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Cytomegalovirus (CMV) seropositivity decreases B cell responses to the influenza vaccine

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Abstract

Cytomegalovirus (CMV)-seropositivity has been shown to have a negative effect on influenza vaccine-specific antibody responses. In this paper we confirm and extend these results showing for the first time a negative association between CMV-seropositivity and B cell predictive biomarkers of optimal vaccine responses. These biomarkers are switched memory B cells and AID in CpG-stimulated B cell cultures measured before vaccination which positively correlate with the serum response to the influenza vaccine. We also found that CMV-seropositivity is associated with increased levels of B cell-intrinsic inflammation and these both correlate with lower B cell function. Finally, CMV-seropositivity is associated with decreased percentages of individuals responding to the vaccine in both young and elderly individuals.

Keywords

Aging; CMV; influenza vaccine; B cell biomarkers

1. Introduction

Aging represents a complex remodeling in which innate and adaptive immune responses deteriorate, leading to greater susceptibility to infectious diseases and reduced responses to vaccination [1]. Aging is characterized by increased levels of circulating pro-inflammatory cytokines, such as TNF- α and IL-6 [2–4], which have been associated with functional disability/mortality of the elderly. The age-related increase in inflammation is called “inflammaging” [5].

Humoral and cellular immune responses are impaired in aged individuals, leading to decreased antibody responses to vaccination and reduced specific titers. Although defects in

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T cells [6, 7] and antigen-presenting cells [8, 9] occur, we [10, 11] and others [12, 13] have shown that intrinsic B cell defects occur in aging. The B cell defects we have identified include a reduction in activation-induced cytidine deaminase (AID), the enzyme necessary for class switch recombination (CSR) and somatic hypermutation and the ability to generate optimal switched memory B cells as well as high affinity antibodies. AID and switched memory B cells, both measured before vaccination, positively correlate with the serum response and therefore are good predictors of the response to vaccines and infectious agents in humans [14–18].

Cytomegalovirus (CMV) is a β -herpes virus, which latently infects a large proportion (40–70%) of the human population and this proportion increases with age [19]. The infection is asymptomatic in immunocompetent individuals, but may cause severe diseases in immunocompromised hosts. CMV has been postulated to be one of the major driving forces of immunosenescence. CMV infection is associated with premature mortality and is a component of the Immune Risk Phenotype, which predicts remaining longevity in the very elderly [4]. CMV induces the production of a variety of pro-inflammatory mediators which in turn induce CMV reactivation [20, 21]. It has been shown that CMV-positive elderly have higher levels of inflammatory mediators than CMV-positive young, may be due to a better control of inflammation by the immune system of young individuals [22].

The CMV-induced changes in vaccine responses of elderly individuals are well documented for T cells [6, 23], but little is known about how CMV infection affects B cell responses. In the present study, we measured CMV effects on in vivo/in vitro B cell responses to the seasonal influenza vaccine and found a significant negative association of CMV-seropositivity with these responses.

2. Materials and methods

2.1. Subjects

Experiments were conducted using blood from 62 healthy individuals, 36 young (20–59 years) and 26 elderly (≥ 60 years), after signed informed consent and were approved with IRB protocol #20070481. We have previously shown [14, 16–18] that those aged ≥ 60 had similar characteristics for the markers we measure (AID). We have also previously shown no gender differences in either the young or elderly groups. The individuals participating in the study were screened for diseases known to alter the immune response or for consumption of medications that could alter the immune response. In particular, the following categories were excluded: diabetes, malignancies, Congestive Heart Failure, Cardiovascular Disease, Chronic Renal/Hepatic Failure, autoimmunity, infectious diseases, recent (<3 mo) trauma or surgery, pregnancy, documented current substance and/or alcohol abuse. Each individual was influenza-free at the time of blood draws.

2.2. Influenza vaccination

The study was conducted during the 2011–2012 seasonal influenza vaccination. Two Trivalent Inactivated Vaccines (TIV) were used: Novartis Fluvirin and GSK Fluarix, which were given in two different centers at UM. Both vaccines gave similar results. Blood samples were collected before (t0), 1 week (t7) and 4–6 (t28) weeks after vaccination. The

2011–2012 vaccine contained the following viral strains: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008. All of our subjects were previously immunized, and therefore seroprotected at t0. The peak of the response was at t7 [15], earlier than what we [14, 17] and others [24] previously found, perhaps due to the repeated vaccine immunization. In most cases, peak titers were maintained through t28.

2.3. Hemagglutination inhibition (HAI) assay

We evaluated the response to the purified proteins from pH1N1, H3N2 and B, provided from Novartis (Siena, Italy). The response was measured by HAI assay and results expressed as fold-increase in the reciprocal of specific titers [14–18, 25].

2.4. Enzyme-linked immunosorbent assay (ELISA)

Plasma TNF- α , IL-6, CRP, LPS and soluble (s)CD14 were measured by ELISA (Life Technologies KHC3013, KHC0062, KHA0032; Lonza QCL-1000; and R&D Systems DC140). CMV-seropositivity was measured in plasma using a CMV IgG ELISA from IBL International (RE58311).

2.5. B cell isolation

PBMC were collected by density gradient centrifugation using Vacutainer CPT tubes which contain sodium heparin (BD 362761). B cells were isolated from PBMC with anti-CD19 Microbeads (Miltenyi Biotec), briefly by incubation for 20 min at 4°C with 20 μ l of beads/ 10^7 cells. Cells were then purified using magnetic columns. After isolation, cells were maintained in serum-free medium for 1 hr at 4°C to abolish potential effects of anti-CD19 antibodies on B cell activation. The main reason to use positive selection is because B cell purity is higher (>90%). We have previously compared positively- and negatively-selected B cells and shown that positive selection does not interfere with activation, as cells without stimulus show no phosphorylation in western blot experiments (not published). In addition, in the experiments herein, we stimulate B cells for 5 days. Because magnetic sorting is performed at 4°C in PBS, we also abolish the effects of anti-CD19 antibodies on B cell activation.

2.6. B cell culture

B cells were cultured in complete medium (RPMI 1640, supplemented with 10% FCS, 10 μ g/ml gentamicin, 2×10^{-5} M 2-ME, and 2 mM L-glutamine) and stimulated for 7 days in 24-well culture plates with 1 μ g/ 10^6 cells of CpG (ODN 2006 In Vivogen). At the end of this time, mRNA was extracted for quantitative (q)PCR.

2.7. RNA extraction and quantitative (q)PCR

The μ MACS mRNA isolation kit (Miltenyi Biotec) was used. The mRNA was extracted from CpG-stimulated B cells to evaluate AID. Reactions were conducted in MicroAmp 96-well plates (Applied Biosystems, ABI N8010560) and run in the ABI 7300 machine. Reagents and primers (Taqman) were from Life Technologies.

2.8. Flow cytometry

One hundred μ l of blood were stained for 20 min rt with anti-CD19 (BD 555415), anti-CD27 (BD 555441), anti-IgD (BD 555778) to measure switched memory B cells (IgD-CD27+). After staining, red blood cells were lysed using the RBC Lysing Solution (BD 555899). For intracellular staining of TNF- α , after membrane staining with anti-CD19, cells were fixed, permeabilized with 1X-PBS/0.2% Tween-20 and incubated with anti-TNF- α (BD 554512). Up to 10^5 events in the lymphocyte gate were acquired on an LSR-Fortessa (BD) and analyzed using FACS Diva (BD) software. Single color controls were included in every experiment for compensation.

2.9. Statistical analyses

Non-parametric analyses of the variables were performed by Mann-Whitney test (two-tailed); correlations were performed by Spearman's tests, using GraphPad Prism 5 software.

3. Results

3.1. Study population

Demographic characteristics of the individuals participating in the study are in Table 1. Pro-inflammatory status of these individuals is also in Table 1. We have measured plasma levels of the following markers of inflammation: TNF- α , IL-6, CRP, Hsp60, Leptin. Results show increased levels of these in CMV-positive elderly individuals only. We also measured plasma LPS and its ligand, sCD14, which indicate microbial translocation following intestinal permeability [26] and found increased levels of LPS and sCD14 only in CMV-positive and not in CMV-negative individuals.

3.2. The serum response to the influenza vaccine is decreased in both young and elderly CMV-positive individuals

We have previously reported an age-related impairment in the serum antibody response to pandemic pH1N1, measured by HAI assay [15, 17], which is a correlate for vaccine protection. During the 2011–2012 influenza vaccine season, we tested the serum response to the purified proteins from the 3 viral strains present in the vaccine preparation: H1N1, H3N2 and B. In this study, we only present data on H1N1 because the response to H3N2 and B was much lower and we wanted to measure the memory response to the H1N1 antigen given the third time. In particular, we present here the effects of CMV-seropositivity on the serum antibodies to the pH1N1 strain present in the vaccine.

Results in Fig. 1A show that CMV-seropositivity is significantly associated with decreased in vivo response to the pH1N1 in both young and elderly individuals. The difference between CMV-seronegative young and elderly in fold-increase shows a trend ($p=0.05$) which is less significant from what we have seen in previous years, due to repeated immunizations with H1N1. The peak of the response was anticipated (t7) as compared to what is usually seen (t28) because of repeated immunizations with a vaccine containing the pandemic (p)2009 H1N1 strain for the third consecutive year. Results obtained at t28 showed the same correlations shown here for t7 (not shown). For the same reason, all individuals were seroprotected at t0. This is why we do not observe significant differences at

t0 between young and elderly individuals (both CMV– and CMV+). Fig. 1B shows that after vaccination the titers are not significantly different between young and elderly individuals due to the high heterogeneity in the response, especially in CMV-seronegative, but the fold-difference is significant in the young from t0 to t7. We have previously observed great heterogeneity in young individuals also and we have seen that the lower response is associated with increased inflammation [25].

3.3. Switched memory B cells, which predict good serum antibody responses, are also decreased in both young and elderly CMV-positive individuals

Vaccine-specific serum antibodies are a proven correlate of protection for influenza infection although their effectiveness in older adults has been questioned [27]. We have previously shown that the percentage of switched memory B cells at t0 is correlated with the serum response and therefore is a valid B cell biomarker able to predict optimal serum antibody responses [14, 17]. Results in Fig. 2A show that the percentage of these cells at t0 is significantly decreased in both young and elderly CMV-positive individuals. There is a strong effect of age with the elderly having fewer switch memory B cells as we have previously shown [11, 14–17], and therefore the effect of CMV-seropositivity on B cell biomarkers of the influenza-specific antibody response to vaccination appears to also decline with age. The percentage of switched memory B cells at t0 in these subjects is also correlated with the in vivo response to the vaccine (Fig. 2B). The differences between results in Fig. 1A and 2A, i.e. different effects of age versus CMV, may be explained as follows: 1) switched memory cells represent many specificities and not just those toward flu but they do give a read-out of the quality of B cell function; 2) the H1N1 antibody response is derived not only from flu-specific switched memory B cells but also from plasma cells residing in the bone marrow, i.e. there are different contributors to these measures, although we have shown that these 2 measures are also correlated (Fig. 2B).

3.4. AID, which also predicts good serum antibody responses, is decreased in both young and elderly CMV-positive individuals

The enzyme AID is another B cell-specific biomarkers which we have shown can be used to predict the in vivo response to the influenza vaccine, because it completely correlates with the ability of human B cells to undergo CSR [14, 17] and affinity maturation [18] of Ig genes. We measured AID mRNA expression induced by CpG in the same individuals as above. The CpG response was measured at t0, as we have shown that this can predict the robustness of in vivo responses [14, 16, 17]. Results in Fig. 3A show that AID at t0 is significantly decreased in both young and elderly CMV-positive individuals and significantly correlated with the in vivo response (Fig. 3B).

3.5. CMV-seropositivity is associated with increased levels of intracellular TNF- α in B cells at t0

To identify a mechanism explaining CMV effects on B cell function, we have correlated serum CMV IgG levels with serum TNF- α and found a significant positive correlation (Fig. 4A). We also found a significant positive correlation between serum TNF- α and icTNF- α measured in unstimulated B cells (Fig. 4B). Representative histograms of icTNF- α staining, one per group of individuals, are shown in Fig. 4C. We have previously shown that

unstimulated, ex vivo isolated B cells make detectable amounts of intracellular TNF- α and more in elderly as compared to young individuals and this correlates with reduced in vitro AID activation [16, 25], and in vitro treatment of B cell cultures with an anti-TNF- α antibody increases AID and CSR [25]. We here show that CMV-seropositivity is associated with increased levels of icTNF- α in B cells of both young and elderly individuals at t0 (Fig. 4D). Both serum TNF- α (Fig. 4E) and icTNF- α (Fig. 4F) negatively impact CpG-induced AID mRNA expression, as we have already demonstrated in both mice [28] and humans [16, 25]. These results altogether suggest that systemic inflammation induces TNF- α production by B cells, more in CMV-positive than in CMV-negative individuals and this suggests a possible mechanism responsible for the lower in vivo and in vitro B cell responses to the vaccine. Levels of icTNF- α are not correlated with the in vivo response measured by HAI, we hypothesize because there are many factors operating in vivo including T cell functions. We are aware that T cells are also targets of systemic TNF- α , but we have clearly demonstrated that intrinsic B cell defects occur [11]. In addition, the lack of correlation between icTNF- α and the in vivo response may be due to a direct effect of systemic inflammation (high levels of CRP/TNF- α /IL-6 and low levels of IL-10) on B cell response.

3.6. B cell and vaccine responses are affected by CMV-seropositivity in young and elderly individuals

In order to correlate the effects of CMV-seropositivity on the influenza vaccine response, and B cell markers of response, we calculated the percentages of both young and elderly responders, which were defined as those who had HAI ≥ 4 , switched memory B cell percentages at t0 ≥ 2 , CpG-AID qPCR at t0 ≥ 0.1 and icTNF- α at t0 ≤ 4 . We propose to include icTNF- α as a measure of B cell response because it negatively correlates with AID.

Results in Fig. 5 and Table 2 show that the immune response of the elderly as measured by HAI, switched memory B cell percentages, CpG-AID and icTNF- α , is also dramatically affected by CMV+. If we look at the absolute HAI response rates to vaccination between CMV+ versus CMV- groups, which in young is 67% in CMV- and 15% in CMV+ and in elderly is 40% in CMV- and 12.5% in CMV+, one could then conclude that CMV status has a greater impact on response rates in young compared to older adults.

4. Discussion

Our knowledge to date about the effect of CMV on B cells is virtually nil. We here show a negative association between CMV-seropositivity and the B cell predictive biomarkers of optimal vaccine responses, switched memory B cells and AID [14, 16, 17]. Another predictive B cell biomarker is TNF- α . Here we show that the levels of intracellular TNF- α in B cells at t0 are increased in CMV-positive individuals, either young or old and this pre-activated status of the B cells negatively impacts their function (AID).

CMV-seropositivity is associated with changes in the immune profile of aged individuals [27], including lower naïve and higher memory T cells percentages [29–32], both of which are hallmark of immunosenescence, leading to the increasingly accepted notion that CMV accelerates T cell immunosenescence. In agreement with this, CMV has been associated

with poor humoral response to influenza vaccination in the elderly [23, 33]. In these studies, CMV-seropositivity was associated with CD27-CD28-CCR7-CD45RA+ [23] or with CD28-CD57+ T cells [33], both identified as late differentiated/exhausted T cells, which produce pro-inflammatory cytokines, therefore having a significant role in age-related immune pathologies [34]. The absence of an effect of CMV-seropositivity on the response of young individuals to the vaccine in the study above as compared to ours may be related to differences in the inflammatory profile, in the vaccine used, and/or history of vaccination.

A mechanism through which CMV affects B cell function may be an increase in systemic TNF- α which induces B cell-derived TNF- α . Moreover, TNF- α is a powerful stimulator of the promoter/enhancer of CMV leading to further upregulation/exacerbation of systemic inflammation [21]. This positive feedback loop, perhaps only in part due to CMV, drives inflammaging more effectively in elderly as compared to young individuals, causing deleterious effects in the immune system of the individual, as we have summarized in Fig. 5. Therefore, just modifying the direct effect of CMV on B cell function would not “fix” the effect of aging on seroprotection rates.

In addition to the mechanism above, other mechanisms through which CMV may down-regulate the antibody response to the influenza vaccine in elderly individuals have been proposed. These include the induction of terminally differentiated T cells and consequent accumulation of senescent T cells [23, 33], leading to reduced stimulation of memory T cells and poorer responses to influenza vaccination in elderly individuals [30, 35].

One of the limitations of the present study is the relative small number of individuals recruited, especially the CMV-positive individuals, and we do not know how long they have been positive. This is important to know, as it is not clear whether inflammation correlates with length of the time that an individual has been CMV-positive, and results in the literature have shown that the age-related increase in chronic systemic inflammation over a 10 year period is not driven by CMV infection, indicating that CMV infection is not a primary causative factor in the age-related increase in inflammaging [36]. Another limitation is that we have not measured the impact that other latent viral infections (EBV/VZV) may have on the inflammatory profile of the CMV-negative participants, although several studies have suggested that the presence of these viruses does not have a significant impact on age-related changes in T cell function.

In conclusion, our results are in line with the previously described impact of persistent CMV infection on humoral antibody responses in elderly individuals and show for the first time a negative association between CMV-seropositivity and B cell predictive biomarkers of optimal vaccine responses.

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Highlights

Influenza vaccination is less effective in CMV-seropositive individuals

B cell biomarkers of optimal vaccine responses are reduced by CMV-seropositivity

CMV-seropositivity is associated with increased intrinsic inflammation in B cells

Fig. 1A

Fig. 1B

Figure 1. The decreased serum response to the influenza vaccine is associated with CMV-seropositivity in both young and elderly individuals

Sera isolated from young and elderly individuals, before (t0) and after vaccination (t7), were collected and analyzed by HAI assay to evaluate antibody production to vaccine. **A.** Results are expressed as fold-increase in the reciprocal of the titers after vaccination, calculated as follows: reciprocal of titer values after vaccination/ reciprocal of titer values before vaccination. A value of 4 or higher is defined as positive for seroconversion. Mean comparisons between groups were performed by Mann-Whitney test (two-tailed). **B.** Results are expressed as reciprocal of the titers before and after vaccination. Mean comparisons between groups were performed by Student's t test (two-tailed). The geometric means of the reciprocal of the titers at t0 and t7 are respectively: 98 and 198 (young CMV+); 61 and 230 (young CMV-); 80 and 104 (elderly CMV+); 49 and 160 (elderly CMV-). The difference between t0 values between CMV+ and CMV- is not significant in both young (p=0.17) and elderly (p=0.15) individuals.

Fig. 2A

Fig. 2B

Figure 2. Switched memory B cell percentages at t0 are decreased in both young and elderly CMV-positive individuals and are positively correlated with the serum response

A. One hundred μ l of blood at t0 were stained as described in Materials and methods.

Results are expressed as percentages of CD19+ B cells. Mean comparisons between groups

were performed by Mann-Whitney test (two-tailed). **B.** Switched memory B cell percentages at t0 and the in vivo response (HAI) are positively correlated. The correlation was performed by Spearman's test ($r=0.45$, $p=0.0005$).

Fig. 3A

Fig. 3B

Figure 3. AID mRNA expression in response to CpG is also decreased in both young and elderly CMV-positive individuals and is positively correlated with the serum response

A. B cells (10^6 cells/ml), isolated from fresh PBMC at t0, from the same subjects as Fig. 2 were cultured with CpG, for 3 days. At the end of this time, cells were processed as described in Materials and methods. Results are expressed as raw qPCR values of AID mRNA normalized to GAPDH. Mean comparisons between groups were performed by Mann-Whitney test (two-tailed). **B.** CpG-AID at t0 and the in vivo response (HAI) are positively correlated in all subjects. The correlation was performed by Spearman's test ($r=0.44$, $p=0.0033$).

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4D

Fig. 4E

Fig. 4F

Figure 4. CMV-seropositivity is associated with increased levels of intracellular TNF- α in B cells of young and elderly individuals at t0 and B cell-derived TNF- α levels correlate with lower B cell response to CpG

A. CMV IgG levels and serum TNF- α were measured by ELISA. Correlations were performed by Spearman's test ($r=0.30$, $p=0.01$). **B.** ELISA (to measure serum TNF- α) and flow cytometry (to measure icTNF- α) were performed as in experimental procedures. Correlations were performed by Spearman's test ($r=0.51$, $p<0.0001$). **C.** Representative histograms of icTNF- α staining are shown, one per group of individuals. One hundred μ l of blood at t0 were stained as described. Results are expressed as percentages of CD19+ B cells. Isotype controls (shaded) are set up to be 1–2% positive. **D.** Mean comparisons between groups were performed by Mann-Whitney test (two-tailed). **E.** CpG-AID at t0 and serum TNF- α levels are negatively correlated. The correlation was performed by Spearman's test ($r=-0.3800$, $p=0.0100$). If the top 2 points for serum TNF- α are removed, significance is maintained ($r=-0.3796$, $p=0.0121$). **F.** CpG-AID at t0 and icTNF- α levels at t0 are negatively correlated. The correlation was performed by Spearman's test ($r=-0.51$, $p<0.0007$).

Figure 5. CMV-seropositivity significantly decreases the percentage of responders in young and more in elderly individuals

Responders are defined as individuals who had HAI 4, CpG-AID qPCR at $t_0 - 0.1$, switched memory B cell percentages at $t_0 - 2$ and icTNF- α at $t_0 - 4$. The difference between CMV-seronegative and CMV-seropositive is significant in both young ($p=0.045$) and elderly ($p<0.0001$) individuals. The difference between young and elderly both CMV-seronegative and CMV-seropositive is also significant ($p<0.001$).

Table 1

Demographics and plasma inflammatory profile of the individuals participating in the study

	Young ^a		Elderly ^a	
	CMV-negative	CMV-positive	CMV-negative	CMV-positive
Number	22	14	10	16
Age in years (range)	41 (21–57)	44 (25–58)	67 (62–75)	66 (60–80)
Gender (M/F)	8/14	3/19	4/6	6/10
Race (W/B)	21/1	12/2	9/1	14/2
Ethnic groups ^b (Hisp/Non Hisp)	12/10	6/8	5/5	9/7
BMI (mean Kg/m ² ±SE)	28±1	25±1	26±1	24±1
TNF-α (pg/ml)	3.4±0.5	6.1±2.2	5.9±0.5 [#]	23.9±5.1 ^{**}
IL-6 (pg/ml)	47.9±24.1	58.1±25.7	67.7±22.4 [#]	224.3±52.3 [*]
CRP (pg/ml)	647.3±122.8	648.0±132.6	1049.4±390.2 ^{###}	2485.7±445.5 ^{**}
Hsp60 (ng/ml)	6.5±3.9	6.3±2.1	5.4±1.8	17.6±6.9 [*]
Leptin (pg/ml)	785±263	541±128	494±110	1450±131 ^{**}
LPS (pg/ml)	116.6±3.9	121.1±5.5	104.4±6.7	135.8±6.9 ^{**}
sCD14 (μg/ml)	0.8±0.3	0.8±0.4	0.8±0.3	1.3±0.2 ^{**}

BMI measures are means±SE. Normal BMI is 24.9.

^aPrevalence of CMV-seropositivity was higher in elderly as compared to young individuals (p<0.05). All subjects were CMV IgM-negative, which indicates chronic CMV infection and no viral reactivation.

^bAll races. Hispanic are individuals from Mexico, Puerto Rico, Cuba, Central/South America and from other countries with Spanish culture or origin.

All plasma inflammatory cytokine results are means±SE. Normal plasma levels of TNF-α, IL-6, CRP, Hsp60, Leptin, LPS and sCD14 are, respectively: 3–10 pg/ml, 30–60 pg/ml, 800 pg/ml, 3–88 ng/ml, 70–110 pg/ml and 0.8–3.2 μg/ml.

^{**}(p<0.01) and

^{*}(p<0.05) refer to differences between CMV-negative and CMV-positive individuals with their age groups.

^{###}(p<0.01) and

[#](p<0.05) refer to differences between Young and Elderly CMV-negative individuals.

Table 2

Number of individuals with a responding phenotype in the different measures

	Young		Elderly	
	CMV-negative	CMV-positive	CMV-negative	CMV-positive
HAI ^a	14/22	2/13	4/10	2/16
Switched memory B cells ^b	20/22	6/14	2/10	1/16
AID ^c	11/15	6/10	3/8	0/11
B cell icTNF- α ^d at t0 (% 4)	10/22	3/14	0/8	0/14

A responding phenotype is as follows.

^a fold-increase in reciprocal of the titers 4;

^b percentage of switched memory B cells at t0 2;

^c qPCR of CpG-induced AID mRNA expression at t0 0.1;

^d percentage of icTNF- α + B cells at t0 4. The cut-off value for the first 3 measures was calculated based on the characteristics in the majority of the young CMV-seronegative individuals. The cut-off for B cell icTNF- α was calculated based on values obtained in almost all elderly individuals. Numbers of individuals responding in the 3 or 4 measures are as follows. CMV-negative young: 15/22 (68%); CMV-positive young: 3/14 (21%); CMV-negative elderly: 11/10 (10%); CMV-positive elderly: 0/16 (0%).