Bruxelles, le 26 juillet 2013

Cher Professeur Englert,

Par la présente, je vous transmets mon dossier de postulation à une bourse de perfectionnement de la Fondation Médicale Horlait-Dapsens pour l'année académique 2013-2014.

Je suis diplômé de l'ULB et en formation en médecine interne depuis 2007. En 2009, j'ai débuté une thèse de doctorat à l'Institut d'Immunologie Médicale (IMI).

Mon travail porte sur l'étude de la réponse humorale lors de l'infection par le CMV pendant la grossesse et la vie fœtale. Lors de ces 4 années, durant lesquelles j'ai été financé par le F.R.S.-FNRS, nous avons mis en évidence un nouveau mécanisme par lequel le CMV manipule la réponse des lymphocytes B promouvant ainsi probablement sa persistance. Ces travaux sont résumés dans 2 manuscrits, l'un a été soumis récemment à la revue *PLoS Pathogens* et le deuxième est en voie de finalisation.

Cette bourse de la Fondation Horlait-Dapsens me permettrait de compléter mon travail en caractérisant finement la réponse des lymphocytes B chez les nouveau-nés congénitalement infectés. Ces travaux seront poursuivis en utilisant les outils développés lors de ces 4 années mais également en collaboration avec un laboratoire renommé, spécialisé dans le clonage des lymphocytes B (Institute for Research in Biomedicine du Pr. Lanzavecchia à Bellinzona).

Je joins un résumé de mes travaux antérieurs, du projet de recherche, mon curriculum vitae avec une liste de mes publications ainsi que la preuve de soumission de mon manuscrit à *PLoS Pathogens*.

Veuillez agréer, Professeur Englert, mes salutations distinguées.

Dr Nicolas Dauby

Nicolas Dauby - Research project - Medical Foundation Horlait-Dapsens

- **Title of the project**:
Memory B cell response to congenital human cytomegalovirus infection

- **Objective of the work**:

The objective of the work is to characterize the response of B lymphocytes to congenital human cytomegalovirus infection, including the impact of CMV infection on fetal B cell subsets and on the B cell repertoire and the study of virus specific B cells at both subsets and clonal levels.

Short summary of previous work :

During the last 4 years, our work has been primarily focused on the B cell response to primary CMV infection in pregnant women. This has allowed us to set up the tools and provide a reference for the work that has been initiated in the fetus. We have assessed the impact of CMV on peripheral blood subsets and characterized the acquisition of CMV specific memory B cells response during the course of primary CMV infection.

In the manuscript attached to this submission, recently submitted to the journal *PLoS Pathogens,* we describe the impact of primary infection on peripheral blood B cells subsets, the phenotype of these subsets and their link with viremia. We observed that primary CMV infection induces a significant and persistent expansion of two B cells subsets: activated memory B cells and atypical memory B cells. We have shown that those two subsets share similarities in term of trafficking receptors expression that are compatible with an effector phenotype. Atypical memory B cells expressed high levels of inhibitory receptors, a phenotype closely related to the one of exhausted B cells, previously described in chronic infection like HIV and *Plasmodium falciparum* malaria(1-2). On the contrary, activated memory B cells express high levels of activation markers and low levels of inhibitory receptors. Both subsets were enriched in CMV specific cells and were found at higher frequencies in subjects with detectable viremia. These results indicate that CMV has a profound impact on B cell subsets in humans and suggest that viral replication induces the regulation of B cell effector functions.

In a second manuscript in preparation, we characterized the B cell response to CMV antigens during primary infection. We observed, as previously described in the literature(3), a delayed acquisition of neutralizing antibodies directed against envelope glycoprotein B (gB) while tegument specific antibodies, that are non neutralizing, were acquired rapidly after primary infection. In order to decipher the underlying mechanisms leading to this differential kinetic, we characterized the phenotype of both gB and tegument specific B cells and observed that they stimulated different B cell subsets. gB specific B cells were mainly of the classical memory phenotype while tegument specific B cells were mainly of the activated memory phenotype, suggesting that the delayed acquisition of neutralizing antibodies may be related to a defective induction of effector memory B cells. Accordingly, ELISPOT experiments suggested that gB specific B cells had higher proliferative potential as compared to tegument specific ones. This work is the first demonstration that different antigens of the same pathogen induce qualitatively distinct B cell responses and opens new avenues for our understanding of the evasion mechanisms used by pathogens to escape B cell responses.

During the course of the PhD, we have also written a third manuscript reviewing the impact of chronic maternal infections on the development of the immune system in the fetus and on the susceptibility to infectious pathogens(4). This review was published in *The Lancet Infectious Diseases* and forms a basis for ongoing and future projects developed by our lab on exposure to maternal HIV and CMV infection during fetal life.

- **Research project**

Background

Infectious diseases remain a major cause of infant mortality worldwide. Vaccination is the most efficient strategy to prevent both morbidity and mortality associated with infections in childhood. However, vaccination effectiveness in infants is dampened as compared to adults. Both T-independent and dependent antibody responses are limited in early life and, consequently, vaccines often induce low levels of antibodies of low affinity and that persist for shorter time as compared to adults. The mechanisms involved are not elucidated and may include a reduced expression of co-stimulatory molecules by B cells as well as defective interactions with antigen presenting cells. Also, studies suggest that the neonatal B cell repertoire is not as diversified as the repertoire of adults and that infections or vaccinations in the early post natal period are associated with a limited number of somatic mutations(5). A better understanding of the mechanisms limiting B cell responses in early life is required to develop improved strategies controlling infectious diseases. The Institute for Medical Immunology has selected congenital CMV infection as a model to study the ontogeny of immune responses very early during human life. CMV is the first cause of congenital infection worldwide, infecting around 40.000 newborns each year in the US only(6). Our lab has previously shown that congenital CMV infection induces strong CD8 and gamma delta T lymphocytes CMV specific responses(7-8). CMV specific CD4 T cells are also observed following congenital infection. These CD4 T cells display signs of immune exhaustion: they express a late-differentiate phenotype and following in vitro restimulation they will secrete less cytokines and proliferate less. In the last 3 years, we have generated original results regarding the B cell response to primary CMV infection during pregnancy. These results indicate that the response of B lymphocytes is regulated during primary infection and that these regulatory mechanisms could favor viral dissemination and the establishment of a viral reservoir. The tools generated to study adult B cell responses and the results obtained in this population allowed us to study the response to fetal B cells to congenital CMV infection. Our preliminary results indicate that the fetus develops a B cell response to CMV that we are currently characterizing. First, we observed the expansion of a particular subset of B cells expressing a phenotype of activated memory cells ($CD19^{+}CD27^{+}CD20^{+}CD21^{low}$) and enriched in class switched IgD negative cells(9). This response is analogous to the one detected in pregnant women with primary infection. Secondly, we detected IgM directed against several CMV antigens in the majority of infected newborns. Finally, CMV specific B cells producing IgG were detected using the ELISPOT assay. The work that is currently ongoing includes: 1) the analysis of the impact of congenital CMV infection of fetal B cell subsets 2) the analysis of the impact of CMV on the fetal B cell repertoire 3) the analysis of the phenotype of CMV-specific fetal B cells.

Material and methods

Subjects

Since 2005, we initiated a collaboration with a network of obstetric clinics allowing the recruitment of pregnant women who acquired primary CMV infection during pregnancy along with their newborns, infected or not. Congenital CMV infection is based on the detection of the virus in the urine by PCR during the first days of life. Samples are cord blood mononuclear cells (CBMC) and B cells isolated from cord blood samples from congenitally infected newborns, newborns of pregnant women with a primary CMV infection but uninfected and newborns unexposed to maternal CMV infection. All samples have already been collected and are cryopreserved.

Study of B cells subsets in congenitally infected newborns

Using multi-parameter flow cytometry, we will assess the impact of CMV infection on fetal B cell subsets. As previously mentioned, we have observed a significant expansion of a subset that shares features with the activated memory subset we observe in adults (CD27+CD20+CD21^{low}). An adapted panel is being set up in order to refine this analysis and study the recently described human equivalent of B1 B cells (10) (CD27+CD20+CD43+CD70-) and the atypical memory B cell subset (CD27-CD20+CD10-CD21-). An expansion of the latter subset was observed in pregnant women with primary CMV infection, suggesting a functional regulation of the B cell response (11). The expression of activation markers and inhibitory receptors will be assessed on the different B cell subsets.

Characterization of CMV specific B cells

Our previous experiments indicated that congenital infection induces the expansion of B cells secreting IgG recognizing HCMV tegument proteins and glycoprotein B. Using fluorescent antigens, we will quantify and characterize the phenotype of glycoprotein B and tegument specific B cells in congenitally infected newborns, uninfected newborns born to infected mothers and CMV unexposed newborns. This will allow us to test the hypothesis that newborns preferentially develop memory rather than effector memory B cell responses(5). These fluorescent antigens have already been used to study the phenotype of CMV specific B cells in pregnant women with primary CMV infection (Dauby et al. manuscript in preparation).

B cell repertoire analysis

High throughput sequencing of the heavy chains of immunoglobulins has been performed using the immunoSEQ technology (Adaptive Technologies, Seattle, WA, USA). This analysis has been performed on cord blood mononuclear cells from 3 congenitally infected newborns and 3

uninfected newborns as controls. The analysis of the dataset is currently ongoing and includes the usage of VDJ families, the presence of specific clonotypes and the presence and number of somatic mutations associated with CMV infection(12). The analysis is performed using the IMGT/HighV-QUEST software (13) in combination with the Immunoglobulin Analysis Tool for Excel(14).

In order to extend the analysis of the B cell repertoire and directly analyze the repertoire of CMV specific B cells, a collaboration with the Institute for Research in Biomedicine (Bellinzona, Switzerland) directed by Pr. A Lanzavecchia has been initiated. B cells from congenitally infected newborns will be cloned and immortalized before screening of the secreted immunoglobulins for reactivity against CMV antigens (15). This technology represents the state of the art for pathogen specific B cell repertoire analyses and has been applied successfully to identify potently neutralizing antibodies against pathogens such as SARS(16) or CMV(17). This technology has not been applied to study the response of fetal B lymphocytes. The samples will be selected and prepared in our lab and sent to IRB in September 2013 for analysis.

Research implications

Memory B cells responses in the neonatal period are poorly characterized. This gap in knowledge represents one of the major limitations for the development of improved vaccines. Our study will provide unique information regarding the ontogeny of B cell responses during early human life and will help identify the factors limiting the quality of these responses. The repertoire analyses could identify clonotypes preferentially induced by CMV and provide critical information for the development of vaccines or monoclonal antibodies against CMV.

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Charleroi, le 26 juillet 2013

Cher Monsieur le Doyen,

Par la présente, je souhaite vivement soutenir la candidature du docteur Nicolas DAUBY à une bourse de perfectionnement de la Fondation Médicale Horlait-Dapsens. Nicolas mène un travail de thèse dans mon équipe, au sein de l'Institut d'Immunologie Médicale, depuis octobre 2009. Son projet vise à caractériser la réponse des lymphocytes B à l'infection primaire par le cytomégalovirus au cours de la grossesse et de la vie fœtale. L'infection congénitale par le CMV représente une thématique centrale pour mon équipe de recherche. Nos recherches sont menées en collaboration avec les équipes de l'Hôpital Erasme et de l'Hôpital Saint-Pierre, à Bruxelles. Notre équipe ne possédait pas une expertise importante dans le domaine des lymphocytes B au commencement du projet de Nicolas. Son travail a permis de mettre au point des modèles et des outils nouveaux pour développer notre expertise. Dans ce processus, Nicolas a fait preuve d'une très grande curiosité, d'une très grande rigueur et de beaucoup de créativité. Je considère que son travail est d'une qualité exceptionnelle. Il lui a permis de développer de nouveaux concepts dans le domaine du CMV en particulier et des infections virales en général. Son travail révèle des mécanismes nouveaux indiquant que la réponse humorale est modulée au cours des premiers mois de l'infection par le CMV, un phénomène qui favorise probablement la dissémination et la transmission du virus. Ces résultats feront l'objet de deux publications dont la première est soumise au journal PLoS Pathogens. La seconde est en préparation et devrait être soumise avant la fin de l'année. En parallèle, Nicolas a rédigé une revue, publiée dans le Lancet Infectious Diseases, sur l'impact des infections maternelles chroniques sur le développement du système immunitaire et des défenses anti-infectieuses en début de vie. Cette thématique de recherche avait été étudiée par Nicolas avant sa thèse et avait fait l'objet d'une publication importante. Elle fait également l'objet d'un projet collaboration entre notre équipe et le département de pédiatrie de l'Hôpital Saint-Pierre dans le cadre due la prise en charge et du suivi des enfants nés de mères infectées par le VIH mais eux-mêmes non infectés.

Au cours de la dernière année, Nicolas a étudié la réponse des lymphocytes B fœtaux à l'infection congénitale par le CMV. A ce stade, il a fourni la preuve de concept qu'une réponse humorale est développée par le fœtus et qu'elle inclut une commutation isotypique et la production d'IgG spécifiques par les lymphocytes B fœtaux. Dans la cadre de cette partie du projet, Nicolas a supervisé très efficacement un mémoire de recherche de quatrième doctorat en médecine. Ce mémoire a fait l'objet du Prix de l'Institut de Biologie Clinique de l'ULB. Ces observations représentent, à ma connaissance, la première démonstration d'une réponse lymphocytaire B fœtale mature à un pathogène. Elles sont à l'origine d'une collaboration importante pour notre équipe avec le laboratoire du Prof Lanzavecchia qui est un des meilleurs experts dans le domaine des réponses immunes adaptatives.

La productivité de Nicolas et l'état actuel de ses recherches me font vivement souhaiter qu'il puisse réaliser une cinquième année de recherche dans notre laboratoire. Je considère que, d'ici à septembre 2014, l'étude des réponses fœtales sera suffisamment avancées que pour permettre l'écriture d'un troisième article qui fera partie de la thèse de doctorat que Nicolas défendra à ce moment.

En restant prêt à vous donner tout complément d'information que vous jugeriez nécessaire, je vous prie de croire, cher Monsieur le Doyen, en mes sentiments dévoués.

Dr Arnaud Marchant

PLOS Pathogens

Primary human cytomegalovirus infection induces the expansion of virus-specific activated and atypical memory B cells

--Manuscript Draft--

Manuscript Number:	
Full Title:	Primary human cytomegalovirus infection induces the expansion of virus-specific activated and atypical memory B cells
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Abstract:	Neutralizing antibodies play a central role in the control of cytomegalovirus (CMV) dissemination. However, little is known about the quality of the response of B lymphocytes to primary CMV infection. In this study, we show that primary CMV infection induces a sustained expansion of activated (CD27+CD20+CD21low) and atypical (CD27-CD20+CD21low) memory B cells (MBC), two subsets previously associated with chronic exposure to high antigen loads. Both activated and atypical MBC expressed an effector phenotype. The highest levels of activation markers were expressed by activated MBC whereas atypical MBC expressed high levels of inhibitory receptors, suggesting functional exhaustion. Fluorescent antigen labeling indicated that activated and atypical MBC were enriched in CMV-specific cells. The intense activation and functional regulation of B lymphocytes during primary CMV infection may limit the production of antibodies and the control of viral dissemination.
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