help us to localize the presence of the raft together with the presence of the virus. The next step will be to determine the MNV-specific vacuole pathway by searching for the presence of caveolin, chlatrin (armoured vacuole), flotillin. Of note, preliminary reports showed that NV entry is dynamin-dependent, and inversely indirect evidence suggest that caveolin is not involved in MNV entry (4, 9).
Confocal observation should be completed with EM observation of infected cells. We believe that EM observation of infected cells should help us to determine what types of structure participate in virus entry.

We anticipate that the presence of inhibitors will not prevent binding of the virus, but will stop its internalization. We will conduct co-localization experiments on MNV using specific markers for rafts such as GM1 ganglioside.

Electron microscopy and confocal microscopy studies will be conducted in the core facility of Burgundy Medical school.

Aim 2c: Should we confirm that NoV entry is raft-dependent, the long-term objective will be to search for NoV receptor(s). For human NoV as well as MNV, carbohydrates involved in the docking of the viral particles have been identified. We hypothesize that these carbohydrates are part of or associated with a bigger molecule(s) of unknown nature (glycoside, lipid or protein). Our efforts will be essentially focus on the analysis of the MNV.

➔ The objective will be to determine whether there is a protein component to the NoV receptor. We first plan to set up an ELISA binding assay of MNV and human NoV VLP by using raft-enriched fractions. The raft will be either treated by proteinase K (removal of the protein component) or sodium periodate (removal of the carbohydrate component) prior to the binding of the VLP. Our preliminary experiments showed that the binding of MNV is entirely abolished after sodium periodate treatment, which confirm previous data showing that the receptor possesses a carbohydrate moiety. ELISA-based competition assay using lectin will be set up to verify whether VLP and virus are specifically bound by the raft-enriched fraction.
Octyl-β-D-glucoside, Triton X-100 and Tween 20 will be used for the solubilisation of the raft fraction at room temperature. Our preliminary experiments conducted with VLP showed that the binding to carbohydrate was resistant to Triton X-100 treatment. Of note, lipid-rafts are resistant to triton X-100 when kept at 4°C.
➔ We anticipate that proteins play a role in the binding and internalization of NoVs. And so, our main objective will be the identification of the proteins that are involved in virus docking and internalization. Pull-down assays will be set up by immunoprecipitation following incubation of purified VLPs and lipid raft. Precipitated materials will first be analyzed by SDS-PAGE. We anticipate that proteins play a key role in the virus entry. The proteins will be identified in collaboration with the proteomic platform of the public hospital of Dijon.

4.6. Collaboration with the Georges Herbein Laboratory

Because of the challenges of such projects and the complexity of the adhesion molecules of the immune cells, this project will be pursued in association with the Georges Herbein laboratory in Besançon. Since the MNV specifically infects immune cells (e.g. macrophage dendritic cells), we will benefit from the experience of the Besançon laboratory with adhesion molecules which are present on immune cells.

5. Aims 3. Internalisation of the NoV

This aims is a long term project and we will proceed after sections 1 and 2 are completed or near completion.

5.1. Introduction

In the longer term, we will study the mechanisms involved after internalization of the virus into the cell. The first studies have been conducted on MNV, and it appears that the penetration of the virus can occur in the absence of caveoline, flotilline or clathrine. The fact that indirect evidence showed the absence of vesicles with caveoline suggests that penetration of the virus does not lead to signal transduction by pathways that bring into play activation of the G protein and kinase cascade as suggested previously (4). These results, however, still need to be confirmed. It is worth mentioning that these data are preliminary and were usually based upon indirect evidence. Our strategy will be to give a more robust demonstration of the signalling events that occur during the virus infection.
Additionally, it is important to mention that the propagation of the MNV is only possible in murine cell lines that lack the IFN response (e.g. RAW).

5.2. Rationale

The entry of the virus into the cells triggers a cascade of events at cellular and molecular levels. Because of the lack of a cell culture system for human NoV and the recent discovery of MNV, the knowledge that we have about the mechanisms involved in NoV entry are somewhat fragmented. For this project, our work will be essentially focused on cultivatable MNV.

5.3. Specific aims

1/ To determine which signalling pathway is triggered by viral infection.
2/ To determine the role of apoptosis during viral infection.

5.4. Research design and methods

5.4.1. Signal transduction during NoV infection

Signal transduction will be studied in collaboration with the virology laboratory of Besançon University Hospital. We will study the signaling pathways and the cytokine response.

- The following signaling pathways will be studied:
  - IKK/NF-kB
  - JNK/AP1
  - P38 kinase pathway
  - Jak/STAT

  Of note, MNV has been adapted to cell culture in cell lines that lack the STAT1 interferon (IFN) response. This observation was further confirmed on MNV and a human NoV-based replicon system. We anticipate that MNV might trigger the STAT-related pathway.

  Dose-response experiments and kinetics studies will be performed. Neutralizing antibodies and replication inhibitors (e.g. ribavirine, 2-thiouridine) will be used to determine whether the formation of RC triggers signal transduction.
- The cytokine response will be determined by treating cell culture supernatant of MNV-infected cells with Th1 (e.g. TNFα, IL-1, IL-6, IL-8 and IFNγ) and Th2 (IL-4) as well as with IFNα and INFβ. The autocrine and paracrine effects of the cytokines will be studied following MNV infection. Cytokine-specific antibodies and antibodies raised against their receptors will be used to determine their function.

5.4.2. MNV infection and apoptosis

It has been shown that infection with MNV induced apoptosis in RAW cells, leading to the activation of caspase 3 and procaspase 9. Our aim is to study in more detail the early stages leading to cell death after MNV infection.

➔ We first propose to perform an annexin A5 affinity assay to determine whether MNV-infected cells undergo apoptosis. Infected and/or apoptotic cell populations will be characterized by flow cytometry.
➔ In the event that apoptosis does occur, we plan to determine whether the apoptosis was directly related to MNV infection or by activation of the TNF/TNFR1 and/or FasL/Fas pathways by measuring the expression of these molecules at the surface of MNV- or VLP-treated cells and by using TNF- and Fas-specific antibodies. Quantification by western blot

analysis of the protein from the Bcl-2 family will enable us to determine the amplitude of the apoptotic response.

5.5. Collaboration with the Georges Herbein Laboratory

The virology laboratory of Franche-Comté Medical School is specialized in the study of immune cells, especially macrophages, during HIV and CMV infection. One of the main fields of research of our colleagues from Besançon is signal transduction during infection. Their knowledge and skills perfectly meet our needs for this project. We believe that their skills and knowledge will help us reach our objectives as quickly as possible.

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Project 2. Intrarectal immunization with rotavirus (RV)-like particles as a model to investigate lymphocyte homing to mucosal surfaces and anti-RV immune response

1. Introduction

The induction of immune response at the level of mucosal surfaces is increasingly important in viral vaccine development. Many viruses enter the organism through mucosal tissues and several of them also replicate in mucosal epithelial cells of gastrointestinal and respiratory tract. Although immunization by parenteral route usually induces a strong systemic immune response but weak or no mucosal response, the induction of an immune response at the mucosal level often requires direct antigen application on a mucosal surface (1). The mucosa-associated lymphoid tissue (MALT) represents a highly compartmentalized immunological system that functions essentially independent from the systemic immune system. Lymphocytes that have been primed in the mucosal inductive sites tend to migrate and exert their effector functions mainly into mucosal tissues, although not necessarily into the mucosa of origin. Immunization by intranasal (i.n.) route stimulates an immune response in the respiratory tract but also in cervicovaginal mucosa and immunization by oral route induces antibody production not only in intestine but also in mammary glands. However, some compartmentalization also exists within the MALT and, for instance, previous study showed that oral immunization is relatively inefficient for inducing an immune response in the genital tract (1).

Rotavirus (RV) is the main etiological agent of severe gastroenteritis in infants and young children worldwide, causing approximately 440,000 deaths and 1,4 billion of episodes of diarrhea per year (2). Vaccination will have a significant impact on the incidence of the disease, and development of a RV vaccine remains a global priority. Since this virus essentially infects and replicates within the enterocytes of small intestine, an anti-RV immune response in the gut seems to be important to provide specific protection against RV infection. To address the safety concerns associated with live vaccines, several immunization strategies using recombinant RV proteins or non-replicating virus-like particles (VLPs) are currently evaluated by several groups. The co-expression of the viral protein (VP) 2 and VP6 using a recombinant baculovirus system, results in their spontaneous assembly into double-layered 2/6-rotavirus-like particles (2/6-VLPs) (3, 4). 2/6-VLPs have been reported to induce protective immunity in mice when administered by oral or i.n. route (5, 6). Immunization by i.n. route requires smaller doses of VLPs and elicits higher serum and intestinal anti-RV antibody levels than immunization by oral route, resulting also in a better protection against RV infection (5).

The intrarectal (i.r.) route for immunization has been mainly investigated for its ability to induce a local immune response on the rectal mucosa, with the aim of protecting against sexually transmitted diseases. Intrarectal immunization in mice, humans and non-human primates induces high antibody titers in rectal secretions and feces (7-10). Other studies showed that i.r. immunization of mice with a synthetic HIV peptide induced cytotoxic T lymphocytes (CTL) in the spleen and in the small intestine, whereas subcutaneous immunization with the same peptide only induced a CTL response in the spleen (11, 12). In macaques, i.r. immunization with an HIV/SIV peptide is more effective against i.r. challenge with simian/human immunodeficiency virus (SHIV) than parenteral vaccination, because of a better clearance of the virus from its major replication site in intestinal mucosa (13).

In a previous work we have shown that i.r. immunization with 2/6-VLPs protects adult mice against RV infection, and this protection, evaluated as reduction in fecal RV shedding,

was even higher in mice immunized with 2/6-VLPs by i.r. route than in animals immunized by i.n. route (14). We found that although i.r. immunization with 2/6-VLPs induces lower anti-RV antibody levels in serum than i.n. immunization, the intestinal anti-RV IgA production is higher in mice immunized by i.r. route. Moreover, mice immunized by i.r. route displayed higher interferon (IFN)-γ production in Payer's patches (PPs) after lymphocyte restimulation with RV whereas mice immunized by i.n. route displayed higher IFN-γ production in spleen. Taken together these results demonstrate that i.r. immunization with 2/6-VLPs shows better efficacy than i.n. immunization for raising an efficient humoral and cellular immune response in the digestive tract.

2. Specific aim 1: Chemokines and adhesion molecules involved in the intestinal homing of antigen-specific B cells induced by i.r. immunization.

In order to understand how antigen administration by i.r. route is able to induce an immune response in the small intestine, we decided to make an extensive analysis of the adhesion molecule and chemokine receptors expressed by antigen-specific lymphocytes induced by i.r. immunization in comparison with immunization by other routes. We have started our investigation by studying the homing properties of antigen-specific IgA antibody secreting cells (ASCs) and we are now going to extend our investigations to memory B cells.

2.1. Background and significance

When we compared the distribution of IgA ASCs after i.r. and i.n. immunization with 2/6-VLP (in absence of RV infection) we found that i.r. immunization induces a higher number of antigen-specific IgA ASCs in small intestinal and colonic lamina propria whereas i.n. immunization induces higher number of antigen-specific IgA ASCs in lung and bone marrow. We started to investigate the molecular basis for this compartmentalization by evaluating the responsiveness of 2/6-VLP-specific IgA ASCs to the chemokine CCL25 that is known to play a central role in the recruitment of IgA ASCs (and also effector T cells) into the small intestinal lamina propria. In fact most IgA ASCs in small intestine are $CCR9^+$ and migrate in response to CCL25, whereas IgA ASCs from other segment of the gut and other mucosal tissues are not responsive to this chemokine (15-17). Moreover CCL25 is expressed at high levels by crypt epithelial cells and endothelial cells in the small intestine but not in other mucosal sites (18). We collected lymphocytes from draining lymph nodes (LNs) of mice immunized three times with 2/6-VLPs by i.r. route at day 5 after the last immunization (when the number of antigen-specific IgA ASCs is at its peak) and we performed an in vitro chemotaxis assay followed by an ELISPOT analysis of the migrated cells to determine the percent of 2/6-VLP-specific IgA ASCs that migrate in response to CCL25. We found that antigen-specific IgA ASCs induced by i.r. immunization were unable to migrate towards CCL25 gradients, suggesting that this chemokine does not play any role in IgA ASCs homing to the small intestine after i.r. immunization. As expected also 2/6-VLP-specific IgA ASCs induced in cervical LNs after i.n. immunization were unable to migrate towards CCL25 and only antigen-specific IgA ASCs induced in mesenteric LNs (MLN) after oral immunization were able to migrate towards CCL25. Not surprisingly 2/6-VLP-specific IgA ASCs induced by both i.r. and i.n. immunization were able to migrate towards CCL28, a chemokine produced by epithelial cells in many mucosal tissues (such as salivary glands, mammary glands, trachea and bronchi, stomach, colon and also small intestine) and known to attract almost all IgA ASCs from intestinal or non-intestinal MALTs (17, 19, 20).

In order to understand why antigen-specific IgA ASCs induced by i.r. immunization have a superior ability to home towards the small intestine, even in absence of CCL25 responsiveness, we evaluated if they express the integrin $\alpha_4\beta_7$. This integrin is expressed by

most intestinal IgA ASCs and binds specifically to the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), present in post-capillary venules of intestinal tissues (21). By performing a static adhesion assay followed by ELISPOT analysis, we found that antigen-specific IgA ASCs present in draining LNs of mice immunized with 2/6-VLPs by i.r. route were able to adhere to MAdCAM-1-coated wells after stimulation with PMA or CCL28. As expected also antigen-specific IgA ASCs induced by oral immunization were able to bind MAdCAM-1, whereas antigen-specific IgA ASCs induced by i.n. immunization did not bind to MAdCAM-1. In conclusion i.r. immunization seems to induce IgA ASCs that migrate more efficiently towards small intestine in comparison with i.n. immunization because, even if they do not respond to CCL25, they express the integrin $\alpha_4\beta_7$. We confirmed the role of $\alpha_4\beta_7$ in the intestinal migration of IgA ASCs induced by our immunization protocol by analyzing the distribution of antigen-specific IgA ASCs in integrin β_7-deficient mice (*Itgb7*$^{-/-}$) (22) immunized with 2/6-VLPs by i.r. route. As expected we found that in comparison with *Itgb7*$^{+/-}$ control mice, β_7-deficient mice displayed a dramatic reduction of antigen-specific IgA ASCs in small intestinal lamina propria. However we found that 2/6-VLP-specific IgA ASCs were surprisingly much less reduced in the cecum and almost normal in the colon of *Itgb7*$^{-/-}$ mice, suggesting that the integrin $\alpha_4\beta_7$ plays just a minor role in IgA ASCs homing to the lower segments of the gut (Agnello D. *et al.*, manuscript in preparation). Similar experiments with mice genetically deficient for the CCL28 receptor CCR10 (*Ccr10*$^{-/-}$) (23) and with *Itgb7*$^{-/-}$*Ccr10*$^{-/-}$ double-deficient mice are currently in progress in our laboratory.

In order to complete our understanding on the regionalization of the mucosal B cell response, we would like to investigate if also memory B cells express different homing molecules when generated through immunizations by different routes, as observed for IgA ASCs.

It is well known that after their development in the bone marrow naïve B cells express receptors for chemokines and adhesion molecules that allow them to migrate into lymph nodes. They use L-selectin (CD62L) to tether and roll on LN high endothelial venules (HEVs) that express L-selectin ligands that are collectively known as peripheral node addressin (PNAd). In addition HEVs present the chemokines CCL19 and CCL21 that bind to CCR7 on naïve B cells and trigger the activation of the integrin $\alpha_L\beta_2$ (LFA-1), leading to lymphocyte arrest on HEVs. After entering in a secondary lymphoid tissue, naïve B cells use CXCR5 to localize into the follicular zone where the CXCR5 ligand CXCL13 is produced by stromal cells. Naïve B cells also express low levels of $\alpha_4\beta_7$ that play an important role for their migration into PPs. In fact PP HEVs do not express PNAd but instead they express high levels of MAdCAM-1 that can interact with $\alpha_4\beta_7$ (and L-selectin) on naïve B cells. In MLNs HEVs express both PNAd and MAdCAM-1, thus supporting both the peripheral LN-like and the PP-like adhesion for lymphocyte homing (24, 25). After activation with their cognate antigen, B cells undergoing germinal center (GC) formation downregulate some of the homing receptors such as L-selectin or $\alpha_4\beta_7$ and are unable to migrate from blood to LNs or PPs (26-28). However sIgA$^+$ and IgG$^+$ class-switched memory B cells are again responsive to the LN-homing chemokines CCL19/21 and CXCL13, lack the mucosal homing receptor CCR10 and heterogeneously express $\alpha_4\beta_7$ or L-selectin (28-30). It is likely that memory B cells activated in gut-associated lymphoid tissue (GALT), such as those primed by oral or i.r. immunization are $\alpha_4\beta_7^+$ whereas the ones that have been induced after immunizations by other routes are $\alpha_4\beta_7^-$, although this has not been experimentally proven yet. If memory B cells that normally express CCR7 and CXCR5, and thus are able to home to secondary lymphoid organs, also express $\alpha_4\beta_7$, they will preferentially home to those LNs that also express MAdCAM-1 such as PPs or MLNs. On the other hand memory B cells such as those induced by i.n. immunization, that probably lack $\alpha_4\beta_7$ and maybe express L-selectin, will be largely excluded from GALTs and will probably recirculate through peripheral LNs. As mentioned

earlier, after i.r. immunization with 2/6-VLPs we found high numbers of antigen-specific IgA ASCs in small intestinal lamina propria but only low to undetectable numbers of 2/6-VLP-specific IgA ASCs in PPs (14). However after oral infection with RV, mice immunized with 2/6-VLP by i.r. route displayed higher number of RV-specific IgA ASCs in PPs than mice immunized by i.n. route or unimmunized mice. It is likely that at least part of those ASCs derive from the conversion of $sIgA^+$ 2/6-VLP-specific memory B cells that were already present in PPs because of their preferential recirculation through the secondary lymphoid organs of the GALT.

2.2. Research design and methods

Mice will be immunized several times with 2/6-VLPs together with cholera toxin (CT) as adjuvant by i.r., i.n. or oral route. One to two months after the last immunization mice will be sacrificed and the distribution and phenotype of 2/6-VLP-specific memory B cells in draining and non-draining LNs and in mucosal organs (small intestinal lamina propria, colon, lung etc.) will be evaluated by FACS analysis. Unfortunately no specific marker for murine memory B cells exists, therefore we will identify them as isotype-switched B cells that lack GC-specific markers and they will be labeled as $B220^{high}$, $sIgD^-$, $sIgA/G^+$, $CD38^+$, $GL7^-$ cells that bind to fluorescent 2/6-VLPs. T lymphocytes and other cells will be eliminated from the analysis by a dump gate. An example of fluorochrome-conjugate antibody combination is the following: anti-IgA (or IgG)-FITC, anti-B220-PerCP-Cy5.5, anti-Thy1.2-Pacific Blue, anti-IgD-Pacific Blue, GL7-Pacific Blue and anti-CD38-PE or APC. The homing molecules expression will be analyzed with specific antibodies directed against the integrin $\alpha_4\beta_7$, L-selectin, CXCR4, CXCR5, CCR6, CCR7, CCR9 and CCR10, and conjugated to PE or APC. All these antibodies in the conjugated form are commercially available from BD Biosciences, eBioscience or R&D Systems, with the exception of GL7 that will be conjugated to Pacific Blue with a commercial kit from Invitrogen/Molecular Probes. Antigen-specific cells will be stained with biotin-conjugated 2/6-VLP and visualized with streptavidin-PE-Cy7. A similar staining method has already been used with satisfactory results in our laboratory for FACS analysis of antigen-specific IgA ASCs ($2/6\text{-}VLP^+$, $B220^{low/-}$, $sIgD^-$, $sIgA^+$, $CD138^+$) in draining LNs and blood of 2/6-VLP-immunized mice.

In order to evaluate the functional activity of the chemokine receptors we will perform a chemotaxis assay in which LN cells from immunized mice will be migrated towards gradients of CXCL12, CXCL13, CCL19, CCL20 or CCL21 (commercially available from R&D Systems or PeproTech) following a protocol similar to that already used for evaluating the migration of IgA ASCs. At the end of chemotaxis, migrated cells will be collected, stained with the cocktail of conjugated antibodies described above, and then analyzed and counted by flow cytometry in presence of counting beads as described (31), in order to determine the number of 2/6-VLP-specific $sIgA^+$ or $sIgG^+$ memory B cells that are responsive to each chemokine. We will also evaluate if 2/6-VLP-specific memory B cells adhere to MAdCAM-1 or VCAM-1-coated plates upon stimulation with CCL19, CCL21 or CXCL13, by performing adhesion experiments in a way similar to that used for IgA ASCs. Adherent cells will be collected by EDTA treatment, then analyzed and counted as described for chemotaxis assay.

Finally we will evaluate the role of specific molecules in the homing of memory B cells by immunizing gene-deficient mice (such as *Itgb7*$^{-/-}$, mice that are already present in our laboratory) and analyzing the distribution of antigen-specific memory B cells in secondary lymphoid and mucosal organs of those mice.

2.3. Preliminary and Expected results

Preliminary results from our laboratory showed that around 60% of $2/6\text{-}VLP^+$ $B220^{high}$ $sIgD^-$ lymphocytes isolated from draining sacral LNs of mice immunized with 2/6-VLPs by

i.r. route two months after the last immunization were $\alpha_4\beta_7^+$. On the contrary only 8% of 2/6-VLP$^+$ B220^{high} sIgD$^-$ isolated from cervical LNs of mice immunized by i.n. route were $\alpha_4\beta_7^+$. Although in this preliminary experiment we did not use the complete antibody panel described above, and we cannot formally exclude that the analyzed population contained some GC cells, it is reasonable to assume that the majority of antigen-specific B220^{high} sIgD$^-$ found in draining LNs two months after the last immunization are memory B cells (32): moreover virtually all 2/6-VLP$^+$ sIgD$^-$ B220^{high} cells found in draining LNs seven days after the first immunization (mostly GC lymphocytes) were $\alpha_4\beta_7^-$, regardless of the route of immunization. Therefore we expect to find high levels of $\alpha_4\beta_7$ expression on 2/6-VLP$^+$ CD38$^+$ GL7$^-$ sIgD$^-$ B220^{high} memory B cells isolated from draining LNs of mice immunized with 2/6-VLP by i.r. route but not on 2/6-VLP$^+$ memory B cells isolated from draining LNs of mice immunized by i.n. route. On the contrary it is possible that memory B cells induced by i.n. immunization express higher levels of L-selectin in comparison with memory lymphocytes induced by i.r. immunization. If those lymphocytes are also CCR7$^+$ and/or CXCR5$^+$ they will migrate towards CCL19/21 and or CXCL13 gradients and in lymphocytes isolated from mice immunized by i.r. route, those chemokines might also stimulate adhesion to MAdCAM-1-coated plates, as reported for human lymphocytes (33).

The distribution of memory B cells is also likely to be different between mice immunized by i.r. and i.n. route. In particular we assume that mice immunized by i.r. route will display higher number of 2/6-VLP-specific memory B lymphocyte in PPs and MLNs, whereas in mice immunized by i.n. route antigen-specific memory B cells will be enriched in peripheral LNs. Conversely we expect to find that in integrin β_7-deficient mice immunized by i.r. route, the number of antigen-specific memory B cells will probably be decreased in PPs and MLNs of in comparison with wild-type mice.

Taken together those results will add a new level of complexity in our view of the compartmentalization and regionalization of the mucosal immune system. The concept of regionalization will be applicable not only to effector lymphocytes, such as IgA-secreting plasma blasts and plasma cells that migrate preferentially to specific mucosal surfaces, but also to memory cells that might recirculate asymmetrically through secondary lymphoid organs in a way to be particularly enriched at those body sites where a second encounter with the antigen carried by an invading pathogen is most probable.

3. Specific aim 2: Mechanisms of protection against RV infection in mice immunized with 2/6-VLPs by i.r. route.

3.1. Background and significance

As previously mentioned, i.r. immunization with 2/6-VLPs and CT as adjuvant protects mice against RV infection and protection is even higher in comparison with mice immunized with the same antigen and adjuvant administered by i.n. route. Both cellular and humoral immune responses (evaluated as IFN-γ and IgA production, respectively) were increased in the gut mucosa of mice immunized by i.r. route, making impossible to understand which immunological mechanisms are really responsible for inhibiting viral replication (14). In fact although 2/6-VLPs do not contain the RV outer layer proteins VP4 and VP7 and therefore they do not induce neutralizing antibodies, it is known that VP6-specific IgA can inhibit RV replication *in vivo*. Murine hybridomas producing anti-VP6 IgA implanted in a backpack model have been reported to protect adult mice from RV infection and clear chronic infection in immunodeficient mice. Based upon these observations, it has been postulated that those immunoglobulins, during their transcytosis inside enterocytes, might interact with partially decapsidated virions and inhibit viral replication (34, 35). On the other hand also T cells have been reported to mediate anti-RV immunity. It is known that

$CD8^+$ T cells together with antibodies play a major role in the clearance of primary RV infection in mice. In fact β_2-microglobulin-deficient mice clear RV infection with a short delay in comparison with normal mice and B cell-deficient *Jh*$^{-/-}$ mice, that usually control infection like immunocompetent mice, become chronically infected with RV when depleted of $CD8^+$ T cells (36, 37). In contrast mice depleted of $CD4^+$ T cells clear infection just like undepleted mice, despite a marked reduction in virus-specific IgA production (38). However $CD4^+$ lymphocytes have been reported to protect against RV infection in mice immunized intranasally with recombinant VP6 (39).

Here we would like to investigate in details which cells of the immune system protect against RV infection in our protocol of immunization by using both immunodeficient mice and lymphocyte depletion with monoclonal antibodies.

3.2. Research design and methods

Since newborn mice are susceptible to RV-induced diarrhea only during the first 15 days of life, it is almost impossible to use this model to study the mechanism that protect against RV infection with an immunization protocol. However in selected strains of mice, adults are susceptible to infection like pups and shed RV in stools for almost a week after oral RV infection, although without any apparent clinical symptom (40). In this model it is possible to evaluate the protection provided by an immunization protocol by measuring the reduction in fecal RV shedding in the 5 to 7 days following infection, with the assumption that the mechanisms that mediate protection from infection in adults are similar to those mediating protection in mouse pups (41).

In order to understand the role of anti-VP6 and anti-VP2 antibodies in the protection against RV infection, we will immunize B cell-deficient *Jh*$^{-/-}$ mice (C.129(B6)-IgH-*J*$^{tm1Dhu\ N?+2}$ purchased from Taconic Farms and fully backcrossed on BALB/c background in our laboratory) and control *Jh*$^{+/-}$ mice three times with 2/6-VLPs and CT as adjuvant by i.r. route. Two other groups of *Jh*$^{-/-}$ and *Jh*$^{+/-}$ mice will be mock-immunized with CT only. Four weeks after the last immunization mice will be orally infected with murine RV (strain EC_w, provided by Dr. H. Greenberg, Stanford University, CA) and viral shedding will be measured in stool samples as copies of RV genomic RNA by real time PCR, as previously described (14).

The role of $CD8^+$ T cells will be evaluated by using BALB/c β_2-microglobulin-deficient (*B2m*$^{-/-}$) mice (C.129P2(B6)-*B2m*^{tm1Unc}/J, available from Jackson Laboratory) that we will backcross to *Jh*$^{-/-}$ mice in order to obtain *Jh*$^{-/-}$*B2m*$^{-/-}$-double deficient and *Jh*$^{-/-}$*B2m*$^{+/-}$ control mice. Mice will be immunized with 2/6-VLPs and CT and infected with RV as described above and fecal RV shedding in immunized and mock-immunized *B2m*$^{-/-}$ and *B2m*$^{+/-}$ (and *Jh*$^{-/-}$ *B2m*$^{-/-}$ and *Jh*$^{-/-}$*B2m*$^{+/-}$) mice will be compared.

The role of $CD4^+$ T cells will be evaluated by depleting this lymphocyte subset in *Jh*$^{-/-}$ *B2m*$^{-/-}$ mice with a cocktail of monoclonal antibodies such as GK1.5 (available from ATCC) and YTS191.1 (available from Dr. David Baker, Centre for Neuroscience and Trauma, Queen Mary University, London, UK). Those antibodies will be produced as concentrated hybridoma cell culture supernatants in protein-free BD Cell MAb Medium (Quantum Yield, BD Biosciences) with the CELLine CL1000 system (Integra Biosciences AG, Chur, Switzerland), as described (42). Depleting anti-CD4 antibodies will be daily injected intraperitoneally to immunized or mock-immunized *Jh*$^{-/-}$*B2m*$^{-/-}$ mice before RV infection and the treatment will be continued until the end of the experiment. The effective depletion of $CD4^+$ T cells will be confirmed by FACS analysis of lymphocyte populations in lymphoid organs and intestinal mucosa. Fecal RV shedding of $CD4^+$-depleted mice will be compared with that of undepleted mice.

Since both antibodies and T cells may contribute to protection in a redundant way, we will also evaluate the role of anti-RV antibodies in absence of T cells by immunizing *Jh*$^{-/-}$

B2m$^{-/-}$ and *Jh*$^{+/-}$*B2m*$^{-/-}$ mice and depleting CD4$^+$ T cells just before RV infection. Fecal RV shedding of depleted *Jh*$^{-/-}$*B2m*$^{-/-}$ mice will be compared to that of depleted *Jh*$^{+/-}$*B2m*$^{-/-}$ mice. In this protocol CD4$^+$ lymphocytes are still present during the immunization phase for helping B cell of *Jh*$^{+/-}$*B2m*$^{-/-}$ to differentiate into ASCs producing high affinity anti-RV antibodies, but they will be eliminated just before the infection, when their helper activity may no longer be essential. Indeed in preliminary experiments we have found that normal BALB/c mice immunized with 2/6-VLPs by i.r. route and depleted of CD4$^+$ T cells just before RV infection, display the same number of RV-specific IgA ASCs in PPs as undepleted mice at the third day of infection (when viral shedding is maximal). Hence with this experimental system we will be able to reveal the protection provided by anti-VP6 (or anti-VP2) antibodies in the absence of the direct antiviral activity of T cells.

3.3. Preliminary and expected results

Preliminary results obtained in the laboratory have shown that immunization with 2/6-VLPs by i.r. route protects against RV infection by multiple and partially redundant mechanisms.

We already know that B cell-deficient *Jh*$^{-/-}$ mice immunized with 2/6-VLPs by i.r. route are equally protected against RV infection as normal mice. Depletion of CD8$^+$ T cells in normal BALB/c mice by using a monoclonal antibody does not affect the protection provided by the immunization and only marginally reduce this protection in *Jh*$^{-/-}$ mice, suggesting that CD8$^+$ T cells play only a minor role in reducing RV replication in mice immunized with 2/6-VLPs. Although the depletion obtained with the anti-CD8 antibody may be incomplete, we presume that both *B2m*$^{-/-}$ and *Jh*$^{-/-}$*B2m*$^{-/-}$ mice immunized with 2/6-VLPs will be at least partially protected from RV infection. On the contrary we expect that CD4$^+$ T cells depletion in *Jh*$^{-/-}$*B2m*$^{-/-}$ mice will completely abolish the protection provided by immunization and will revert viral shedding at the levels of mock-immunized mice. In fact we have already observed that CD4$^+$ T cells depletion with GK1.5 antibody significantly reduced protection in *Jh*$^{-/-}$ mice immunized with 2/6-VLP, suggesting that in this model CD4$^+$ T cells not only behave as helper cells for antibody production but also exert a “direct” antiviral activity.

Mock-immunized *B2m*$^{-/-}$ mice are expected to clear RV infection with a short delay in comparison with *B2m*$^{+/-}$ mice, whereas mock-immunized *Jh*$^{-/-}$*B2m*$^{-/-}$ mice might be even unable to control RV infection leading to a chronic viral shedding, in agreement with previous results (36, 37). Therefore *Jh*$^{-/-}$*B2m*$^{-/-}$ might represent an interesting strain of mice that cannot control primary RV infection but can still block viral replication if immunized with 2/6-VLPs, and this protection is likely to be mediated by CD4$^+$ T cells. If this hypothesis is correct, it will be interesting to elucidate why immunization with 2/6-VLPs induces CD4$^+$ T cells with protecting activity, whereas in infected unimmunized mice CD4$^+$ T cells alone are unable to clear RV infection.

Finally, if complete depletion of CD4$^+$ T cells will completely abrogate protection in immunized *Jh*$^{-/-}$*B2m*$^{-/-}$ mice but not in *Jh*$^{+/-}$*B2m*$^{-/-}$ mice, we will demonstrate that anti-VP6 (or anti-VP2) antibodies elicited by 2/6-VLPs actually play a protective role against RV infection. In fact although we did not detect any difference in viral shedding between immunized *Jh*$^{-/-}$ and *Jh*$^{+/-}$ mice, it is possible that in this experimental settings the protective role of anti-RV antibodies was just masked by the redundant antiviral activity of T cells. Conversely if depletion of CD4$^+$ T cells will abrogate protection in both *Jh*$^{-/-}$*B2m*$^{-/-}$ and *Jh*$^{+/-}$*B2m*$^{-/-}$ mice, we will conclude that although IgA antibodies directed against VP6 have been shown to prevent RV infection when produced at high levels by hybridomas in the backpack tumor model (34), they do not have a significant effect when produced in physiological conditions by intestinal IgA ASCs.

4. References

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