INFECTIOUS DISEASE

Cytomegalovirus infection enhances the immune response to influenza

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Cytomegalovirus (CMV) is a β-herpesvirus present in a latent form in most people worldwide. In immunosuppressed individuals, CMV can reactivate and cause serious clinical complications, but the effect of the latent state on healthy people remains elusive. We undertook a systems approach to understand the differences between seropositive and negative subjects and measured hundreds of immune system components from blood samples including cytokines and chemokines, immune cell phenotyping, gene expression, ex vivo cell responses to cytokine stimuli, and the antibody response to seasonal influenza vaccination. As expected, we found decreased responses to vaccination and an overall down-regulation of immune components in aged individuals regardless of CMV status. In contrast, CMV-seropositive young adults exhibited enhanced antibody responses to influenza vaccination, increased $CD8^+$ T cell sensitivity, and elevated levels of circulating interferon-γ compared to seronegative individuals. Experiments with young mice infected with murine CMV also showed significant protection from an influenza virus challenge compared with uninfected animals, although this effect declined with time. These data show that CMV and its murine equivalent can have a beneficial effect on the immune response of young, healthy individuals, which may explain the ubiquity of CMV infection in humans and many other species.

INTRODUCTION

Cytomegalovirus (CMV) is a common β-herpesvirus that infects most of the population worldwide. Primary infection often occurs during childhood and induces a strong immune response that, while neutralizing viral spread, does not prevent the virus from persisting in a latent form, defined by a reversibly quiescent state in which viral genomes are maintained, but viral gene expression is highly restricted and no virus is produced (*1*). In immunocompromised individuals, CMV can reactivate and cause serious clinical complications and death (*2*). In healthy individuals, CMV reactivation can also occur during the differentiation of myeloid cells, a reservoir for latent CMV (*3*), causing a state of chronic infection where the virus is persistently shed at low levels and for extended periods of time (*4*). In the immunocompetent host, this state of chronic infection is asymptomatic and is believed to occur intermittently, which appears to account for the substantial changes in the phenotype of T cells observed in the infected individuals (*5*), with up to 10% of the latter being specific for CMV epitopes (*5*–*9*). Because this increase in both $CD4^+$ and $CD8^+$ memory T cells is also generally observed in aged individuals, it has been suggested that CMV promotes accelerated immunological aging (*10*–*13*). However, the T cell compartment comprises only a fraction of the immune response, and very little is known about the effect of CMV on other immune system components. Furthermore, the effect of these changes on immune function remains controversial (*14*, *15*). For instance, in young humans and mice, CMV may improve immune responses to unrelated antigens (*16*–*19*), whereas in older human cohorts, a number of association studies suggest that CMV seropositivity is linked to immune dysfunction and chronic inflammatory diseases including immunosenescence, cancer, cardiovascular disease, atherosclerosis, frailty, and early mortality (*11*, *20*–*31*). These observations suggest that the effect of CMV on the immune system may be highly dependent on an individuals' age.

To study the influence of age and CMV in the immune system in a broad and relatively unbiased fashion, we undertook a systems biology approach where we measured hundreds of immune variables. This type of approach enables comprehensive characterization of biological processes taking into consideration the diversity and interaction of the components involved, and thus, it is often used to study complex systems. To do this, we obtained peripheral blood from 91 young and older individuals and measured the antibody responses to seasonal influenza vaccination, as well as serum cytokines and chemokines, immune cell phenotyping by multiparametric flow cytometry, gene expression, and high-throughput analysis of cell function by determining cellular responses to multiple cytokine stimuli. As expected, we found evidence of decreased immune cell function with age including lower antibody responses to influenza vaccination, but there was no significant effect of CMV status in the older members of the cohort. In contrast, young CMV-infected adults exhibited an overall up-regulation of immune function, including enhanced antibody responses to influenza vaccination, increased $CD8^+$ T cell sensitivity, and elevated levels of circulating interferon-γ (IFN-γ) compared to uninfected individuals. These differences may be unique to CMV, because we did not observe any significant changes in these parameters with Epstein-Barr virus

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(EBV) seropositivity, another lifelong herpes virus infection. In parallel experiments, mice infected with murine CMV (MCMV) showed improved T cell responses to influenza virus challenge and markedly reduced influenza virus titers. This effect was IFN-γ–dependent and declined with time. These data indicate that CMV can boost the immune response in younger individuals and thus has features of a mutualistic agent, that is, one that confers benefits on the host (*32*).

RESULTS

Positive contribution of CMV to immune function in young, but not older adults

To determine the influence of age and CMV in the immune system, we undertook a systems analysis of peripheral blood from 91 young and older individuals from the Stanford-Ellison cohorts (table S1) (*33*–*35*). We focused on immunological parameters representing different layers of the immune system (fig. S1) including the level of soluble cytokines and chemokines, cell subset frequencies and function by measuring the phosphorylation levels of signal transducer and activator of transcription (STAT) proteins in response to multiple stimulations (fig. S2), and genome-wide mRNA expression (gene modules, see http://cs.unc.edu/~vjojic/fluy2-upd/). A gene module corresponds to a set of coexpressed genes sharing transcriptional regulatory programs (*33*, *36*, *37*).

We first determined the changes associated with CMV latency and aging across a total of 236 baseline immune parameters. To do so, we used a classifier to identify features that best separate each of the following categories: young CMV-uninfected (yCMV−), young CMV-infected (yCMV+), older CMV-uninfected (oCMV−), and older CMV-infected (oCMV+) individuals. To minimize false positives and avoid overfitting, we conducted cross-validation, a machine learning procedure that allows for variable selection in an unbiased fashion. We identified a number of features that separate with good accuracy all combinations of groups of individuals, with the exception of oCMV− versus oCMV+ (63% model accuracy compared to 60% baseline accuracy, table S2). This indicates that in older individuals, the effect of CMV is negligible, because oCMV− cannot be distinguished from oCMV+ using the immunological parameters studied here. To isolate the contribution of age or CMV in young individuals, we focused on the comparisons of yCMV− versus yCMV+ individuals (CMV effect independent of age) and yCMV− versus oCMV− individuals (age effect independent of CMV). We also analyzed EBV-infected (EBV+) versus uninfected (EBV−) individuals, but the classification model was unable to distinguish EBV+ from EBV− subjects (the accuracy for the logistic regression model is lower that 50%), and multiple regression analysis (adjusted for age and sex) showed that the most significant EBV-related immune feature is only detected at a false discovery rate (FDR) of 75% (Q = 0.75) (fig. S3A), suggesting no significant correlations with the immune biomarkers measured in this study.

The accuracies of the computational models to distinguish yCMV− versus yCMV+ and yCMV− versus oCMV− were 79% and 91.7%, respectively (table S2), indicating that aging has a more profound effect on the immune system than latent CMV. Strikingly, the effects of CMV and aging on the immunological variables measured here were almost entirely different (Fig. 1). The only exceptions to this were the $CD8^+$ effector memory (T_{EM}) and $CD8^+$ $CD28^-$ cell frequencies, both being positively correlated with age and CMV. This suggests that, in general, the aging process and CMV infection have very different influences on the human immune system. Strikingly, although expression of most parameters (71%, 17 of 24) decreased with age, the majority (88%, 14 of 16) increased with CMV seropositivity in young individuals (Fig. 1 and table S2), indicating an overall down-regulation of the immune response and associated parameters during aging and an up-regulation of several components of the immune system in young subjects with latent CMV.

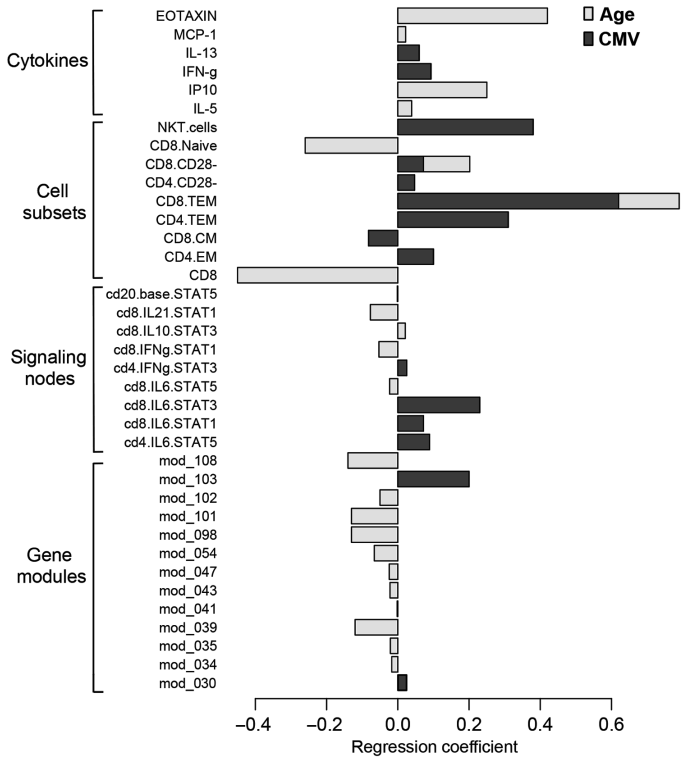

Fig. 1. Different immunological profiles in aging versus CMV seropositivity. The contribution of age and CMV to immunological and gene expression profiles was estimated by a combination of nuclear norm and the elastic net methods (see Materials and Methods). The magnitudes of the regression coefficients used to separate the classes yCMV− and oCMV− (CMV-independent age effect) or yCMV− and yCMV+ (age-independent CMV effect) are shown in light and dark gray bars, respectively. Only two parameters, the frequency of $CD8^+$ $CD28^-$ and $CD8^+$ T_{EM} cells, overlapped between these classification tasks. Fourteen of 16 (87.5%) of the parameters used to separate the yCMV− from yCMV+ classes were up-regulated in CMV; in contrast, most parameters used to separate the yCMV− from oCMV− classes (16 of 23, 69.5%) were down-regulated in aging.

In particular, we found an elevation of circulating interleukin-13 (IL-13) and IFN-γ cytokines and higher $CD8^+$ pSTAT1 and pSTAT3 responses to IL-6 in CMV+ individuals in the younger cohort compared to the CMV− subjects (Fig. 1). This indicates that the former group has a generally activated immune system involving increased T helper 1 (T_H1) and T_H2 cytokines, and also suggests that CMV improves the $CD8^+$ response to IL-6 in young adults. Note that compared to yCMV+ subjects, the oCMV+ individuals are defective in this pathway (table S2, yCMV+ versus oCMV+), which suggests a degree of adaptation to chronic levels of inflammatory cytokines in older CMV-infected subjects. At the gene expression level, the CMV effect

independent of age was an up-regulation of genes associated with immune activation. For example, expression of module 103 (antigen processing and presentation, $P < 0.00001$; natural killer (NK) cell–mediated cytotoxicity, $P < 0.00001$) was elevated in yCMV+ (Fig. 1), and this module includes several *KIR2* and *KIR3* genes as well as *GZMH* (see http://cs.unc.edu/~vjojic/fluy2-upd/mod103.html), genes typically highly expressed in NK cells and in $CD4^+$ and $CD8^+$ T lacking CD28 (*38*, *39*). The expression of the gene module 30 was also elevated in yCMV+ (Fig. 1), and this module is composed of *HLA-DOA* and *HLA-DOB* genes, which clustered with *APOD*, *LAMC1*, and *MIR600*, among other genes. Intriguingly, HLA-DOA and HLA-DOB have been recently shown to confer susceptibility to hepatitis B virus infection and clearance (*40*). The age effect independent of CMV (yCMV− versus oCMV−) revealed down-regulation of several gene modules (Fig. 1) including those associated with cell cycle (modules 34 and 101, $P = 0.019$), protein synthesis (modules 35 and 39, $P = 0.0009$ and $P < 0.0001$, respectively), amino acid metabolism (module 43, $P = 0.0023$), cell death (modules 47 and 54, $P = 0.0021$ and $P < 0.0001$, respectively), the ubiquitination pathway (module 98, $P = 0.0092$), hypoxia-inducible factor 1α signaling (module 101, $P < 0.0001$), LXR/RXR activation, which is involved in cholesterol and lipid metabolism (module 102, $P = 0.007$), and the metabolism of carbohydrates (module 108, $P = 0.00074$). Many of these observations are consistent with a series of previous studies in diverse models of aging and in aged humans (*41*–*43*). These results indicate a relatively restricted contribution of CMV to the expression of genes associated with immune activation and a broader contribution of age to critical aspects of cell function, such as cell cycle, protein synthesis, and metabolism.

Negative contribution of age and positive contribution of CMV in the serological response to influenza vaccination

Although aging typically has a negative effect on vaccination (*33*, *44*), the influence of CMV infection on influenza vaccine responses has been controversial (*14*, *45*, *46*). To probe the immune system's response in a standardized manner, we used seasonal trivalent inactivated influenza vaccination (IIV) and assayed antibody responses using the standard hemagglutination inhibition (HAI) assay at day 0 and 28 ± 7 days after vaccination (fig. S1) in two consecutive years, with 30 young (20 to 30 years old) and 61 older individuals (60 to >89 years old) in year 1 and 25 young and 52 older individuals in year 2. We computed the pre- and postvaccine geometric mean titer (GMT) for all three strains in the vaccine and calculated a response score as the delta post- minus pre-GMT. As expected, a negative effect of age was observed in the antibody response to IIV ($P < 0.0001$) (Fig. 2A). Surprisingly, in the yCMV+ group, the antibody response was higher than in yCMV− subjects ($P = 0.03$) (Fig. 2A). These CMV-related differences were not significant in the older cohort (Fig. 2, A and B). A similar positive effect of CMV in the young ($P = 0.04$), but not the older, cohort was observed in the 77 individuals who returned 1 year later during the 2009 to 2010 influenza season (Fig. 2B). In addition, these findings were validated in an independent cohort of 37 young individuals recruited during the 2010 to 2011 influenza season ($P = 0.017$) (Fig. 2C). These results demonstrate that in young individuals, CMV infection may be beneficial, because it improves the serological response to influenza vaccination and possibly other vaccines and infection.

Single-nucleotide polymorphisms associated with CMV-related alteration in the $CD4^+$ $CD28^-$ T cell pool

The alterations in the frequency of immune cell subsets, cytokines, and other immune measurements observed in CMV+ subjects may contribute to improved response to vaccination. Indeed, yCMV+ subjects exhibited increased levels of IFN-γ, which was shown to mediate cross-protection in mice (*16*) (see below). Notably, the $CD4^+$ T cell response to CMV involves an increase in the fraction of cells lacking the CD28 receptor, which exhibit antigen-primed phenotypes and express cytolytic molecules including granzyme B and perforin. These cells emerge after the peak viral load, produce substantial levels of IFN-γ, and are observed only in CMV-infected persons (*47*). Thus, the variation in the $CD4^+$ $CD28^-$ cell subset may be important for the immune status of the individuals in our study and likely contribute to the variation in other immune responses. To find genetic variations that may confer susceptibility to important immunological alterations in CMV+ subjects, we used a single-nucleotide polymorphism (SNP) genotyping approach (ImmunoChip), which analyzes ~200,000 immune-related SNPs (*48*). We restricted our analyses to SNPs with minor allele frequency greater than 5% (116,405 SNPs) (see Materials and Methods) and focused on the frequency of $CD4^+$ $CD28^-$ cells, a hallmark of CMV infection (*49*) and the level of which is not significantly affected by age. Also, these cells often arise as a result of selective clonal expansions of a CMV-specific T cell response (*47*, *50*), and in our data, they showed the lowest P value for comparison of variance between CMV− and CMV+ individuals ($P = 1.6 \times 10^{-7}$, by one-tailed F test). We identified 35 SNPs (on chromosomes 6, 9, 12, 14, 15, 17, and 19) associated with the frequency of $CD4^+$ $CD28^-$ cells at an FDR lower than 5% ($P < 5 \times 10^{-6}$, FDR $Q < 0.05$) (fig. S4). One of the three SNPs on chromosome 6 [rs7744001 (6p21.32)] is located less than 3.5 kb from

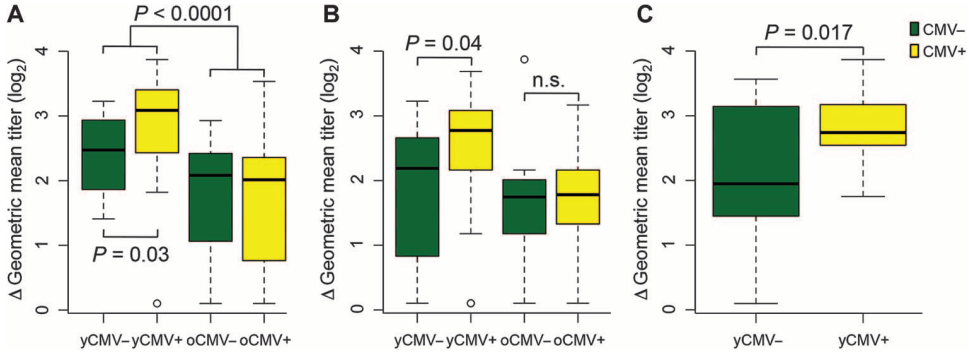

Fig. 2. Young but not old CMV+ individuals have a better response to influenza vaccination. The GMT for all three strains in the vaccine was calculated for each individual in the study and a standardized score [delta (Δ) post-pre GMT] for response was computed as described in Methods (*y* axis). (**A** to **C**) A higher response is observed in yCMV+ compared to yCMV− in the first (A) and second (B) year, as well as in an independent validation study conducted during the 2010 to 2011 influenza season (C). No significant differences (n.s.) were observed between oCMV− and oCMV+. Green bars, CMV−; yellow bars, CMV+. The age ranges for young and older individuals were 20 to 30 and 60 to >89 years, respectively (A); 22 to 32 (young) and 62 to >89 (older) years (B); and 19 to 44 years (C).

HLA-DQB1 and 22 and 140 kb from *HLA-DQA1/DRA*, *HLA-DRB5*, and *HLA-DRB1* (fig. S5), which is in agreement with two recent studies showing multiple genomic loci at 6p21 in the human leukocyte antigen (HLA) region associated with the total lymphocyte count (*51*) and with the levels of $CD8^+$ $CD28^-$ cells (*52*). A cluster of 27 SNPs spanning a region in chromosome 9 from position 122,705,118 to 122,748,094 is located in the vicinity and within *TRAF1* and 55 kb centromeric from *C5* (fig. S6), both of which have been found to be associated with rheumatoid arthritis, where an elevated frequency of $CD4^+$ $CD28^-$ T cells is a hallmark. These results indicate that polymorphisms in important immune-related genes could be responsible for the variation observed in the $CD4^+$ T cell response to CMV.

IFN-γ–dependent, improved response to influenza challenge in mice previously infected with MCMV

To determine whether the specific phenomenon observed in our human cohorts could be modeled in mice, we tested early and established latency with MCMV (5 and 12 weeks of MCMV infection, respectively) versus long-standing latency (9 months of MCMV infection) in C57BL/6 mice and then challenged them with the influenza A virus (IAV) strain x31. Both in early and established latency, we observed better protection against influenza in the MCMV-infected (IAV+ MCMV+) versus the mock-infected (IAV+ MCMV–) mice at day 7 after IAV challenge as seen by the reduced influenza viral titers in the lungs (Fig. 3, A and B). In contrast, in older mice exposed to long-standing MCMV infection, these differences were lost (Fig. 3C). Consistent with this observation, the influenza-specific $CD8^+$ T cell responses against the three major influenza epitopes [$D^bPA_{224–233}$ (PA), $K^bPB1_{703–711}$ (PB1), and $D^bNP_{366–374}$ (NP)] in BAL cells were higher in animals with early and established latency but not after long-standing latency compared with the mock-infected group (Fig. 3, D to F). Our previous results showing increased circulating levels of IFN-γ as well as better influenza vaccine responses in yCMV+ subjects suggested that the observed CMV-mediated cross-protection might be mediated by IFN-γ. To test this, we monitored the influenza viral titer in the lungs and also the number of influenza-specific $CD8^+$ T cells in IFN-γ knockout (KO) or control mice challenged with IAV at 5 weeks after MCMV infection, as described earlier. Significantly higher viral titers and lower influenza-specific responses were observed in the IFN-γ KO compared to control mice (Fig. 4, A and B), demonstrating that IFN-γ is essential for the MCMV-induced cross-protection against influenza in young mice during early MCMV latency and probably also contributes to this effect directly through its antiviral activity.

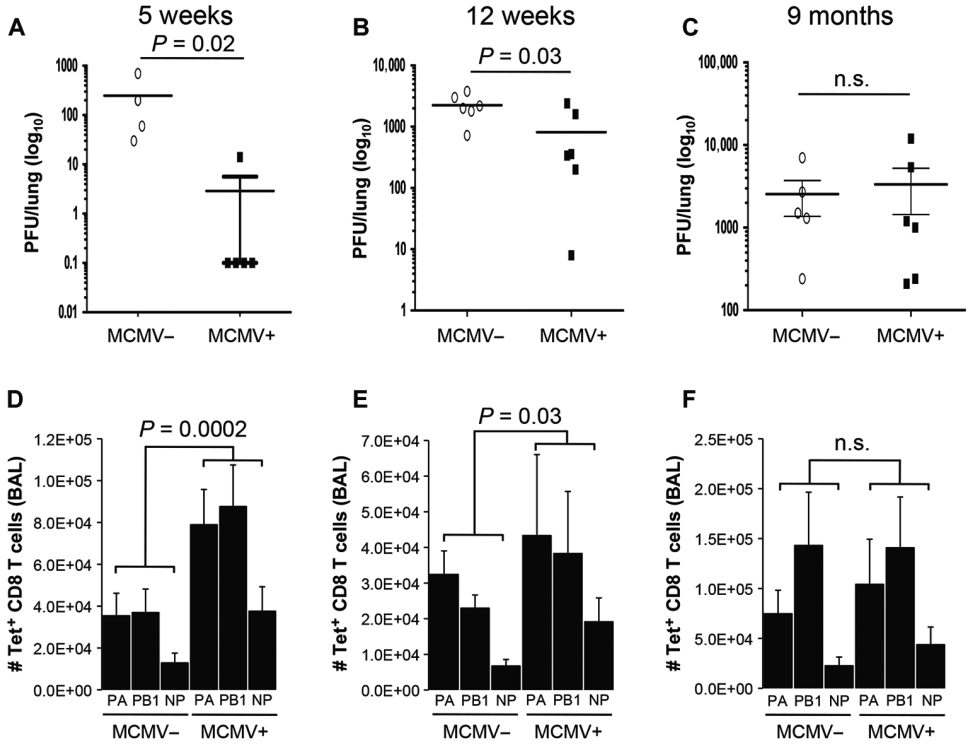

Fig. 3. Reduced viral titer and enhanced IAV-specific $CD8^+$ T cell responses in early and established but not long-standing MCMV latency. (**A** to **F**) Groups of C57BL/6 mice were mock-infected or infected with 4×10^4 plaque-forming units (PFU) of MCMV Smith strain intraperitoneally and challenged with 10^6 EID_{50} (mean egg infective dose) of IAV x31 intranasally 5 weeks (early latency) (A and D), 12 weeks (established latency) (B and E), or 9 months (long-standing latency) later (C and F). Seven days after IAV infection, influenza viral titer was determined (upper panel) and IAV-specific T cells were enumerated from the bronchoalveolar lavage (BAL) of IAV+ MCMV– (MCMV–) or IAV+ MCMV+ (MCMV+) mice by tetramer staining for NP-, PA-, and PB1-specific responses. Data are representative of three independent experiments with four to six mice per group in each experiment. Significance was determined by *t* test for viral titer and using the Fisher's combined probability test for comparison of specific T cell responses.

DISCUSSION

Here, we identify major changes in the immune system of healthy individuals with age and CMV infection. Aging had a predominant and negative contribution on most immune parameters including the serological response to influenza vaccination, and CMV had a positive contribution. This effect was not observed for another widespread member of the Herpesviridae family, EBV. This observation contrasts with previous findings in mice showing that both murine γ-herpesvirus 68 and MCMV, which are genetically similar to human EBV and CMV, can induce cross-protection against unrelated pathogens (*16*) but is consistent with studies in humans and monkeys showing that CMV induces much larger changes in immune cells than EBV, including the chronic activation of effector T cells (*53*). More specifically, because we show here that cross-protection in mice is IFN-γ–dependent, it is possible that these differences are caused by the high frequencies of IFN-γ–producing $CD4^+$ $CD28^-$ cells observed in persistent CMV but not EBV infection (*54*). Furthermore, increased percentages of circulating $CD8^+CD45RA^+CD27^-$ T cells, which also produce high levels of IFN-γ, have been detected in CMV carriers but not after EBV or varicella-zoster virus infection, nor after vaccination with the MMR

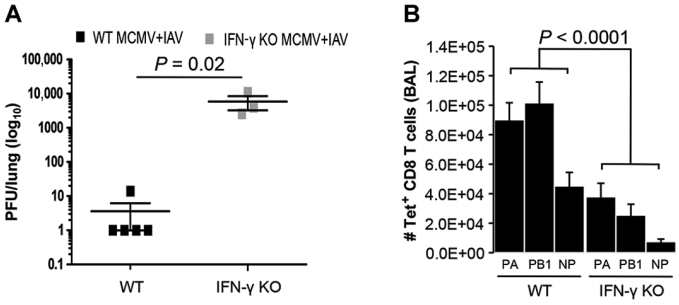

Fig. 4. The effect of MCMV on cross-protection against influenza is IFN-γ–mediated. Groups of C57BL/6 mice or IFN-γ–deficient mice (on the C57BL/6 background) were mock-infected or infected with 4×10^4 PFU of MCMV Smith strain intraperitoneally and challenged 5 to 6 weeks later with 10^6 EID_{50} of IAV x31 intranasally. (**A**) IAV lung viral titers from control [wild-type (WT)] or IFN-γ–deficient co-infected (IAV+ MCMV+) mice were determined at day 7 after IAV infection in early MCMV latency (>5 weeks). (**B**) IAV-specific $CD8^+$ T cells were enumerated from the BAL of WT and IFN-γ–deficient MCMV and IAV co-infected mice by tetramer staining for NP-, PA-, and PB1-specific responses 7 days after IAV infection. Significance was determined by *t* test. Data are representative of two independent experiments with three to five mice per group. Significance was determined by *t* test for viral titer and using the Fisher's combined probability test for comparison of specific T cell responses.

(measles-mumps-rubella) vaccine (*55*). It is plausible that the differences between the CMV- and EBV-associated changes in immune cells' phenotype and function are due to the site of virus latency. For example, CMV reactivation occurs upon differentiation of myeloid precursors with systemic viral shedding and seemingly far-reaching effects, whereas latent EBV is found in differentiated memory B cells (*56*) with reactivation apparently occurring only locally upon recirculation of EBV-infected memory B cells into mucosa-associated lymphoid tissue (*57*). More studies are needed to address this possibility.

Young CMV+ subjects exhibited an increase in the circulating levels of T_H1 and T_H2 cytokines as well as a stronger $CD8^+$ pSTAT1 and pSTAT3 responses to IL-6 compared to CMV− subjects. Older CMV + subjects were defective in this pathway. It is possible that the hyperresponsive T cells observed in young CMV+ lose the ability to respond to further stimuli in the older CMV+ individuals, possibly the result of long-term in vivo exposure to inflammatory mediators and cell desensitization. In support of this hypothesis, CMV infection was found to induce high levels of IL-6 (*58*), which may affect T cell responses to cytokine stimuli in vivo in a manner dependent on the duration of infection. This has an implication for the immune response to infection and for vaccination strategies in humans of different ages, for example, in prime-boost regimens, where the transient elevation of inflammatory mediators and their receptors is required for vaccine efficacy (*59*).

At the gene level, aging was associated with the reduced expression of genes participating in critical aspects of cell function, such as cell cycle, protein synthesis, metabolism, autophagy, and the stress response, which is consistent with a series of previous studies in diverse models of aging and in aged humans (*41*–*43*). In contrast, the higher expression of module 103 in yCMV+ versus yCMV− could be attributed to the known CMV-associated immune activation observed in infected individuals (*60*).

In agreement with the negative contribution of age and positive contribution of CMV to the immune components studied here, vaccine responses were reduced in older subjects and improved in young CMV-infected subjects. Whereas vaccine responses are known to be reduced in aging populations, the effect of CMV has been controversial, with some studies in older adults showing a negative effect of CMV, whereas others showed no difference in CMV+ versus CMV− subjects (*14*, *45*, *46*). Notably, in young individuals, a negative association between the CMV antibody titer and the serological response to the influenza vaccine was found in one study; however, this effect was weak ($R = 0.16$) and observed only for one of four strains of the virus (*61*). In contrast, our analysis takes into account the response to all influenza strains in the vaccine for a given year and makes use of longitudinal and validation cohorts as well. Thus, our results indicate that CMV has no apparent effect on the serological response to influenza vaccination in older individuals and is consistent with a recent report analyzing more than 700 older subjects in long-term care facilities (*45*). Also in our studies, CMV clearly boosts the immune responses of younger individuals, because yCMV+ subjects exhibited elevated serum levels of IFN-γ, stronger $CD8^+$ T cell responses to cytokine stimuli, and elevated antibody responses to the influenza vaccine. Consistent with these results, mice infected with MCMV showed a greater response to influenza challenge that is IFN-γ–dependent and wanes with time in infected animals, much as older humans show no benefit. These results are consistent with long-standing experiments termed "heterologous immunity" in mice where infection with one pathogen can enhance the response to another (*16*, *62*) and also has parallels with human work in which vaccination strategies directed against a specific pathogen have been shown to decrease mortality to different infectious diseases (*63*–*65*).

Other mechanisms can also be implicated in the CMV-mediated cross-protection. For example, Welsh and colleagues (*62*) showed a degree of cross-reactivity of $CD8^+$ T cell epitopes between influenza and CMV in mice and between influenza and EBV in humans. Furthermore, recently, Su *et al.* (*66*) have proposed that T cell cross-reactivity could be a factor based on the observation that healthy adults can have large numbers of memory phenotype $CD4^+$ T cells specific for viral epitopes to which those individuals had never been exposed. However, we note that heterologous cross-reactivity has been directly examined for human CMV–specific responses (*9*) and was found to be rare, arguing against this mechanism in this situation. In addition, here, we did not find influenza-specific cells in the lung during the early stages of MCMV infection when IFN-γ–producing MCMV-specific cells were present, suggesting that cross-protection in MCMV+ mice is not due to cross-reactivity in our system. Nonetheless, there may be other T cell epitopes or antibodies that are cross-reactive, which were not assessed here and that could contribute to the protective response.

Alternatively, the presence of high levels of circulating IFN-γ in young humans and mice infected with CMV strongly suggests that a bystander effect is one mechanism of cross-protection, because the elimination of this gene shows that this cytokine is essential for the protective effect. Also, in agreement with our observations in both humans and mice, in one study, CMV induced cross-protection to a bacterial infection in young mice that is lost in older animals (*17*).

In conclusion, these data demonstrate that the effects of CMV infection and aging on the immune system are almost entirely independent of each other, and offer no support for the hypothesis that this virus accelerates immunosenescence. In addition, and quite unexpectedly, we found that CMV infection enhances the immune responses

of younger adults, as it also in an inbred mouse model. The fact that it did not enhance the responses of older adults indicates that this effect requires a robust immune system, which is interesting in that it largely confines the benefits of infection to those of child-bearing age. Finally, although CMV is clearly a pathogen for immunodeficient individuals and some infants, the data presented here indicate that it is beneficial to a great many more people.

MATERIALS AND METHODS

Study design, subjects, and sample collection

Ninety-one healthy donors (ages 20 to >89 years) were enrolled in an influenza vaccine study at the Stanford-LPCH (Lucile Packard Children's Hospital) Vaccine Program during the fall of 2008, of which 89 completed the study (*33*). The validation study consisted of the 77 individuals who returned during the fall of 2009 and an additional independent cohort of 37 individuals vaccinated in another study during the 2010 and 2011 influenza seasons. Given this sample size and six related tasks of sizes corresponding to classification tasks between four age/CMV combinations, an effect size exceeding 0.5 is detected with a probability of 73.03%. We synthesized data with randomly distributed weights across six tasks such that rank of weight matrix is 2. We generated 100 such synthetic data sets. We ran our method 100 times on these data sets. We computed power of our method to detect an effect in excess of 0.5. One outlier for cytokine data was removed from the analysis. Because all the individuals were vaccinated, no randomization or blinding was done for this study. The protocol for this study was approved by the Institutional Review Board of the Research Compliance Office at Stanford University. Informed consent was obtained from all subjects. All individuals were ambulatory and generally healthy as determined by clinical assessment. Volunteers had no acute systemic or serious concurrent illness, no history of immunodeficiency, nor any known or suspected impairment of immunologic function, including clinically observed liver disease, diabetes mellitus treated with insulin, moderate to severe renal disease, blood pressure >150/95 at screening, chronic hepatitis B or C, or recent or current use of immunosuppressive medication. In addition, none of the volunteers were recipients or donors of blood or blood products in the past 6 months and 6 weeks, respectively, nor showed any signs of febrile illness on day of enrollment and baseline blood draw. Peripheral blood samples were obtained at day 0 (prevaccine) and 28 ± 7 days after receiving a single intramuscular dose of trivalent seasonal influenza vaccine Fluzone (Sanofi Pasteur). Each dose of the vaccine contained 15 μg of HA each of H1N1, H3N2, and B strains of the virus. Whole blood was used for gene expression analysis (below). Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation (Ficoll-Paque) and frozen at −80°C for 24 to 48 hours before transferring to LN_2. Serum was separated by centrifugation of clotted blood and stored at −80°C before use. Whole blood, PBMCs, or serum from the first visit (baseline, day 0) was processed and used for determination of gene expression, leukocyte subset frequency, signaling responses to stimulation, serum cytokine and chemokine levels, and CMV and EBV serostatus. Serum samples from days 0 and ~28 were used for HAI titer determination.

Determination of CMV and EBV seropositivity

Determination of CMV and EBV seropositivity was conducted by enzyme-linked immunosorbent assay (ELISA) using the CMV immunoglobulin G (IgG) ELISA kit (Calbiotech, catalog no. CM027G). Serum samples were thawed at room temperature, and dilutions were prepared according to the manufacturer's recommendations. Calculation of results was done on the basis of the controls provided by the vendor. Six individuals could not be classified because they exhibited an antibody index between 0.9 and 1.1 ("borderline positive" according to the manufacturer). The category (seropositive versus seronegative) for these unclassified individuals was imputed using the "Impute" package (R Bioconductor), which performs nearest neighbor averaging based on the entire cohort's immune measurements and gene expression values.

Whole-blood microarray analysis of gene expression

Total RNA was extracted from PAXgene Blood RNA Tubes (PreAnalytiX) using the QIAcube automation RNA extraction procedure according to the manufacturer's protocol (Qiagen). The amount of total RNA, and $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios were assessed using the NanoDrop 1000 (Thermo Fisher Scientific). RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). For each sample, 750 ng of total RNA was hybridized to Beadchips (HumanHT-12 v3 Expression BeadChip, Illumina) that contain 48,771 probes for around 25,000 annotated genes. The hybridized Beadchips were scanned on an Illumina BeadScan confocal scanner and analyzed by Illumina's GenomeStudio software version 2.0. After checking the quality of each individual array, the feature extraction files were imported into R Bioconductor and analyzed using the BeadArray package for probe filtering, quantile normalization, replicate probe summarization, and $\log_2$ transformation. The original microarray probe-level data files were entered into the Gene Expression Omnibus (GEO) repository under accession number GSE41080.

Leukocyte subset frequency determination

PBMCs were thawed in warm media, washed twice, and stained with three separate anti-human antibody cocktails containing (i) anti-CD3 AmCyan, CD4 Pacific Blue, CD8 allophycocyanin (APC)–H7, and CD28 APC; (ii) CD3 AmCyan, CD4 Pacific Blue, CD8 APC-H7, CD27 phycoerythrin (PE), and CD45RA PE-Cy5; (iii) CD3 AmCyan, CD19 Alexa Fluor 700, CD56 PE, CD33 PE-Cy7, and T cell receptor (TCR) APC, all reagents from BD Biosciences. Additional information for these antibodies can be found on ImmPort (https://immport.niaid.nih.gov/) under accession number SDY212. Incubation with antibodies was performed for 40 min at 4°C. Cells were washed with fluorescence-activated cell sorting (FACS) buffer [phosphate-buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) and 0.1% sodium azide] and resuspended in 200 μl of FACS buffer. Data were collected using DIVA software in an LSRII instrument (BD Biosciences). Analysis was performed using FlowJo 8.8.6 by gating on live cells based on forward versus side scatter profiles, then using double gating for singlet discrimination, followed by cell subset-specific gating.

Phosphorylation of intracellular proteins by phosphoflow analysis

Cells were thawed in warm media and rested at 37°C in RPMI with 10% FBS. Cells were then distributed in 96-deep well blocks (2 ml) and stimulated with IFN-γ, IL-2, IL-6, IL-7, IL-10, or IL-21 (50 ng/ml) or with IFN-α (10^4 U/ml) for 15 min. After stimulation, cells were immediately fixed with 1.5% paraformaldehyde (PFA) for 10 min at room temperature, washed with an excess of plain PBS, and permeabilized with 95% ice-cold methanol for 20 min on ice. Different stimulus

conditions were barcoded using a 3 × 3 matrix with Pacific Orange and Alexa Fluor 750 (Invitrogen) at low (0.03 and 0.04 μg/ml) and high staining (0.2 and 0.3 μg/ml), respectively. Incubation with barcoding dyes was performed at 4°C for 30 min. After several washes with FACS buffer, stimulated and barcoded cells were pooled into single tubes and stained for 30 min at 4°C with an antibody cocktail containing anti-pSTAT1 Alexa Fluor 488, pSTAT3 Alexa Fluor 647, pSTAT5 PE, CD3 Pacific Blue, CD4 peridinin chlorophyll protein (PerCP)–Cy5.5, CD20 PerCP-Cy5.5, and CD33 PE-Cy7 (all from BD Phosflow). Additional information for these antibodies can be found on ImmPort (https://immport.niaid.nih.gov/) under accession number SDY212. After washing, cells were resuspended in FACS buffer, and acquisition was performed on an LSRII instrument (BD Biosciences). Data were collected using DIVA software. Data analysis was performed using FlowJo 8.8.6. by gating on live cells, then using double gating for singlet discrimination, followed by cell subset-specific gating. Phosphorylation of STAT1, STAT3, and STAT5 proteins in B cells, $CD4^+$ or $CD4^+$ $CD3^+$ T cells, or monocytes was analyzed by deconvolution of stimuli-specific gating. Baseline levels and fold increase between stimulated and unstimulated conditions were calculated using the 90th percentile fluorescence intensity of the pSTAT1, pSTAT3, or pSTAT5 signals.

Phosphorylation of Akt and phospholipase C–γ was assessed in B cells by cross-linking of the B cell receptor. After resting PBMC samples at 37°C (as conducted for cytokine stimulations), cells were distributed in V-bottom 96-well plates at 0.5×10^6 cells per well and incubated for 4 min at 37°C in CO_2 incubator with anti-IgM and anti-IgG (10 μg/ml; BD Biosciences) and 3% H_2O_2 for phosphatase inhibition. Cells were then fixed with 1.5% PFA for 10 min at room temperature. After washing twice with plain PBS, cells were permeabilized by 20-min incubation in 95% ice-cold methanol. Cells were then washed with FACS buffer and stained with an antibody cocktail containing CD3 Pacific Blue, CD20 PerCP-Cy5.5, CD27 PE-Cy7, PLGγ2 (BD Biosciences), and pAkt-S473 Alexa Fluor 488 (Cell Signaling Technology). After 30-min incubation at 4°C, cells were washed in FACS buffer and analyzed by flow cytometry (as for cytokine stimulation). Median fluorescence intensity (MFI) was recorded and used for the calculation of baseline levels of phosphorylated proteins and fold increase after BCR stimulation. Additional information for all the antibodies used in this study can be found on ImmPort (https://immport.niaid.nih.gov/) under accession number SDY212.

Serum cytokine level determination

Cytokines were measured using a Luminex system (Luminex Corp). 50-Plex kits were purchased from Millipore and used according to the manufacturer's recommendations with modifications as described below. Briefly, serum samples were mixed with antibody-linked polystyrene beads on 96-well filter plates and incubated at room temperature for 2 hours followed by overnight incubation at 4°C. Plates were then vacuum-filtered and washed twice before the 2-hour incubation with biotinylated detection antibody. Samples were filtered as above, washed twice, and incubated with streptavidin-PE for 40 min, then filtered and washed twice again before resuspending in reading buffer. Each sample was measured in duplicate. Plates were read using a Luminex LabMap200 instrument with a lower bound of 100 beads per sample per measured cytokine. The Luminex LabMap200 outputs the fluorescence intensity of each bead measured for a given cytokine in a sample. For each well, we considered the MFI of all beads measured for a given cytokine and averaged the MFI of the two replicates. Values were normalized to a control sample ran in each of the plates.

HAI assay

The HAI assay was performed on sera from days 0 and 28 using a standard technique (*67*); serially diluted 25-μl aliquots of serum samples in PBS were mixed with 25-μl aliquots of virus matching the vaccine strain composition for those years, corresponding to 4 HA units, in V-bottom 96-well plates (Nunc) and incubated for 30 min at room temperature. At the end of the incubation, 50 μl of 0.5% chicken red blood cells was added and incubated for a minimum of 45 min before reading for HAI activity. The HAI titer of a given sample was defined as the reciprocal of the last serum dilution with no HA activity. A titer of 5 was assigned to all samples in which the first dilution (1:10) was negative. The GMT of all three strains in the vaccine was computed for each individual. Wilcoxon rank sum test was used to compare responses across age groups. To estimate vaccine response in young and older CMV– and CMV+, post-vaccination GMT was subtracted from prevaccination GMT (delta post-pre), and Wilcoxon rank sum test was used to compare vaccine responses between age and CMV infection categories.

Mice and virus infections

Five- to 6-week-old female control C57BL/6 and IFN-γ KO mice were obtained from The Jackson Laboratory. All mice were cared for under specific pathogen–free conditions in an approved animal facility at St. Jude Children's Research Hospital (SJCRH). All animal work was reviewed and approved by the appropriate institutional animal care and use committee at SJCRH (protocol #098), following guidelines established by the Institute of Laboratory Animal Resources, and approved by the Governing Board of the U.S. National Research Council. The mouse-adapted IAV HKx31 was grown in the allantoic fluid of 10-day-old embryonated chicken eggs (SPAFAS). The MCMV Smith strain (American Type Culture Collection) was grown in mouse embryonic fibroblasts and passaged through BALB/c mice, where infectious virus was extracted from salivary glands at day 12 after infection.

For MCMV virus infections, mice were infected intraperitoneally with 4×10^4 PFU of MCMV. For co-infections, at the indicated time after MCMV infection, avertin (2,2,2-tribromoethanol)–anesthetized animals were challenged intranasally with 1×10^6 EID_{50} of HKx31. Mice were considered in early latency at >5 weeks, established latency at 12 weeks, and long-standing latency at 9 months after MCMV infection.

Tissue sampling

BAL fluid was recovered from infected animals challenged with 1×10^6 EID_{50} of HKx31 at the indicated time points. BAL samples were obtained by intratracheal Hanks' balanced salt solution wash in individual mice as described previously (*68*). Cells in the BAL were collected by centrifugation following standard procedures. For quantification of influenza in infected lungs, snap-frozen lungs were stored at –80°C until further processing.

Tetramer and phenotypic staining of CD8 T cells

Influenza A peptides $D^bPA_{224–233}$ (SSLENFRAYV), $K^bPB1_{703–711}$ (SSYRRPVGI), and $NP_{366–374}$ (ASNENMETM) and MCMV peptides $M45_{985–993}$ (HGIRNASFI), $M139_{419–426}$ (TVYGFCLL), and $M38_{316–323}$ (SSPPMFRV) were synthesized by the Hartwell Center, SJCRH. Class

I monomers [H-2D^b and H-2K^b major histocompatibility complex (MHC) class I glycoprotein complexed with the IAV (PA, PB1, NP)] were synthesized through a collaboration with the Trudeau Institute and multimerized at SJCRH. Staining was done as described previously (*69*).

Quantification of IAV in infected lung tissue

Tissues were disrupted by chopping with scissors, homogenized, and centrifuged at 10,000 rpm for 15 min. Lung homogenates were titered by plaque assay on Madin-Darby canine kidney (MDCK) cells. Near-confluent 25-cm^2 monolayers of MDCK cells were infected with 1 ml of homogenate or dilution of homogenate (in general, six 10-fold dilutions of lungs were tested) for 1 hour at 37°C. Cells were washed with PBS, 3 ml of minimum essential medium containing L-1-tosylamido-2-phenylethyl chloromethyl ketone–treated trypsin (1 mg/ml; Worthington Biochemical), 0.9% agarose was added, and cultures were incubated at 37°C with 5% CO_2 for 72 hours. Plaques were visualized with crystal violet.

SNP assay

All samples were genotyped using the custom-designed ImmunoChip array (Illumina). The ImmunoChip array is focused on genome-wide association study (GWAS)–reported risk loci for immune-mediated diseases and includes 186 risk loci covered by 196,524 SNPs. Genotyping arrays were processed using 400 ng of genomic DNA according to the manufacturer's protocols. Genotype calls were generated using the Gentrain2 Algorithm implemented in Illumina GenomeStudio software.

Statistical analysis of experiments conducted in mice

Data were analyzed using Prism 5.0 software (GraphPad). Experiments were repeated two to three times as indicated. We tested for homoscedasticity versus heteroscedasticity (equal versus unequal variance) of each group of mice (CMV versus mock-infected) by the Breusch-Pagan test using the R package "lmtest" (http://cran.at.r-project.org/web/packages/lmtest/lmtest.pdf) and found that the groups had equal variance. The data presenting the differences between the groups were assessed using two-tailed unpaired Student's *t* tests or by two-way analysis of variance (ANOVA) with Bonferroni post hoc settings. $P < 0.05$ indicates that the value of the test sample was significantly different from that of relevant controls. The Fisher's combined probability test (*70*) was used for analysis of influenza-specific CD8$^+$ T cell responses. To ensure reproducibility of our findings, we conducted three independent experiments with four to six mice that were randomized in each group for each experiment for the early versus long-standing latency experiments, and two independent experiments with three to five mice that were randomized in each group for the IFN-γ KO versus wild-type mice experiments. No blinding was done for these experiments.

Detailed statistical methodology can be found in the Supplementary Materials.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Fig. S1. Study design.

Fig. S2. Gating strategy for phosphoflow assays.

Fig. S3. No significant effect of EBV in immune measures and response to influenza vaccine.

Fig. S4. Manhattan plot showing genetic variants that associate with CMV-related phenotypic alteration.

Fig. S5. SNAP plot of notable SNPs found to be associated with the CD4$^+$ CD28$^-$ cell frequency on chromosome 6.

Fig. S6. SNAP plot of notable SNPs found to be associated with the CD4$^+$ CD28$^-$ cell frequency on chromosome 9.

Table S1. Subjects' baseline characteristics.

Table S2. Immune parameters computationally selected in all six classification problems.

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Cytomegalovirus infection enhances the immune response to influenza

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CMV boosts immune response in the young

Cytomegalovirus (CMV) has long been thought of as a sleeper agent—present in a latent form in most people but dangerous when activated in immunosuppressed individuals. Now, Furman *et al*. look more closely at the effects of CMV infection in young, healthy people. They find that in contrast to aged individuals where CMV infection decreased response to flu vaccine, CMV infection actually enhanced the response to flu vaccine in young adults. This beneficial effect was also seen in mice. These data suggest that latent CMV infection may be beneficial to the host, and provide a possible explanation for the prevalence of CMV infection worldwide.

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