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## **Pathogenesis of congenital cytomegalovirus infection : finding prognostic markers and correlates of protection**

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# CHAPTER

GENERAL DISCUSSION

7



This thesis aimed to identify prognostic markers, for short- and long-term clinical outcome, and correlates of protection, for future vaccine development. In order to identify such biomarkers, a retrospective nationwide cohort of children with (n=125) and without congenital CMV infection (cCMV) (n=263) was used. The findings of this thesis allowed us to get more insights into cCMV pathogenesis, and into the potential processes leading to immune dysfunction, and therefore to a worse clinical outcome. Thus, Chapter 7 delves into some important aspects of each chapter first, with particular attention to the possible mechanisms of cCMV pathogenesis and its clinical implications, i.e. *Summarising Discussion*. This is followed by a paragraph that attempts to integrate all the findings in one final model, with special emphasis on what could be done in the future research to complete the findings of this thesis, i.e. *Concluding Discussion*.

## 7.1. SUMMARIZING DISCUSSION

### 7.1.1. Neonatal T and B cell markers in relation to symptoms, LTI and viral load

In **Chapter 2** the immune system of the neonates was assessed by means of quantification of the most common DNA rearrangements occurring at the receptor level of B cells,  $\alpha\beta$  and  $\gamma\delta$  T cells. Our findings suggested that the intrauterine infection may have an effect on thymopoiesis, leading to a reduced thymic production of  $\alpha\beta$  T cells. Because CMV can infect thymic epithelial cells (1, 2), and because smaller thymuses have been described in CMV infected newborns (3), our hypothesis seems plausible. Infected thymic epithelial cells, which are fundamental for T cell development and maturation, could impair the output of naïve T cells. T cells play a central role in controlling CMV infection and disease, therefore, if the initial impairment was permanent one could assume a reduced long-term control of the infection, with a consequent worse long-term outcome. However, the reduced thymic output at birth was not associated to long-term impairments (LTI), suggesting that it is temporary and that other mechanisms are in place to compensate such initial impairment. In our cohort, the infected group had higher number of  $\gamma\delta$  T cells. The role of  $\gamma\delta$  T cells during cCMV has already been shown, but in the context of primary maternal infection, with antiviral activity when incubated with CMV-infected cells (4). Therefore, they could be potential candidates for such compensation, at least in the early phase of the infection, because they develop earlier than  $\alpha\beta$  T cells during the immune ontogeny, and react rapidly upon activation (5). Having higher levels of  $\gamma\delta$  T cells at birth did not correlate with a better long-term outcome, but positively correlated with CMV viral load, further suggesting their role in the early phase of cCMV. Interestingly, the reduced thymic output did not get worse with higher viral load. However, we do not know if higher blood viral load corresponds to higher viral load in the thymus. Therefore, we cannot exclude that a more severe local infection leads to a more reduced thymic output. The infected group had an increased number of circulating and newly derived bone marrow B cells compared to the control group. Therefore, even though we know that CMV can infect the bone marrow, a different pathogenetic mechanism may be assumed between the  $\alpha\beta$  T cell and B cell compartment, because, overall, cCMV does not induce a reduced B output as for  $\alpha\beta$  T cells. As the number of circulating and newly derived bone marrow B cells were comparable in all analyses, we concluded that cCMV leads to an increased B cells production in the fetal period, rather than to an extensive proliferation.

And such a B cell production positively correlated with viral load, suggesting a causal relationship between high viral loads and cell activation, as previously suggested (6). Although, overall, infected neonates had higher number of B cells at birth, for those who developed LTI, this number was lower, comparable to non-infected controls. It is tempting to speculate that this difference in numbers reflects a difference in e.g. the capacity to generate long-lived plasma cells, memory B cells, Abs or support effector functions of immune cells. What is the trigger to this dysfunction is difficult to determine in our cohort, as it seems that viral load is not related to LTI development. Finally, none of the immunological markers included in this study was associated with symptoms at birth.

### 7.1.2. Neonatal metabolic markers in relation to symptoms, LTI and viral load

In **Chapter 3** the metabolism of neonates was assessed by means of quantification of the metabolites extracted from dried blood spots (DBS) for the screening of the rare genetic metabolic disorders (essential amino acids, hormones, carnitines and enzymes). During infections, there is a general increase of metabolic requirements. The virus is a considerable burden on cells because they are dependent on the host's energy and biosynthetic pathways to replicate (7). For example, CMV envelope is enriched for longer chain fatty acids, and its infectivity is reduced by the inhibition of the host enzyme involved in their biosynthesis. This is why we found a positive correlation between palmitoicarnitine, which reflects the level of palmitate (precursor of longer chain fatty acids) and viral load. Additionally, the immune system increases the proteins biosynthesis to allow a broad-spectrum of anti-microbial functions (7). In our cohort, cCMV influenced the metabolites only in premature neonates. If cCMV occurs in premature neonates, they may not be able to cope, or at least not as good as term neonates, with the general increased energetic and biosynthetic demand during an infection, with a consequent lower level of essential aminoacids reflected in whole blood. However, since a certain degree of correlation has been shown between cCMV and prematurity (8), we cannot establish which was first, and most likely it is a combination of factors. Although we did not find a significant difference in the number of premature between cCMV+ and cCMV-, we found more prematurity in the infected group that developed LTI than in the infected group that did not. Interestingly, prematurity, both early, moderate and late, has been shown to be associated with LTI similar to those reported for cCMV (9). Obviously, prematurity, even if not caused by cCMV, will not help to fight the effect of a CMV infection.

Finally, in past pediatric cohorts more female were reported with neurological impairments and cerebral ultrasounds findings, even though the number of infected males was higher than the number of females (10). This bias was explained by assuming that the most severely affected fetuses died in utero and that the damage in the survivors was caused by the immune inflammatory response to CMV and by direct cytopathic effect (CPE) (10). Since in our cohort neonates were not selected based on their abnormal ultrasonographic state, or on the maternal primary infection, we did not enrich for severe cases. And this may be the reason why we did not detect differences in male/female proportion between symptomatic and asymptomatic. However, we found a higher proportion of infected males that developed LTI, whereas this was not observed in the control group (unpublished observations). The same differences that lead to higher resistance to bacterial and viral

infections in female, or higher susceptibility to autoimmune diseases when an inadequately high immune response occurs, might be involved in the long-term response to CMV as well. The overall morbidity rate is higher in males than females (11) and, in absence of immunopathology, the sex-related differences might lead to a more effective long-term CMV control in female. Interestingly, infections as measles, mumps and RSV, other relevant problems in neonates, can cause more extensive complications in male (12). Such differences certainly merit more research because sex-specific data on long-term CMV response in congenitally infected newborns are missing.

### 7.1.3. Neonatal transcriptome in relation to LTI and viral load

In **Chapter 4** neonatal transcriptome was assessed by means of sequencing RNA extracted from DBS. Overall, the differences in whole blood gene expression profile were too small to be able to identify prognostic markers. This happened for a number of reasons extensively discussed in the paper. However, since gene expression analysis captures a snapshot of the cellular activity, considered to be the result of the response to genetic, environmental and epigenetic factors (13), we could make important observations on cCMV immunoregulation. Indeed, DBS mainly reflect the neonatal immune system because they are produced by spotting whole blood on filter paper. Three main observations characterized this study. First of all, high CMV viral load is the main initiator of the transcriptional differences observed in whole blood. Consequently, the differences between CMV+ and CMV- are diluted out because of the presence of low viral load individuals in the CMV+ group. Second of all, numerous antiviral and NK cells activation genes were positively associated with CMV viral load, suggesting the involvement of the innate immune system in response to higher CMV viral load in the fetus. This has been shown before in human first-trimester maternal-decidual tissues. Here, CMV infection upregulated genes related to antiviral innate immune response pathways, with particular emphasis on immune cell activation, proliferation, and trafficking pathways (14). Therefore, what occurs at the maternal-fetal interface may be reflected at birth. Third of all, anti-inflammatory markers, such as the cytokine IL-4, were associated with congenitally infected children that did not develop LTI, suggesting that LTI pathogenesis may be partially attributable to an uncontrolled inflammation. The molecular mechanisms of LTI development are largely unknown. In neonates with neurological impairments and cerebral ultrasounds findings at birth, an uncontrolled inflammation together with a direct CPE are believed to be the responsible (10). Whereas, the late-onset hearing loss is believed to be the result of a chronic productive infection that occurs throughout childhood (15, 16). This suggests that the long-term immune response against cCMV is dysfunctional. Therefore, it cannot be excluded that such a dysfunctional immune response can lead to an uncontrolled inflammation that additionally contributes to tissue damage. Similarly to IL-10, IL-4 has been shown to possess similar capacity of down-regulating the production of pro-inflammatory mediators by microglia, both in humans and in mice (17-19), and its neuroprotective effect is believed to be based on the inhibition of brain inflammation (20). Additionally, in a cohort of healthy CMV infected individuals, the CD4 T-cell response associated with a protective immunity included the production of IL-4 (21). Considering that half of the neonates (in this chapter) developing LTI had microcephaly at birth, it is tempting to speculate that anti-

inflammatory markers may help in limiting the long-term tissue damage. Finally, what causes this immune long-term immune dysfunction is unknown, and T cell exhaustion could be one potential mechanism. However, in our cohort, T cell exhaustion that characterized the infected group, and was more marked with high CMV viral load, did not seem to play a role in the development of LTI, further supporting the reversibility of such phenomenon.

#### 7.1.4. Maternal-child HLA, expressed at the placenta, in relation to symptoms, LTI and viral load

In **Chapter 5** the maternal and fetal HLA background was assessed by means of HLA-C, HLA-E and HLA-G typing in both mothers and neonates, and the mothers were additionally typed for KIRs. We wanted to evaluate whether the maternal-child combination of HLAs expressed at the placenta, as well as the individual HLAs, may influence cCMV outcome. cCMV pathogenesis, and consequently its clinical outcome, is the result of a complex interplay between viral, maternal, placental, fetal and child factors. Within each compartment there are many aspects to be taken contemporarily into consideration, and given the complexity of the placental immune cross-talk, a short introduction is preparatory to this section's discussion.

*Maternal-fetal immune cross-talk summary:* the mother has a complicated task, she has to guarantee the immune tolerance to the fetus, by definition a semi allograft, and at the same time has to protect the fetus from infections. These two tasks involve opposite mechanisms of the same cellular populations, e.g. CD8+ T cells. Therefore, a proper compromise should be established. T cells represent the 5-20% of total decidual lymphocytes in the early phases of gestation, whereas this percentage becomes 40-80% at the end of pregnancy (22, 23). The majority of CD8+ T cells are activated effector memory T cells with naïve T cells almost absent (24-26), therefore cells that are highly Ags experienced/differentiated (27). Maternal fetus-specific T cells, i.e. specific for paternal Ags such as HLA-A, HLA-B and HLA-C, have been shown both in the maternal peripheral blood, and at the maternal-fetal interface (28, 29). The frequency of B cells in the decidua is very low (30), and this allows a certain degree of protection from Abs-mediated effects (31, 32). Monocyte are recruited to the decidua through interaction with the trophoblast-derived chemokine receptor ligands, they can differentiate into DCs (33), and are approximately <1% of total cells in the first trimester (23). Finally, decidual natural killer cells (dNK) cells are the most abundant leucocyte population, at least during the first trimester of pregnancy (34), and can also be generated locally (27). NK cytotoxicity is controlled by a combination of both activating and inhibitory receptors (35-38). In normal conditions, several mechanisms are in place to prevent a detrimental maternal immune response against the fetus. However, endogenous and exogenous factors can alter such delicate balance. An endogenous factor may be a not so favorable maternal KIR/fetal HLA-C combination (27), that skews dNK response towards an inhibitory response, which in turn negatively affects trophoblast invasion (39). An exogenous factor may be an infection, such as cCMV, which triggers an inflammatory response. This alters normal trophoblast invasion, induces apoptosis and placental dysfunction, with a consequent fetal damage, e.g. IUGR (40-42).

Our findings added to this picture the following: if cCMV occurs at the backdrop of certain maternal-fetal HLA combinations such inflammation may be worsened, with a consequent worse



clinical outcome at birth. In particular, a reduced maternal control over cCMV, due to HLA-G del/del genotype which leads to higher protein, may induce an increased viral and cellular burden at the placenta. Here, a fetal allo-Ags recognition by the maternal immune system may contribute to placental inflammation and dysfunction through direct damage. This was suggested by the increased percentage of HLA-E mismatches (mm) and HLA-C mm in the group of symptomatic neonates. Since in normal healthy pregnancies a shift towards more placental activated T cells in the presence of HLA-C mm has been shown (29), our theory seems plausible. Additionally, considering that HLA-E and HLA-C can present CMV Ags, a suboptimal viral clearance seems reasonable because maternal cells would not efficiently recognize CMV presented in the context of the allo-HLA by fetal cells.. This is further supported by the higher percentage of mm in the high viral load group. Finally, the absence of maternal HLA-C belonging to the C2 group was associated with symptoms at birth, most likely because this skews NK cell activation towards a pro-inflammatory state as the HLA-C1 has less inhibitory capacity on dNK cells. Indeed, dNK cells do not interact only with fetal ligand, but also with maternal ligands as part of the internal immune homeostasis. Taken together, these factors may contribute to placental inflammation, dysfunction and a worse outcome at birth. Finally, prematurity has been associated to chronic placental inflammation in the absence of infections (43), and given the relatively high percentage of prematures in our cohort, the possibility of an inflammatory effect independent of CMV needed to be considered as well. Excluding the premature from the analysis, the results showed slight changes of p-values, but not of trends in the percentages. Removing 11 individuals from the symptomatic group led to lack of statistical power as we are left with only 8 individuals, and since we observed the same trends of percentages we could conclude that the associations we found were not influenced by prematurity. Although this is logical, from a statistical point of view we cannot fully exclude it. However, taking into account the association between cCMV and prematurity (8), it is plausible to assume that prematurity is an effect of the combination of cCMV and aforementioned HLAs.

Finally, as previously mentioned, LTI development can be considered the result of a long-term immune dysfunction that leads to an uncontrolled viral replication and inflammation. Our findings added to this picture the following: HLA-C non-inherited maternal antigens (NIMAs) may support a long-term uncontrolled viral replication by means of the tolerance induced in the fetus towards NIMAs, which indirectly induce a tolerance to CMV. The NIMA effect has been shown in transplantation because it improves the outcome. Despite the exact molecular mechanism is largely unknown, several observations led to an hypothetical mechanism, which will be described in the paragraph below. Additionally, as previously mentioned, prematurity has been shown to be associated with LTI similar to those reported for cCMV (9). Given the relatively high percentage of premature in our cohort, we considered the possibility of an effect independent of CMV. In our cohort, the results did not change, suggesting that prematurity does not influence the association we found between HLA-C NIMAs and LTI. By removing 6 individuals from the group with LTI, and 5 in the group without, we are left with 21 vs 64 which does not lead to small numbers, as observed for symptoms at birth.

*Hypothetical mechanism of the NIMA effect in cCMV:* in order for this effect to occur, the fetal immune system has to “see” the maternal cells carrying NIMA. This is due to a mutual exchange of

cells between mother and fetus that starts during pregnancy and continues with breast feeding, leading to microchimerism (44, 45). These allogeneic cells seed in different organs in both mothers and children. In the latter, they can reach several fetal tissues such as bone marrow, blood, spleen, heart, lungs, pancreas and lymph nodes (46-49). The NIMA “recognition” can occur through different mechanisms, direct and indirect pathways. Fetal T cells can recognize NIMAs directly as intact antigens expressed on maternal cells or indirectly as peptides presented by the shared HLA. Fetal T cells can recognize NIMA derived peptides presented by fetal antigen-presenting cell (APC) in HLA Class I and Class II, and fetal APC can “meet” maternal cells in many fetal tissues. However, this recognition will not lead to immune activation but to immunomodulation of both T- and B-cells. When fetal CD4 T cells, that have already been modulated by NIMA, interact with APC’s expressing both NIMA and peptides derived from CMV, this will lead to downregulation of the immune response to CMV (linked immune suppression) (50, 51).

### **7.1.5. Maternal-child HLA, not expressed at the placenta, in relation to symptoms, LTI and viral load**

In **Chapter 6** the maternal and fetal HLA background was assessed by means of HLA-A, HLA-B, HLA-DR and HLA-DQ typing in both mothers and neonates. We wanted to evaluate whether the maternal-child combination of HLAs not expressed at the placenta, as well as the individual HLAs, may influence cCMV outcome. We decided to study the HLAs that are not expressed at the placenta because an allogeneic response can still occur (28, 52-54). Viral infections can increase the levels of pro-inflammatory cytokines and chemokines at the maternal-fetal interface, and intensify the decidual T cell influx (22, 55). Therefore, the increased level of allogeneic maternal cells specific for fetal Ag that are not expressed by the trophoblast, may contribute to placental immunopathology by increasing inflammation. This hypothesis seemed plausible because in Chapter 5 placental immunopathology seemed to be responsible for placental dysfunction, and a worse outcome at birth. However, mm and NIMAs for those HLAs not expressed at the placenta were not associated with a worse short- and long-term outcome in our cohort. However, it is worth to mention the trend of higher HLA-A and HLA-B mm percentages in the symptomatic group, which therefore are considered to have a small effect in the context of cCMV. Furthermore, HLA-C NIMA can have a more important role compared to NIMA of other HLAs. CMV has developed strategies to evade host immunity, and to establish latency by down-regulating classical HLA molecules and up-regulating non-classical HLA, while maintaining HLA-C expression in order to avoid NK cells activation. The important role of HLA-C during CMV infection is further supported by the fact that certain HLA-C-restricted CD8 T cells have more efficient antiviral functions than HLA-A or HLA-B (56). Thus, if an HLA molecules is not expressed, the NIMA Ag cannot be processed, and the NIMA-specific regulatory mechanisms do not occur as efficiently as if the Ag is expressed. Additionally, the degree of the NIMA effect may also vary according to the percentage of microchimeric cells that engrafted fetal tissues. In fetal lymph nodes a predominance of hematopoietic cells was observed, with a frequency between 0.0035% to 0.83% (49), and others have reported similar % for non-lymphoid organs both from fetuses, neonates and adults (46, 57).

In this chapter, we additionally evaluated the individual, maternal and child, HLAs in relation to neonatal CMV viral load. The main finding is that HLA-DRB1\*04 seemed to be protective, or at least to better control the infection, as its frequency was increased in the neonates with lower viral load. Such an effect did not lie in the maternal-fetal combination, but in the presence of these Ags in the child. HLA Class II is involved in the support of CTL and humoral response, which are fundamental players in controlling CMV infection. This suggests that a favourable CMV-HLA II combination can have an important dual effect.

## 7.2. CONCLUDING DISCUSSION

Several approaches have been used to explore prognostic markers for long-term outcome, but not all of them seemed promising, although all of them were useful to get more insights into cCMV pathogenesis. An important aspect should be kept in mind, this is an associative study, and, as such, it needs confirmation, both in other associative studies and experimentally. The first would allow us to determine whether the findings of this thesis, mainly those genetic, can be reproduced in other comparable cohorts. The second would allow the determination of the molecular mechanisms, which is fundamental to design any innovative intervention. The neonatal immune markers, through DNA quantification of the most common TCR and BCR rearrangements from DBS, together with the maternal-child HLA background, through typing DNA from buccal swabs, seemed to be quite promising for prognostic markers, and certainly merit further evaluation. Whereas the small effect of cCMV on the transcriptome profile from DBS should first be confirmed with fresh material before excluding such an approach. Finally, the neonatal metabolism, which was assessed when the material was fresh, did not look like an encouraging approach for finding prognostic markers in the context of cCMV. In the following paragraphs, an attempt to integrate all the findings in one final model is presented in relation to cCMV, symptoms at birth and LTI development (Fig. 1). A final paragraph on the role of CMV viral load is included because it was a recurrent topic in all chapters.

### 7.2.1. Congenital CMV infection

cCMV affected the neonatal immune system by reducing the thymic output of  $\alpha\beta$  T cells, increasing circulating  $\gamma\delta$  T cells as well as newly formed and circulating B cells (Chapter 2). cCMV did not induce extensive proliferation of B cells, rather increased the general production (Chapter 2), and induced exhaustion of T cells (Chapter 4). CMV viral load was an important determinant for shaping the immune system not only in relation to the number of circulating  $\gamma\delta$  T cells, newly formed and circulating B cells, but also in relation to the whole blood transcriptome of innate immune system and NK cell activation (Chapter 4). This raises a central point, i.e. the presence of low viral load neonates in the CMV+ group may mask important effects in the immune system. The only immune marker not associated with viral load was the thymic output of  $\alpha\beta$  T cells, however, we cannot conclude that the viral load would not influence this compartment because we do not have a marker for circulating  $\alpha\beta$  T cells. And we do not have a marker for the number of newly formed  $\gamma\delta$  T cells in order to see whether, similarly to  $\alpha\beta$  T cells, cCMV induces a reduced output. Therefore, in future research it would be important to include these two markers to have the complete picture. The innate immune

system, NK cell activation and  $\gamma\delta$  T cells may be important mechanisms to control CMV in the early phase of infection, where the output of  $\alpha\beta$  T cells is reduced, and the CMV-specific CTL and humoral responses are still developing. As both the CTL and the humoral responses are fundamental for controlling CMV infection, a favorable HLA Class II-CMV peptide complex would support more efficiently both arms of the adaptive immune system with a consequent more efficient viral control (Chapter 6). Therefore, in future studies, it would be important to determine which CMV peptide is presented in the context of HLA-DRB1\*04 as this could be exploited in future vaccine development. Moreover, although both the virus and the immune system activation impose an important burden on the cellular metabolism, as it has been shown *in vitro*, overall the effect was not strong enough to be reflected in whole blood in the general population but only in premature neonates (Chapter 3). Premature neonates may not be able to efficiently cope with the general increased metabolic requirements, with a consequent lower level of essential aminoacids, fundamental for the correct development. Therefore, future studies are needed to evaluate the clinical implications of cCMV in this special category of neonates. Finally, similarly to the neonatal immune system, CMV viral load appeared to be an important determinant in shaping the neonatal metabolism. Indeed, C16, which reflects the level of palmitate, i.e. precursor of longer chain fatty acids for which CMV is enriched for, was positively correlated to CMV viral load.

### 7.2.2. Symptoms at birth

The number of various immune cells was not associated with a worse outcome at birth (Chapter 2). One plausible explanation may be the fact that the clinical signs included in the definition of symptoms at birth in our cohort are rather general. Indeed, the mechanisms that lead to neonatal hepatosplenomegaly, petechiae, purpura, thrombocytopenia, neutropenia or elevated ALAT may not be due entirely to a dysfunction of the neonatal immune system per se. Whereas in those symptoms that are more specific of cCMV, such as microcephaly, one could assume that the neonatal immune system may have a more important role. E.g., in solid organ-transplanted patients,  $\gamma\delta$  T cell expansion was associated with resolution of CMV infection and less symptomatic CMV disease, and late  $\gamma\delta$  T cells expansion was correlated with a more intense and durable CMV infection (58). This can potentially be also the case in our cohort if only we could assess all immunological markers in relation to the individual clinical signs. In our cohort, symptoms at birth seemed to be mainly caused by a placental dysfunction as a result of a multifactorial process that starts in the mother and continues in the placenta (Chapter 5). This seems plausible because a placental dysfunction can lead to general fetal harm. The genetic factors involved are: maternal HLA-G del/del genotype that increases the cellular and viral burden at the placenta; HLA-E mm and HLA-C mm that induce a fetal allo-Ags recognition by the maternal immune system, and a suboptimal viral clearance; absence of maternal HLA-C belonging to the C2 group that skews dNK cells activation towards a pro-inflammatory state (Chapter 5). Here, mm that are specific for HLA not expressed at the placenta, such as HLA-A mm and HLA-B mm, may partially contribute to placental inflammation (Chapter 6). Evaluating the molecular mechanisms behind the influence of such genetic factors during cCMV in relation to symptoms at birth would be crucial for creating any interventions. This is not easy in

a prospective study, but to start with maternal blood samples would be useful to compare the CMV-specific immune system in relation to HLA-G genotype. Additionally, placental specimens would be necessary to evaluate the effect of the maternal HLA-G and HLA-C genotypes, as well as maternal-fetal HLA-C mm and HLA-E mm, in the cellular and structural composition of the placenta. And the CMV viral load should always be taken into consideration.

### 7.2.3. Long-term impairments

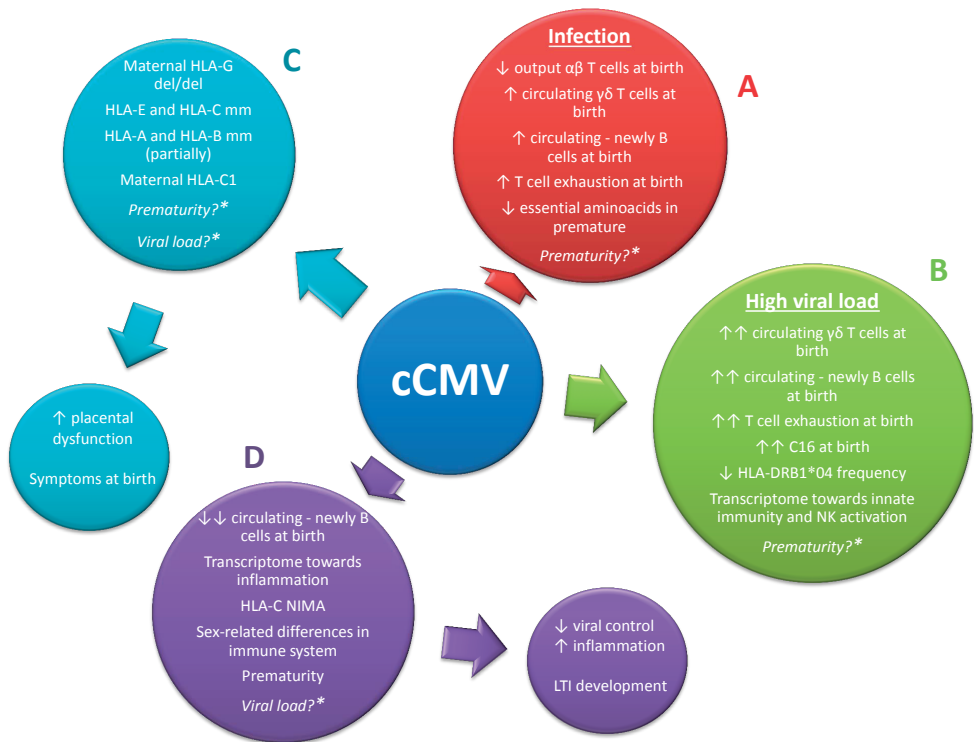
In our cohort, a dysfunctional immune response that leads to an uncontrolled viral replication, combined with an uncontrolled inflammatory response, appeared to be responsible for LTI development. This most likely involves both the effector and regulatory mechanisms. Neonates developing LTI had lower numbers of newly formed and circulating B cells at birth, suggesting some sort of long-term dysfunction (e.g. in the capacity to generate long-lived plasma cells, memory B cells, antibodies (Abs) or support effector functions of immune cells), or reduced capacity to control the infection (Chapter 2). As both CTL and humoral response are necessary to control CMV infection, it would be extremely interesting in future research to see whether infected neonates that developed LTI had lower number of circulating  $\alpha\beta$  T cells at birth, both total and CMV-specific. What is the trigger of this difference in B cells number between infected neonates that develop LTI and those who do not is impossible to establish in our cohort. Importantly, the exhaustion that we described for T cells (Chapter 6), but that has also been described for B cells in the context of other chronic infections such as HIV (59), did not seem to be so permanent to cause a long-term dysfunction. The exhaustion temporariness is not surprising because in a murine model of chronic infection, the blockade of PD-1 resulted in reversion of the exhaustion (60). A genetic factor may contribute to a long-term dysfunction. In our cohort, HLA-C NIMA appeared to be partially responsible for LTI development (Chapter 5). The so-called NIMA effect, i.e. immune tolerance to NIMA, indirectly can induce a tolerance to the infection with a consequent reduced viral control. This effect, that can influence both T and B cells, is due to microchimeric cells that persists at least till early adulthood (49, 61). Demonstrating the role of a genetic factor for LTI development is more difficult because of the late onset of certain LTI, and of different pathogenetic mechanisms that may be responsible for different impairments such as hearing loss and motor impairment. One way to start would be to compare the neonatal CMV-specific immune response with and without HLA-C NIMA, and repeat this over time, in relation as well to CMV viral load. The immune dysfunction that leads to an uncontrolled viral replication may be followed by dysfunctional compensatory mechanisms that in turn lead to an uncontrolled inflammatory response. Indeed, in our cohort, anti-inflammatory markers characterized infected neonates that did not develop LTI, further supporting the role of inflammation as additional determinant for tissue damage (Chapter 4). In the context of development of LTI, the sex-related differences in the immune system should be taken into consideration as well (Chapter 3). Finally, prematurity has been shown to be associated with LTI similar to those reported for cCMV (9), as well as to chronic placental inflammation also in the absence of infections (43). However we believe that it is not the prematurity per se that is responsible for the LTI in our cohort. First of all, although prematurity is not always used as a criterion

in the definition of “symptoms at birth”, it has been shown that cCMV is frequently accompanied by prematurity (8). When focusing on symptomatic groups (with prematurity not used as a criterion) the percentage of prematurity is usually around 36% (62). The percentage of symptomatic children in the infected group in our cohort, including those that were premature, was 18%, which is similar to that found in previous studies. The percentage of prematures in the infected group (10.8%) was similar to that found by others (and only slightly higher than in the general population), although others have found lower percentages (8, 63). Despite the fact that the percentage of premature neonates were relatively high in our cohort, our data showed that the association we found between HLAs and cCMV clinical outcome is not confounded by prematurity. However, there is no doubt that prematurity will not be beneficial for the undisturbed child development, but cCMV and HLAs may have a synergistic effect on prematurity, and these three factors may all contribute to LTI development.

#### 7.2.4. CMV viral load and DBS testing

CMV viral load in our cohort was not significantly associated to symptoms at birth nor to LTI. The role of CMV viral load in the cCMV clinical outcome still needs to be elucidated as some previous studies have related the viral load to a worse outcome (62, 64), whereas others have not (65-67). However, there is no standardized way of defining symptoms at birth or LTI, and the CMV viral load in blood of infected neonates may vary depending on the timing of infection, whether there was a maternal primary or secondary infection, or on the specific compartment impaired. Indeed, it is tempting to speculate that between a neonate with cCMV that has hearing loss at birth and a neonate with cCMV that has elevated liver transaminases, the peripheral CMV viral load differs. Unfortunately, due to lack of statistical power we could not evaluate how whole blood CMV viral load changes in relation to the different compartments impaired. For all aforementioned reasons, if any role of viral load in blood exists, it may have been diluted in our cohort. In future research, defining the role of viral load in relation to individual clinical outcomes, standardizing the definition of symptomatic disease and LTI development is absolutely essential in order to be able to draw any conclusion on pathogenesis. Indeed, what we concluded in this thesis can very well not be the case in another cohort just because different definitions are used. Nevertheless, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI to symptoms at birth with severe LTI. This would be the situation we will have to deal with in a universal screening setting. Additionally, DBS testing for cCMV diagnosis may be seen as a potential limitation. Indeed, the golden standard method for cCMV diagnosis is the urine testing because of the high viruria observed in congenitally infected infants (65), but saliva is considered equally reliable (68), and CMV viral load is usually lower in blood than in urine (65). DBS can be considered a proper and reliable alternative specimen to blood, because a positive correlation between CMV viral load measured on DBS and CMV viral load measured on whole blood/ plasma, both in the context of cCMV and CMV in transplantation, was shown (69, 70). These studies suggested that DBS may be used not only for diagnosis but also for monitoring response to therapy. Moreover, DBS testing has a reputation of lower sensitivity, and the DNA/RNA content is believed to be more prone to degradation. Importantly, even considering

the relative reduction of CMV viral load on DBS in time (69), the specimens that could be potentially more affected from the aforementioned differences between blood and urine/saliva are the ones with low viral load. This implies that low-positive neonates may be found in the CMV negative group. However, with the high sensitivity of our PCR (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (71). Therefore, the influence of the sensitivity of the CMV PCR on DBS on the conclusions in all chapters can be considered negligible. Despite the aforementioned potential limitations, the use of DBS, that are normally collected at birth for the screening of rare genetic metabolic disorders, has several advantages over other specimens. Since the DBS are routinely collected, they are available almost worldwide, and allow large-scale retrospective studies for long-term cCMV outcome, which would be challenging in a prospective setting. Additionally, CMV detection on DBS does not require special facilities, is not expensive and an automation can be performed in order to include many specimens at the same time further reducing the price. However, efforts in improving these aspects and the standardization of such a method are necessary for the introduction in the screening program.



**Figure 1.** Final model of cCMV pathogenesis. A) Overall effects of cCMV in relation to the neonatal immune system and metabolism. B) Effects of higher CMV viral loads in relation to the neonatal immune system and HLA Class II. C) Genetic factors that in presence of a cCMV may lead to placental dysfunction, and consequently to symptoms at birth. D) Genetic and immune factors that in presence of a cCMV may lead to reduced viral control and increased inflammation throughout childhood, and consequently to LTI development.

\* Indicates factors whose role in the pathogenesis of cCMV and cCMV-related disease need further evaluation.

**To conclude**, any future intervention should focus on supporting both arms of the adaptive immune response in order to efficiently control the chronic viral replication, with particular attention to regulating the inflammatory response. This seems achievable with a vaccine, though the target population may vary depending on the goal of such vaccine. However, as the way to a licensed vaccine seems to be long, the *tertiary prevention* sounds like a good compromise. By means of *tertiary prevention* we would like to prevent, or at least positively affect, the short- and/or long-term impairments. How can we efficiently intervene to limit a progressive permanent damage that has a late onset manifestable in years after birth? We have to understand the pathogenesis of cCMV, and to identify a biomarker that differentiate these groups. The majority of congenitally infected neonates are asymptomatic at birth and have a good prognosis for a normal long-term development, therefore the comparison of their immunological condition with that of the symptomatic infected neonates with a worse long-term outcome, as well as those asymptomatic that develop the same LTI, should be the focus in future research. Importantly, the approaches used would need to be powerful enough to get subtle differences that are likely to be expected between asymptomatic that develop LTI and asymptomatic that do not. In parallel with this, it would be essential to determine whether treatments offered to symptomatic neonates, ranging from antivirals to audiological and motor follow-up, are beneficial in those asymptomatic neonates that develop LTI. For example, whether (val)ganciclovir treatment of asymptomatic neonates with higher probability to develop hearing problems actually has a positive effect on the hearing compared to a similar group that did not get such treatment. Maybe the side effects of such treatment would exceed the positive effect in a way that would be better to somehow boost their immune system. However, an even earlier goal should be the standardization of the definitions of symptomatic disease and LTI development, as well as the role of viral load in the clinical outcome, without which no reliable conclusion on the actual cCMV pathogenesis, and its clinical consequences, can be drawn. Concluding, understanding the pathogenesis of cCMV would allow to figure out why certain children develop LTI and others do not. In turn, this would provide the necessary biomarkers to predict outcome and stratify patients according to the risk. On one hand we could define who benefits most from certain clinical interventions; and on the other hand we could define correlates of protection to be used in future vaccine trials.



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