Immunological profile of pregnant women with preconception immunity with or without vertical transmission of human cytomegalovirus to the fetus: a retrospective observational study

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Summary

Background Vertical transmission of human cytomegalovirus (CMV) to the fetus (congenital CMV infection) can occur in pregnant women with preconception immunity. Maternal immunological features associated with congenital CMV infection have been poorly investigated. We aimed to characterise the immunological features of pregnant women with preconception immunity in cases with and without vertical transmission of human CMV to the fetus.

Methods In this retrospective cohort study, we included pregnant women (aged >18 years) with preconception immunity enrolled between Sept 1, 2017, and Oct 15, 2020, in the Congenital Human Cytomegalovirus Infection in Lombardy (CHILd) study (Italy) with (congenital CMV group) and without (no-congenital CMV group) intrauterine human CMV transmission. Blood was collected at 13 weeks of gestation and at delivery. The following immune parameters were measured and compared between groups: serum neutralising titres, antibody-dependent cell cytotoxicity, human CMV-specific long-term memory T cells (interleukin [IL]-7R$^+$ and IL-2$^+$), and effector memory CD45RA$^+$ (TEMRA) cells. Immune parameters were also compared with those of two groups of pregnant women with human CMV primary infection enrolled at Fondazione IRCCS Policlinico San Matteo (Pavia, Italy) in two previous studies: primary infection group 1 for antibody response (followed up for 12 months; enrolled between Feb 23, 2011, and Sept 1, 2015,) and primary infection group 2 for T-cell response (followed up for 24 months; enrolled between July 10, 2016, and March 18, 2020).

Findings 128 women were included in this study: 56 women from the CHILd study (16 women in the congenital CMV group and 40 women in the no-congenital CMV group) and 72 pregnant women with primary infection (40 in primary infection group 1 [15 with and 25 without vertical transmission] and 32 in primary infection group 2 [11 with and 21 without vertical transmission]). Higher neutralising activity ($p \leq 0{\cdot}022$) but lower antibody-dependent cell cytotoxicity ($p=0{\cdot}0004$) was observed in the congenital CMV group versus the no-congenital CMV group. Additionally, the congenital CMV group had a lower percentage of long-term memory T cells than the no-congenital CMV group ($p \leq 0{\cdot}022$). No significant difference was found for TEMRA cells between congenital CMV and no-congenital CMV groups. Immunological parameters of the congenital CMV group were similar to those observed in primary infection groups 1 and 2 within 12–24 months after infection.

Interpretation Immune women with intrauterine human CMV transmission have distinct immunological parameters (different from immune women without CMV transmission, but similar to women with primary infection) and might be those who had a primary human CMV infection within a few years earlier before maternal immunity development was completed. A vaccine able to elicit a fully developed maternal immunity to human CMV is likely to be protective for the fetus.

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Introduction

Human cytomegalovirus (CMV) is the primary cause of congenital infections in high-income countries, with a worldwide birth prevalence of 0·6%.[1] Among infants with congenital CMV, 20–25% are symptomatic at birth or develop long-term sequelae. Although preconception maternal immunity greatly reduces the risk of congenital CMV infection, this immunity does not provide complete protection against maternal non-primary infection and fetal infection. The rate of intrauterine transmission of human CMV is around 32% after maternal primary infection; however, it has been estimated to be less than 3·5% after a

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Research in context

Evidence before this study

Human cytomegalovirus (CMV) is the leading infectious agent responsible for congenital infections, causing intellectual disability, psychomotor delay, hearing loss, speech and language disabilities, behavioural disorders, and visual impairment. The prevalence of congenital CMV infection is around 0·6%, and about 20% of infected newborns are symptomatic at birth or will develop long-term sequelae. The role of preconception immunity in protecting from congenital human CMV infection is debated, since congenital CMV can also occur in neonates born to immune women who develop non-primary infection (by reactivation of the latent virus or reinfection) during pregnancy. We searched PubMed from Jan 1, 1990, to April 20, 2023 using the terms ((("cytomegalovirus [ti]" OR "cmv[ti]") AND ("non-primary*[tiab]" OR "reinfection* [tiab]" OR "reactivation*[tiab]" OR "shedding*[tiab]") AND ("pregnancy" OR "maternal" OR "congenital") AND ("immune" OR "immunity" OR "immunological")) AND "English[lang]") NOT review. Few studies investigated the immune response to human CMV in women with preconception immunity who transmit the virus to the fetus, and these studies were limited to the antibody response to polymorphic sites of glycoproteins B and H. Immune factors associated with the higher susceptibility to maternal non-primary infection and subsequent fetal infection were not investigated in the identified studies.

Added value of this study

To our knowledge, this retrospective cohort study is the first to analyse the human CMV-specific antibody and T-cell responses in pregnant women with preconception immunity with or without viral transmission to the fetus. The results show that mothers of infected newborns or fetuses had a higher serum neutralising activity, lower antibody-dependent cellular cytotoxicity, and lower percentage of long-term memory human CMV-specific T cells than mothers of uninfected newborns. The immunological features observed in women for whom there was human CMV transmission to the fetus despite a preconception immunity were similar to those of pregnant women with human CMV primary infection.

Implications of all the available evidence

The findings of this study suggest that a fully developed maternal immune response to human CMV confers protection from viral transmission to the fetus. Therefore, a vaccine able to elicit such a maternal immunity is likely to be protective for the fetus. Our data suggest that immunological endpoints for human CMV vaccine evaluation should not be limited to neutralising antibodies, but should comprise Fc-mediated antibody functions, such as antibody-dependent cellular cytotoxicity, and analysis of memory T-cell immunity. Clinical trials assessing the efficacy of the human CMV vaccines currently under development should be actively pursued.

non-primary infection.[2–4] Prevalence of congenital CMV in pregnant women with preconception immunity is around 0·2% in Europe,[5,6] whereas a higher rate (0·47%) was observed in Brazil.[7]

A meta-analysis showed that the rate of symptoms at birth or long-term sequelae (eg, sensorineural hearing loss) in children with congenital CMV infection born to mothers with human CMV primary infection is similar to that of children born to mothers with non-primary infection.[8]

Human CMV non-primary infection can emerge after reactivation of a latent human CMV strain or reinfection with a different human CMV strain.[9] Approximately 21·5% (range 12·7–75·0) of pregnant women with preconception immunity to human CMV develop a non-primary infection (defined by virus shedding in at least one bodily fluid among urine, saliva, or vaginal secretions during gestation).[10] However, the prevalence of human CMV non-primary infection varies based on the population demographic characteristics (eg, ethnicity, socioeconomic status, age, and close contact with young children).[10] Furthermore, the prevalence of human CMV shedding in saliva, urine, and vaginal secretions of seropositive pregnant women with non-primary infection increases with advancing gestation.[2,3]

The host immune response appears to be involved in the prevention of viral transmission to the fetus during primary infection. In particular, a fast appearance in peripheral blood of some phenotypes of human CMV-specific $CD4^+$ and $CD8^+$ T cells, such as interleukin (IL)-2 production, IL-7R expression, and re-expression of $CD45RA^+$ (TEMRA) cells, appear to be associated with a reduced risk of human CMV vertical transmission.[11–13] For instance, IL-7R expression and IL-2 production characterise long-term memory T cells, whose appearance in blood is associated with controlling viral replication and antigen clearance.[14,15]

However, knowledge about the immune response during non-primary infection is scarce, and the potential association between defects in the immune control of human CMV and fetal infection during non-primary infection has not been investigated yet. The aim of this study was to characterise the human CMV-specific immunological features of pregnant women with preconception immunity in cases with and without vertical transmission of human CMV to the fetus.

Methods

Study design

In this retrospective cohort study, human CMV-specific B-cell and T-cell immunity was analysed in a subgroup of women with female sex assigned at birth (aged >18 years) enrolled between Sept 1, 2017, and Oct 15, 2020, in the Congenital Human Cytomegalovirus Infection in Lombardy (CHILd; NCT03973359) study,[6] the aim of which was to investigate prevalence, outcome, and prevention of congenital CMV in neonates born to women with preconception immunity in the Lombardy region (Italy). Here, we

included all women with preconception immunity for whom there was vertical transmission (congenital CMV group), excluding women who were taking immunosuppressive drugs, and women with preconception immunity for whom there was no vertical transmission of human CMV, with sufficient availability of serum and peripheral blood mononuclear cells (PBMCs; no-congenital CMV group).

In parallel, pregnant women who had human CMV primary infection with and without vertical human CMV transmission between Feb 23, 2011, and Sept 1, 2015, at a median time of 13 weeks of gestation (range 1–26), were also included in the analysis of the human CMV-specific antibody response (primary infection group 1), and pregnant women who had primary infection with and without vertical human CMV transmission between July 10, 2016, and March 18, 2020, at a median time of 7 weeks of gestation (range 0–23) were included in the analysis for T-cell immunity (primary infection group 2; appendix pp 2, 5). Women with primary infection were enrolled at Fondazione IRCCS Policlinico San Matteo (Pavia, Italy) in the context of other studies on immune response to primary CMV infection.

Study procedures were approved by the IRCCS Policlinico San Matteo Ethics Committee (P-20170011101). IRCCS Policlinico San Matteo Ethics Committee also approved the original cohort studies of primary infection group 1 (P-20100035854) and of primary infection group 2 (P-20180075214). All participants provided written informed consent at enrolment in the original studies, including for the use of residual samples for future studies on CMV infection and immune response).

Procedures

In the congenital CMV group, blood was collected at delivery.[6] In the no-congenital CMV group, blood, urine, saliva, and vaginal swabs were collected at 13, 20, and 30 weeks of gestation as well as at delivery.[3] In primary infection group 1 and primary infection group 2, blood was collected, whenever possible, at months 1, 2, 3, 6, and 12 after infection and, for group 2, month 24 after infection. Congenital CMV infection was diagnosed by detection of human CMV DNA in a saliva swab and subsequent confirmation in urine (congenital CMV and no-congenital CMV groups) or by detection of human CMV DNA in amniotic fluid collected at 20–22 weeks of gestation or in urine collected at birth in primary infection group 1 and primary infection group 2. Demographic, anamnestic, and clinical data were collected in electronic case report forms (REDCap platform).

Human CMV neutralising antibodies

The serum neutralising activity against infection of epithelial and fibroblast cells was analysed at delivery in the congenital CMV and no-congenital CMV groups. Women in the no-congenital CMV group were divided based on their evidence of non-primary infection, and their neutralising activity was compared both at 13 weeks of gestation and at delivery, and in each group, the two timepoints were compared. Serum neutralising activity of the congenital CMV and no-congenital CMV groups were compared with those of primary infection group 1 at 1, 2, 3, 6, and 12 months after the onset of infection. For the neutralisation assay, we used VR1814, a clinical isolate, for epithelial cells, and AD169 (American Type Culture Collection [ATCC], Manassas, VA, USA), a reference laboratory-adapted strain, for fibroblast cells. After 1 h of incubation at 37°C and 5% CO_2, 50 µL per well of virus (100 focus-forming units per well) and human serum antibody mixtures were added onto monolayers of epithelial cells (ARPE-19, ATCC) or human embryonic lung fibroblast cells (human embryonic lung fibroblast [HELF], isolated in-house) in 96 well plates, and centrifuged at 600 × *g* for 30 min. After incubation for 48 h at 37°C and 5% CO_2, cells were fixed and stained using a p72-specific murine monoclonal antibody (produced in-house). Serum dilution inhibiting virus infectivity by 50% was considered the 50% neutralising antibody titre. Titres of IgG specific to glycoprotein B (gB) and the gHgLpUL128L pentamer were also determined (appendix p 3).

See Online for appendix

Antibody-dependent cell cytotoxicity (ADCC)

Aside from the neutralising response, antibodies also potentially contribute to protection against human CMV infection with other functions. The capacity of antibodies to induce natural killer cell degranulation in vitro was investigated as a surrogate of the capacity of antibodies to induce ADCC, as already described previously (appendix p 3).[16] ADCC was determined both in the total and memory ($NKG2C^+$) natural killer populations. Only women with sufficient available serum were included in these analyses; therefore, we compared human CMV-specific ADCC in the congenital CMV group and in the no-congenital CMV group at delivery. Moreover, we compared the no-congenital CMV group with or without non-primary infection at 13 weeks of gestation and at delivery. We analysed ADCC in the primary infection group 1 at 1, 2, 3, and 6 months after infection (insufficient serum was available at 12 months).

Human CMV-specific T-cell response

We compared the human CMV-specific T-cell response at delivery in women who had PBMCs available in the congenital CMV group and no-congenital CMV groups. Moreover, we compared the no-congenital CMV group with or without non-primary infection at 13 weeks of gestation and at delivery. T-cell responses of women in the congenital CMV group and no-congenital CMV group were compared with primary infection group 2 at 1, 2, 3, 12, and 24 months after infection. PBMCs were isolated by standard density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway) and stored in liquid nitrogen. After thawing and overnight incubation of PBMCs, $CD14^+$ monocytes were isolated using MicroBeads conjugated to monoclonal anti-human CD14 antibody and MACS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$ monocytes were incubated for 7 days in RPMI 1640 (Euroclone, Milan, Italy) supplemented with 10% inactivated fetal bovine

serum (Euroclone) to generate dendritic cells. The $CD14^-$ fraction was incubated for 7 days in RPMI supplemented with 10% fetal bovine serum and non-essential amino acids (7·5–15 µg/µL; Euroclone), 1 mM pyruvate sodium (Gibco, Waltham, MA, USA) and 1 µM β-mercaptoethanol (Gibco), without T-cell proliferation or differentiation stimuli, before overnight co-culture with human CMV-infected (VR1814) dendritic cells, in the presence of 10 µg/mL Brefeldin A (Merck, Darmstadt, Germany), as previously described.[17] Monoclonal antibody CD127 (IL-7R) PE (clone hIL-7R-M21, BD Biosciences, San Jose, CA, USA) was added during overnight co-culture. PBMCs were then stained and analysed with flow cytometry (appendix p 4). Human CMV-specific $CD4^+$ and $CD8^+$ interferon (IFN)-γ-producing T cells were measured and among them the percentages of IL-$7R^+$, IL-2^+, and TEMRA ($CD45RA^+$) cells were calculated.

Statistical analysis

Study sample size was not determined by an a-priori power calculation but was based on sample availability.

The Mann–Whitney U test was used to compare antibody and T-cell responses between: (1) the congenital CMV group and no-congenital CMV group, (2) women in the no-congenital CMV group with non-primary infection and those in the same group without non-primary infection, and (3) the congenital CMV group and no-congenital CMV group with the primary infection group (with correction for multiple comparisons in this last case); the Wilcoxon signed-rank test was used to compare antibody and T-cell responses in women of the no-congenital CMV group with non-primary infection and no-congenital CMV group without non-primary infection between 13 weeks of gestation and delivery. Demographic, anamnestic, and clinical characteristics were compared with the χ^2 test. Age between the different groups was compared with the Kruskal–Wallis test. Analyses were performed with Prism 8·3·0 software (Graph Pad Software, San Diego, CA, USA). $p<0·050$ was considered statistically significant.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Of 128 women included in this study, 16 women were in the congenital CMV group, 40 women in the no-congenital CMV group (20 of whom had evidence of non-primary infection and 20 of whom did not), 40 women in the primary infection group 1 (15 with vertical human CMV transmission and 25 without vertical human CMV transmission), and 32 women in the primary infection group 2 (11 with vertical human CMV transmission and 21 without vertical human CMV transmission; figure 1). As reported in the table, no significant differences in age, parity, and mode of delivery were observed between pregnant women with preconception immunity with or without fetal infection. However, a significantly higher frequency of concomitant medical conditions (p=0·0052) was observed in the group of women with fetal infection. In particular, eight (50%) of 16 women in the congenital CMV group had concomitant medical conditions compared with five of (13%) 40 women in the no-congenital CMV group. The association between concomitant medical conditions and congenital CMV in immune pregnant women was already described in the overall population of the CHILd study.[6]

The congenital CMV group had higher concentrations of neutralising antibodies against the infection of ARPE-19 (p=0·022) and HELF (p=0·0003) cells than did the no-congenital CMV group (figure 2A, B).

In the no-congenital CMV group, neutralising antibody titre against the infection of ARPE-19 was significantly higher in women with non-primary infection than in those without non-primary infection, both at 13 weeks of gestation (p=0·011) and at delivery (p=0·034; see appendix p 8). No significant difference was observed in the neutralising antibody titres against HELF, both at 13 weeks of gestation and at delivery (appendix p 8). Additionally, no significant change in neutralising antibody titres against infection of ARPE-19 or HELF was observed from 13 weeks of gestation to delivery in either group (appendix p 8).

We reported in parallel the neutralising activity in the congenital CMV group and no-congenital CMV group with that observed in primary infection group 1 at months 1, 2, 3, 6, and 12 after infection (figure 2C, D). The congenital CMV group exhibited a similar neutralising titre against the infection of ARPE-19 cells to primary infection group 1 at the late timepoint examined (12 months), whereas the no-congenital CMV group had a significantly lower titre of neutralising antibody against ARPE-19 than did primary infection group 1 at 12 months after infection (p<0·0001; figure 2C).

Similar results were observed for the neutralising antibodies against the infection of HELF cells. The congenital CMV group had a titre similar to primary infection group 1 at 12 months after infection, whereas the no-congenital CMV group had a significantly lower titre of neutralising antibody than primary infection group 1 at 12 months after infection (p=0·0042; figure 2D).

IgG antibodies to gB were significantly lower in the congenital CMV group than the no-congenital CMV group (p=0·0003). No significant difference between the two groups was found for the IgG antibody to gHgLpUL128L pentamer (appendix p 9).

When comparing the ability of serum to promote human CMV-specific ADCC, we observed a higher ADCC in the no-congenital CMV group (n=25) than in the congenital CMV group (n=16; p=0·0004; figure 2E). No significant difference was observed when comparing women with or without non-primary infection in the no-congenital CMV group, both at 13 weeks of gestation and at delivery (appendix p 10). No significant change in ADCC was observed from 13 weeks of gestation to delivery.

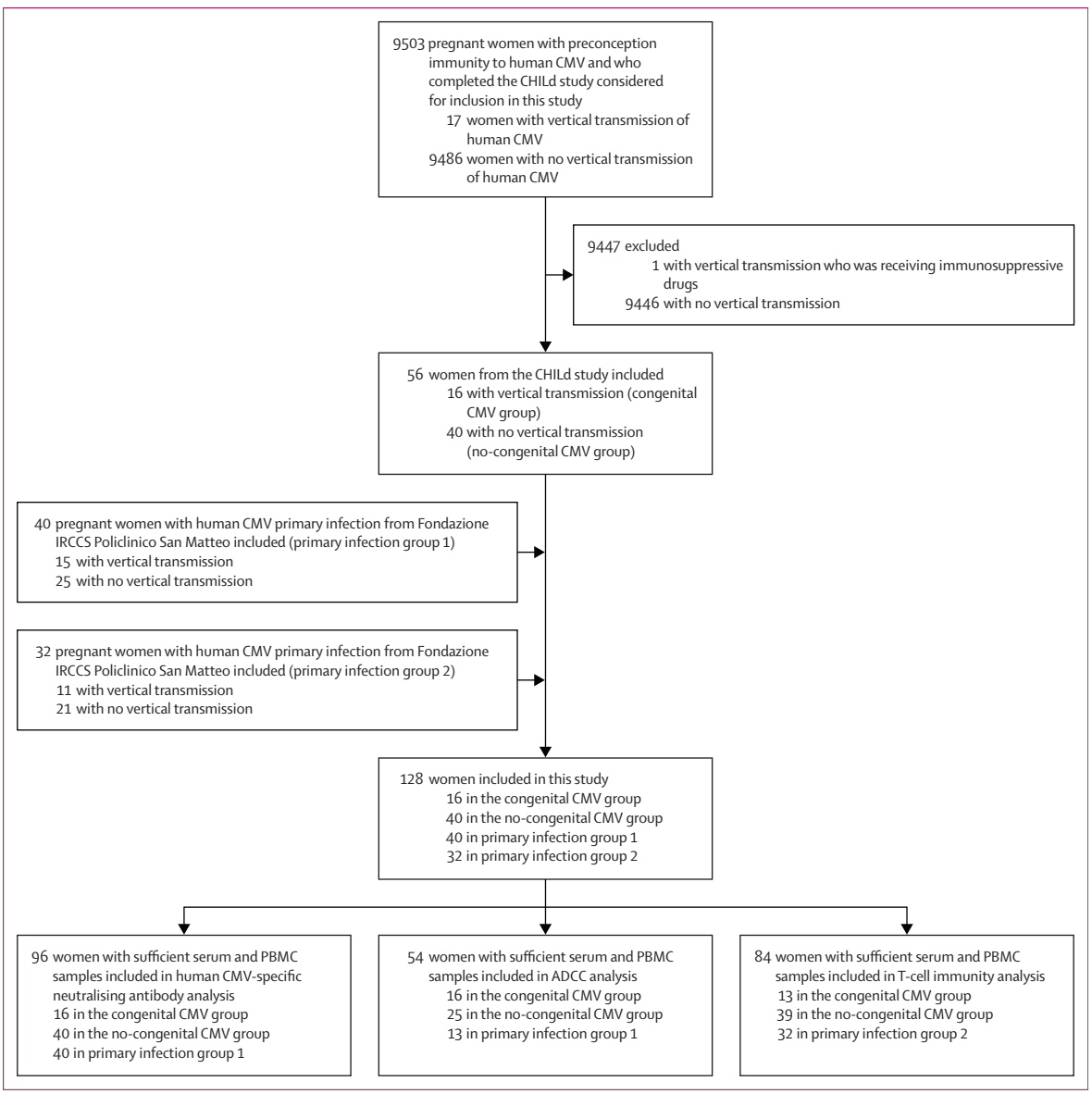

Figure 1: **Study profile**
CHILd=Congenital Human Cytomegalovirus Infection in Lombardy. CMV=cytomegalovirus. PBMC=peripheral blood mononuclear cell. ADCC=antibody-dependent cell cytotoxicity.

Finally, serum from the no-congenital CMV group showed significantly enhanced ability to induce ADCC against infected cells, compared with primary infection group 1 at 6 months after infection (p=0·0006; figure 2F). When comparing the congenital CMV group with the primary infection group 1, this difference was not observed. Similar results were observed when ADCC was determined in the memory (NKG2C$^+$) natural killer cells, instead of in the total natural killer population (data not shown).

No significant difference was observed between the congenital CMV (n=13) and the no-congenital CMV (n=39) groups for total (IFN-γ$^+$) CD4$^+$ and CD8$^+$ T cells (figure 3A, B). Conversely, the congenital CMV group had a lower percentage of IL-7R$^+$ human CMV-specific CD4$^+$ and CD8$^+$ T cells than the no-congenital CMV group (p=0·022 and p=0·018, respectively; figure 3C, D). Moreover, the congenital CMV group had a lower percentage of IL-2$^+$ human CMV-specific CD4$^+$ and CD8$^+$ T cells than the no-congenital CMV group (p<0·0001 and p=0·0005, respectively; figure 3, F). Instead, no statistically significant difference was found for CD4$^+$ and CD8$^+$ TEMRA cells (figure 3G, H). No significant difference was observed for any parameter examined at 13 weeks of gestation and at delivery in women with or without non-primary infection in the no-congenital CMV group (appendix p 11). Additionally, no significant change in human CMV-specific T-cell response was observed from

	Congenital CMV group (n=16)	No-congenital CMV group with non-primary infection (n=20)	No-congenital CMV group without non-primary infection (n=20)	p value*	Primary infection group 1 (n=40)	p value†	Primary infection group 2 (n=32)	p value†
Age, years	34 (23–40)	32 (20–41)	33 (22–40)	0·37	31 (15–42)	0·51	33 (22–41)	0·32
Ethnicity	..	..	..	..	..			
White	15 (94%)	20 (100%)	20 (100%)	0·28	40 (100%)	0·74	32 (100%)	0·58
Arab	1 (6%)	0	0	..	0	..	0	..
Parity	0 (0–2)	0 (0–2)	0 (0–2)	0·23	NA‡	..	NA‡	..
Multiple pregnancy	2 (13%)	0	0	0·077	NA‡	..	NA‡	..
Medical conditions	8 (50%)	1 (5%)	4 (20%)	0·0052	NA‡	..	NA‡	..
Syphilis	1 (6%)	0	0	..	..	..		..
Hypothyroidism and diabetes	2 (13%)	0	0	..	..	..		..
Hypothyroidism	2 (13%)	0	2 (10%)	..	..	..		..
Toxoplasmosis	1 (6%)	0	0	..	..	..		..
Sideropenic anaemia and diabetes	1 (6%)	0	0	..	..	..		..
Autoimmune diseases	0	1 (5%)	1 (5%)	..	..	..		..
Hypertension	0	0	1 (5%)	..	..	..	..	..
Mitral valve prolapse	1 (6%)	0	0	..	..	..	..	..
Mode of delivery	..	..	..	0·74	NA‡	..	NA‡	..
Vaginal	11 (69%)	18 (90%)	12 (60%)	..	..	..	..	..
Caesarean section	5 (31%)	2 (10%)	8 (40%)	..	..	..	..	..

Data are median (IQR) or n (%) unless otherwise specified. CMV=cytomegalovirus. NA=not available. *Statistical analysis between the congenital CMV group, no-congenital CMV group with non-primary infection, and no-congenital CMV group without non-primary infection. †Statistical analysis between the congenital CMV group, no-congenital CMV group with non-primary infection, no-congenital CMV group without non-primary infection, and primary infection group 1 and primary infection group 2. ‡These data are missing because they were not collected in the primary infection studies.

Table: **Characteristics of the women in the congenital CMV group, no-congenital CMV group with or without non-primary infection, and in primary infection groups 1 and 2**

13 weeks of gestation to delivery in the no-congenital CMV group (appendix p 11).

Finally, we reported in parallel the T-cell parameters observed in the primary infection group 2 at months 1, 2, 3, 12, and 24 after infection with those of the congenital CMV or no-congenital CMV group (figure 4). No significant difference was observed between the primary infection group 2 at the late timepoint examined (24 months) and the congenital CMV group. Conversely, the no-congenital CMV group had a higher percentage of IL-7R$^+$ human CMV-specific CD8$^+$ T cells and of IL-2$^+$ human CMV-specific CD4$^+$ and CD8$^+$ T cells than the primary infection group 2 examined 24 months postinfection (figure 4D–F). Although human CMV-specific IL-7R$^+$ CD4$^+$ T cells were not significantly higher in the no-congenital CMV group than in primary infection group 2 at 24 months after infection, they were higher than that in primary infection group 2 at 12 months after infection (p=0·0006; appendix p 13).

Discussion

Although generally protective, maternal immunity does not always prevent congenital CMV, either in cases of reactivation of the latent virus or reinfection with a distinct strain. Results of the present study show the differences in anti-human CMV antibodies and T cells observed in women with preconception immunity in cases of congenital CMV or no-congenital CMV infection. Mothers of newborns or fetuses with congenital CMV infection had a higher serum neutralising activity, lower capacity of antibodies to induce ADCC, and lower percentage of long-term memory (IL-7R$^+$ and IL-2$^+$) human CMV-specific T cells than did mothers in the no-congenital CMV group. Despite having preconception immunity, women in the congenital CMV group had human CMV-specific antibody and T-cell features similar to those of pregnant women with human CMV primary infection.

The immune response in women with preconception immunity and intrauterine human CMV transmission has been scarcely investigated in the past, and the few available studies are limited to the antibody response. Two studies conducted in Alabama (USA) and São Paulo (Brazil) suggested that reinfection with a distinct strain might have a major role in these populations.[9,18] However, immune factors associated with the higher susceptibility to maternal reinfection and subsequent fetal infection were not investigated. A more recent study conducted on the highly seroprevalent population of São Paulo (Brazil) did not find significant differences in the titres of trimer-specific and pentamer-specific neutralising antibodies in mothers for whom vertical transmission of human CMV to the fetus did versus did not occur.[19] In the present study, we observed even higher serum neutralising titres in the congenital CMV group compared with the no-congenital CMV group. Conversely, a previous study[20] suggested a protective role against congenital CMV of rapid development of antibodies against neutralisation sites of the pentamer complex after primary infection. It is possible that anti-pentamer antibodies might confer protection with mechanisms other than neutralisation, or that the immune responses that

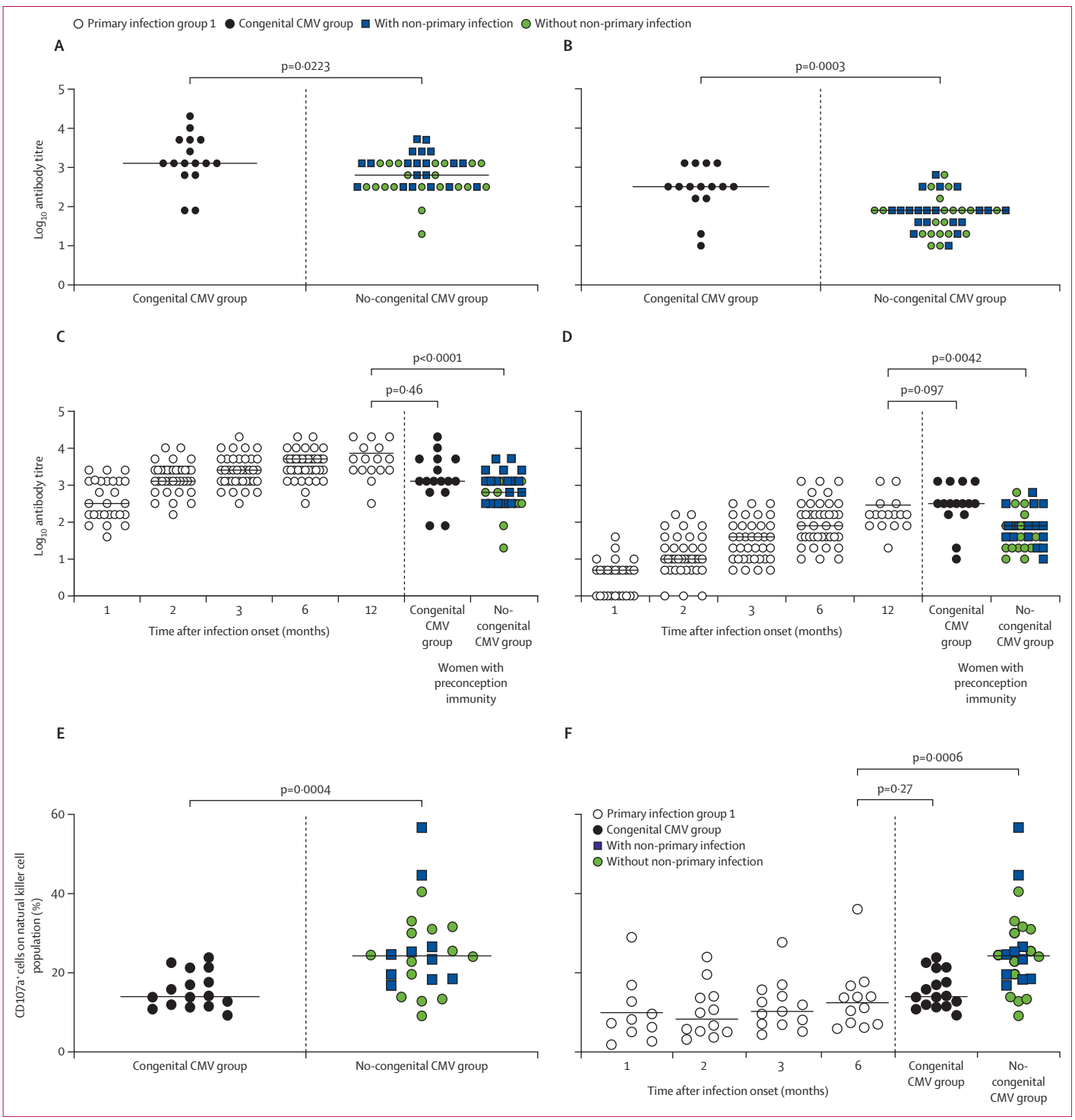

Figure 2: **Comparison of serum antibody titres neutralising human CMV infection**

Serum antibody titres neutralising human CMV infection in (A) human retinal pigmented epithelial (ARPE-19) or (B) HELF cells between immune women in the congenital CMV group or in the no-congenital CMV group tested at delivery. Serum antibody titres neutralising human CMV infection of ARPE-19 (C) or HELF (D) cells in women in primary infection group 1, compared with women in the congenital CMV group or no-congenital CMV group. Comparison of CD107a expression by anti-human CMV serum-activated natural killer cells (E) between women in the congenital CMV group and no-congenital CMV group tested at delivery. CD107a expression by anti-human CMV serum-activated natural killer cells (F) in women in the primary infection group 1, compared with women of the congenital CMV group or no-congenital CMV group. p values were calculated with the Mann–Whitney U test (correction for multiple comparisons was used in panels C, D, and F) and only the p value relevant to the comparison of the last timepoint of primary infection group 1 is shown; p values for the other timepoints are specified in the appendix (p 12). CMV=cytomegalovirus. HELF=human embryonic lung fibroblast.

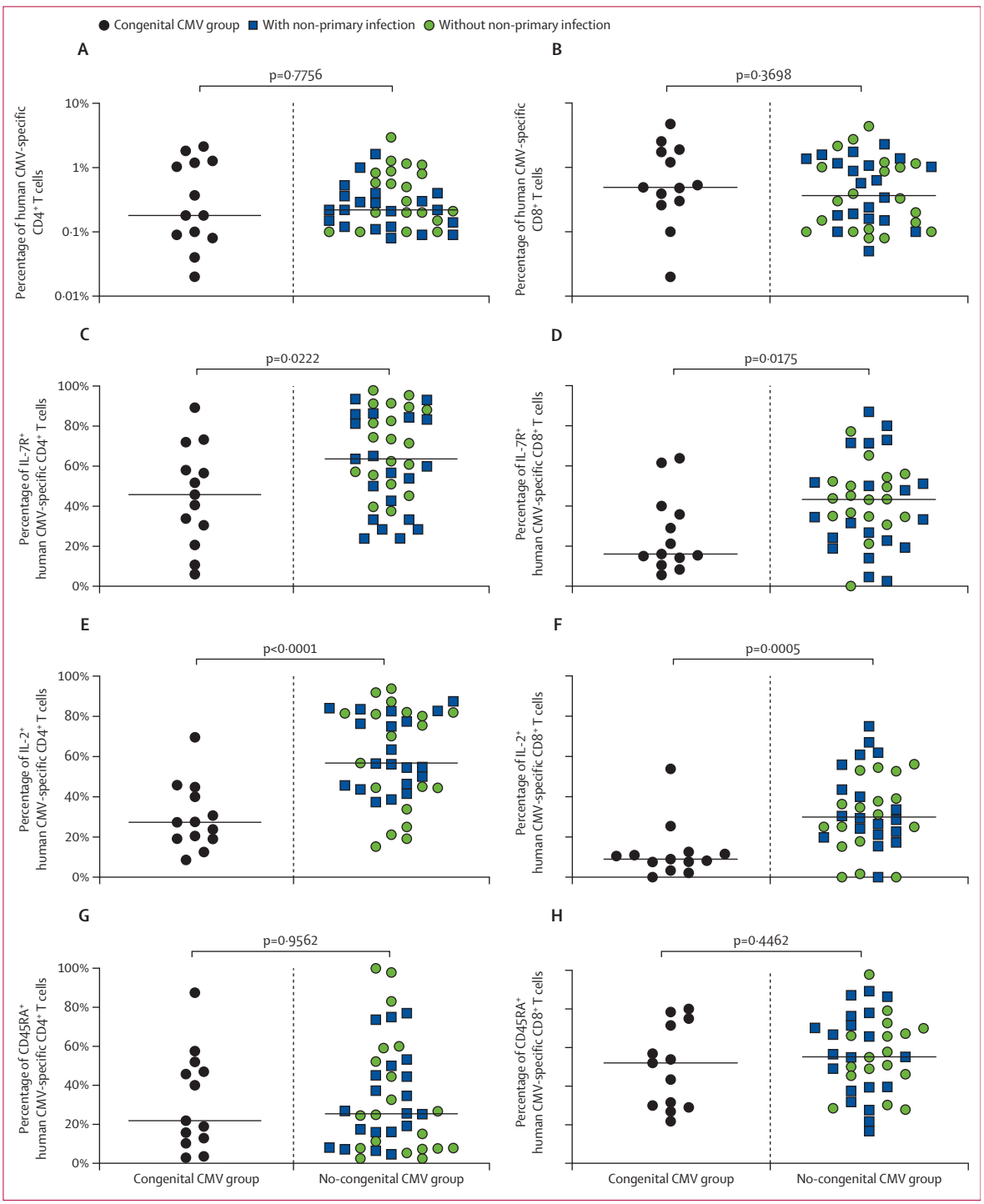

Figure 3: **Comparison of human CMV-specific T-cell response between women in the congenital CMV group or the no-congenital CMV group tested at delivery**
The frequency of human CMV-specific $CD4^+$ and $CD8^+$ IFN-γ^+ T cells (A, B), IL-$7R^+$ (C, D), IL-2^+ (E, F) and $CD45RA^+$ (G, H) was reported. Statistical analysis was performed with the Mann–Whitney U test. CMV=cytomegalovirus. IFN=interferon. IL=interleukin.

protect against congenital CMV after primary infection and non-primary infection are different. Our results suggest that the serum neutralising antibodies do not have a major role in protection from fetal infection in immune pregnant women. Lack of correlation between serum neutralising titres and protection from human CMV was also observed in the transplantation setting.[21]

Although the neutralising capacity of antibodies does not appear to be associated with protection from fetal infection in immune pregnant women, the ability of antibodies to

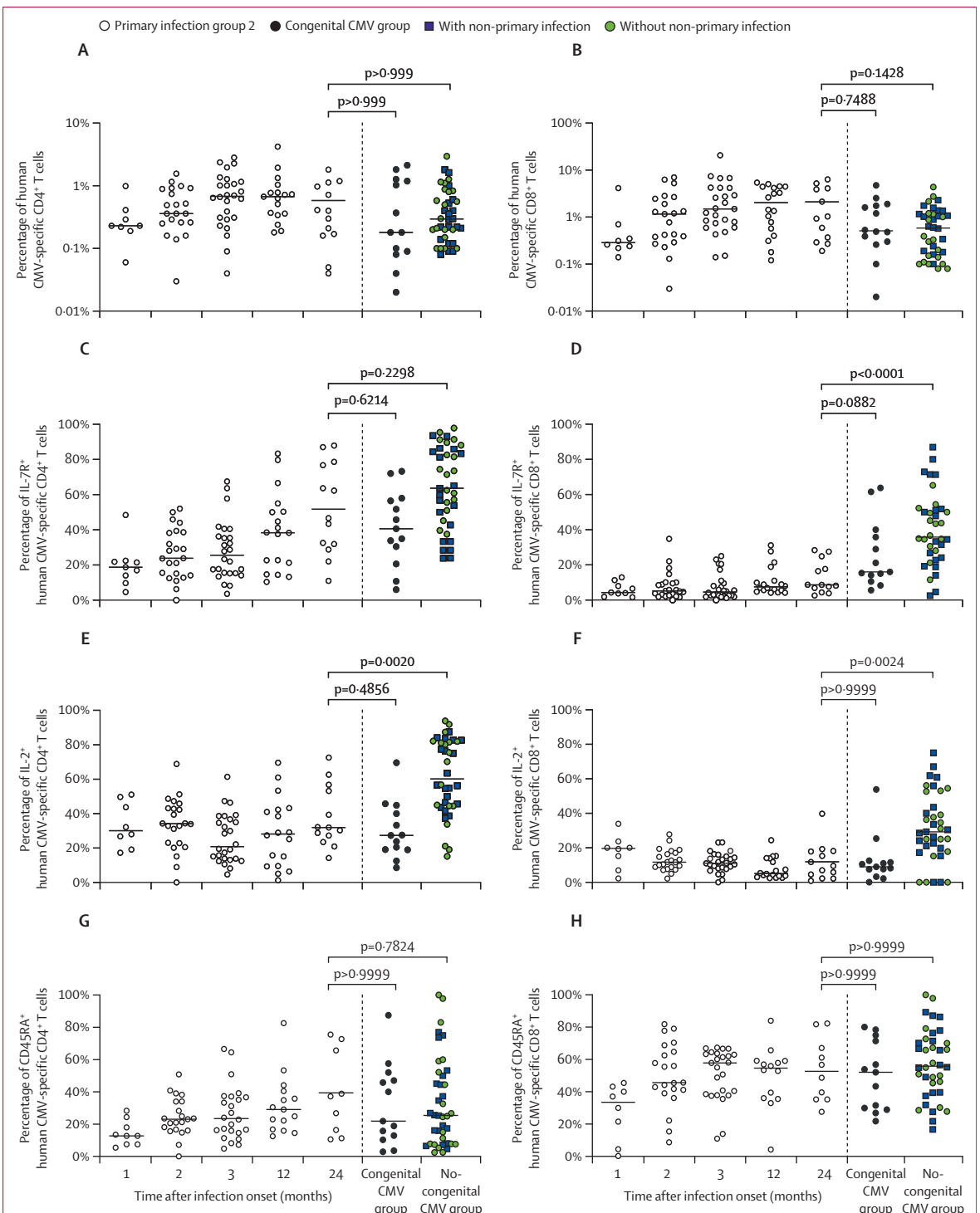

Figure 4: **Human CMV-specific T-cell response in women in primary infection group 2 compared with in the congenital CMV group or in the no-congenital CMV group tested at delivery**

Percentage of human CMV-specific $CD4^+$ and $CD8^+$ IFN-γ^+ T cells (A, B), IL-$7R^+$ (C, D), IL-2^+ (E, F), and $CD45RA^+$ (G, H). p values were calculated with the Mann–Whitney U test (correction for multiple comparisons was used) and only the p value relevant to the comparison of the last timepoint of primary infection group 2 is shown; p values for the other timepoints are specified in the appendix (p 13). CMV=cytomegalovirus. IFN=interferon. IL=interleukin.

activate natural killer cells and, in turn, induce ADCC, does. A potential role of non-neutralising Fc-mediated antibody functions (eg, ADCC) in protection against fetal infection has been suggested by two studies,[22,23] which also reported higher serum neutralising titres in mothers of newborns with congenital CMV. However, these studies did not identify maternal human CMV primary infection versus non-primary infection or the timing of maternal infection. Therefore, the differences observed might have been biased by a higher rate of primary infection among women in the congenital CMV group. Conversely, we analysed women with serological evidence of preconception immunity,[6] showing that women with preconception immunity in the congenital CMV group have antibodies with lower capacity to activate natural killer cells than women in the no-congenital CMV group. The higher protective effect of ADCC versus neutralisation could be explained by the fact that human CMV dissemination is mainly cell associated, thus evading the neutralising activity of antibodies.[24] Therefore, immune functions controlling cell-associated viruses are likely to have a major role in protection from fetal infection. Different studies in relevant models, such as rhesus macaque, have highlighted the importance of maternal antibodies in protection from vertical transmission.[25,26] Nelson and colleagues showed how pre-existing maternal humoral immunity might affect systemic and intrauterine rhesus CMV replication, thereby influencing the severity of congenital infection, not excluding the role of non-neutralising antibodies.[25]

Corrales-Aguilar and colleagues[27] and Kolb and colleagues[28] showed that human CMV avoids Fcγ receptor activation by concomitant expression of viral Fcγ-binding glycoproteins such as gp34 and gp68. This finding indirectly highlights the relevance of Fc receptor-mediated immune responses by showing a selection pressure that led to the evolution of such viral immune antagonists.[27,28] In terms of human CMV vaccine development, our data suggest that neutralising antibodies do not represent a correlate of protection from congenital CMV in women with preconception immunity, whereas ADCC-eliciting antibodies might do.

Regarding the T-cell immunity, we did not find a significant difference in the total concentration of circulating human CMV-specific T cells between women in the congenital CMV group and no-congenital CMV group. Conversely, the percentages of both IL-7R$^+$ and IL-2$^+$ CD4$^+$ and CD8$^+$ T cells (ie, T cells with long-term memory characteristics) were lower in women from the congenital CMV group than in those from the no-congenital CMV group. The low frequency of virus-specific long-term memory T cells is a hallmark of people with human CMV primary infection, whereas people with remote infection (ie, who were infected since more than 2 years before) are characterised by a higher frequency of long-term memory cells, especially among the CD4$^+$ subset.[11,13] Interestingly, we observed that differentiation of human CMV-specific memory T cells is a slow process that requires several months or even a few years.[29] Futhermore, human CMV replication and shedding in bodily fluids persists for at least 1 year after primary infection.[30] The establishment of long-term memory T cells is associated with the extinction of virus replication, whereas short-term effector cells are destined to die after antigen clearance.[14,31]

Our data do not provide evidence that long-term memory are protective per se, but rather suggest that the establishment of a high abundance of long-term memory cells indicates that a sustained control of human CMV systemic dissemination has been achieved, resulting in a negligible risk for fetal infection. Women in the no-congenital CMV group had high concentrations of long-term memory T cells similar to women with remote infection, whether or not they had detectable human CMV replication at local body sites. This finding indicates that peripheral human CMV replication might occur regardless of the establishment of high long-term memory frequency. During primary infection, the percentage of IL-7R$^+$ long-term memory T cells correlates inversely with viral load in blood but not in peripheral bodily fluids,[30] supporting the possibility that this T cell population is associated with control of systemic virus dissemination rather than control of replication at peripheral sites.

It is well known that the risk of human CMV transmission to the fetus in women with a primary infection is around 30%, whereas it is about 100 times lower (0·2–0·5%) in women with previous infection;[5–7] the risk is estimated to be <3·5% in women with previous infection and signs of human CMV replication (ie, women with non-primary infection).[2–4] Based on the data on the immune response observed in women in the congenital CMV group, no-congenital CMV group, and primary infection groups 1 and 2, we speculate that women with immunity who are at risk for viral transmission to the fetus might be those who had a human CMV primary infection within a few years before pregnancy (likely <5 years before). In these women, human CMV replication has not definitively been controlled and specific immunity is not completely developed. This hypothesis is based on the following observations: (1) pregnant women with fetal infection despite preconception immunity have a human CMV-specific antibody and T-cell response similar to that observed in pregnant women with primary infection, (2) pregnant women with preconception immunity and no fetal infection show a fully developed human CMV-specific immune response, and (3) the time period required for the appearance of a fully developed immunity is of several months or a few years (likely 2–5 years).

In support of this hypothesis, a study investigating the risk of congenital CMV in women who seroconverted before pregnancy showed that this risk is increased even when maternal primary infection occurred months and perhaps few years before conception.[32]

To our knowledge, this is the first study to comprehensively analyse the human CMV-specific antibody and T-cell responses in pregnant women with preconception immunity and intrauterine human CMV transmission, in

comparison with a control group. Limitations of the study include the quite small sample size and the retrospective nature of the analysis; therefore, it is not possible to precisely define the prevalence of immune women without a fully developed immune response to human CMV at the beginning of pregnancy or their risk for congenital CMV. Other parameters that should be analysed to improve the characterisation of protective immunity are the frequency of cytolytic T cells and other Fc-mediated antibody functions, such as antibody-dependent cell phagocytosis[21] and complement deposition. Additionally, the study of maternal immunity and congenital CMV should be extended to other geographical areas and populations in different socioeconomic settings or with genetic backgrounds, to validate our results and assess their generalisability.

Nevertheless, our data indicate that, at least in a European, primarily White population, a fully developed maternal immune response to human CMV, characterised by a high frequency of long-term memory T cells and antibodies capable of inducing ADCC, confers protection from viral transmission to the fetus, despite the potential occurrence of non-primary infection during pregnancy. Therefore, a vaccine able to confer such an immunity to the mother is likely to be protective for the fetus. Although studies on maternal immunity and congenital CMV in low-income countries with high seroprevalence rates will be necessary, our data suggest that immunological endpoints for human CMV vaccine evaluation should not be limited to neutralising antibodies, but should comprise T-cell immunity, with memory subsets analysis, and Fc-mediated antibody functions, such as ADCC.

Contributors

PZ and PdA verified and analysed the data and drafted the manuscript; PdA and CF performed serological analyses; PZ, PdA, FZ, and CF performed immunological analyses; CA, BT, AR, LPu, SO, PIC, MC, EF, AL, SC, SR, EM, VS, MDG, FP, LPe, and CP enrolled participants and collected data; MF, AA, and AS performed clinical follow-up; DL conceived the study and drafted the manuscript; AS and FB supervised the study; all authors had full access to all the data in the study and accept responsibility for the decision to submit for publication. All the authors critically revised the manuscript.

Declaration of interests

We declare no competing interests.

Data sharing

The data that support the findings of this study (immunoassays raw data) are available on request from the corresponding author after ethics assessment. The data are not publicly available due to privacy or ethical restrictions.

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