Cytomegalovirus-associated accumulation of late-differentiated CD4 T-cells correlates with poor humoral response to influenza vaccination

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A R T I C L E   I N F O

Article history:
Received 6 July 2012
Received in revised form 9 November 2012
Accepted 13 November 2012
Available online 26 November 2012

Keywords:
Influenza vaccination
Antibody response
CMV
T-cell subsets
Intanza
Late-differentiated CD4 T-cells

A B S T R A C T

Influenza vaccination is less effective in the elderly compared to the young. Studies that have attempted to identify immune parameters correlating with satisfactory vaccine responses have yielded inconclusive results. Here, we correlate the distribution of different circulating CD4+ and CD8+ T-cell phenotypes with the humoral response to vaccination with Intanza, an intradermal seasonal vaccine, in 54 individuals of different ages. Subjects were stratified according to age (below or over 60) and presence of a latent infection with Cytomegalovirus (CMV). CMV-seropositivity was significantly associated with a lower response rate to the vaccine in people over but not below 60 yr of age. Unlike reported data, late-differentiated (CD45RA+CCR7−CD27−CD28−) CD4+, but not CD8+ T-cells associated with a poorer vaccine response. Thus, latent CMV infection has a deleterious effect on influenza antibody responses in the elderly, which might be mediated through CD4 T-cells lacking CCR7, CD27 and CD28 and re-expressing CD45RA.

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1. Introduction

Seasonal influenza is a disease with serious clinical and economic burdens, estimated to be the cause of an average of 226,000 hospitalizations and 36,000 deaths per season between 1979 and 2001 in the United States, with 90% of deaths occurring in individuals over the age of 65 [1,2]. Annual influenza vaccination is the most effective method for preventing influenza and its complications [3]. However, exactly the group most susceptible to influenza morbidity and mortality, the elderly, does not respond as well as the young to vaccination. While in the young, the clinical vaccine efficacy is estimated at 70–90%, this corresponds to only 17–53% in the elderly, depending on the vaccine matching with the viruses in circulation [4]. This is widely believed to be due to immunosenescence, the diminished state of the immune system observed in the elderly [5,6].

The specific mechanisms responsible for the decreased ability of the elderly to respond to influenza vaccination are still poorly understood. CD8+ T-cells lacking the costimulatory receptor CD28 have been associated with poor humoral and cellular response to influenza vaccination in the elderly [7–9]. CD28 down-regulation is the result of several rounds of T-cell division in response to antigenic challenge. Thus, CD8+ T-cells specific for chronic antigens, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV) and to a lesser extent Epstein–Barr virus (EBV) lack the expression of this receptor [10–12]. CMV is an almost-ubiquitous β-herpes virus present in 30–90% of the population in developed countries with a rising seroprevalence with increasing age [13]. Although asymptomatic in immunocompetent individuals, it has an enormous impact on the immune system of the host – more than 30% of CD4+ and CD8+ T-cells of a healthy middle-aged individual can be specific for this virus [14]. A latent CMV infection is associated with lower levels of naïve T-cells and accumulation of memory T-cells, both hallmark features of immunosenescence [15–18]. This has led to the increasingly accepted notion that CMV accelerates T-cell immunosenescence [19]. Accordingly, CMV has been associated with poor humoral response to influenza vaccination in the elderly in some [20,21], but not all studies [22].

Thus, if and how CMV-seropositivity and the accumulation of CD28−late-differentiated T-cells associated with it contribute to poor vaccination outcome in the elderly is still unclear. The few studies demonstrating a negative correlation between CD28−CD8+ T-cells and poor vaccination outcome [7–9] did not take CMV-serostatus into account, thus failing to demonstrate a direct link.

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0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.11.041
between accumulation of these cells and poor responsiveness to influenza vaccine.

Here we have correlated the detailed phenotype of circulating CD4+ and CD8+ T-cell subsets with humoral responses in young and old individuals receiving Influvax, an intradermal influenza vaccine specially designed for the elderly [23]. Our data demonstrate a negative impact of the presence of CD8+ but not CD4+ T-cells with a more late-differentiated phenotype on responsiveness to the vaccine in individuals over the age of 60. This suggests that the negative impact of CMV on vaccination outcome, observed in our study, might be mediated through CD4 T-cells lacking the expression of CCR7, CD27 and CD28 and re-expressing CD45RA.

2. Materials and methods

2.1. Study population and design

The current study was embedded in an open-label uncontrolled multicenter phase III trial (UTN U1111-1112-2795) that evaluated the humoral immunogenicity and safety of the Northern Hemispheric 2010–2011 formulation of an intradermal inactivated split-virion influenza vaccine (Intanza®, Sanofi Pasteur) in adults (18–59 yr) and elderly (>60 yr), as required for marketing authorization. Participants at the University of Antwerp were invited after the last visit of the clinical trial (June–July 2010) to take part in a subsequent study looking at CMV status and cellular phenotypes. Approval for this subsequent study was obtained from the Ethical Review Board of the Antwerp University Hospital. Both the clinical trial and the subsequent study were performed according to ICH/GCP guidelines.

Exclusion criteria for enrollment in the clinical trial were contra-indications for influenza vaccination (such as systemic hypersensitivity to a vaccine component), pregnancy or breastfeeding, history of pandemic H1N1 infection or vaccination, history of influenza vaccination within the preceding 6 months (12 months for adjuvanted vaccines), immunodeficiency, receipt of blood products in the past 3 months, seropositivity for HIV, hepatitis B or C, and risk factors that might affect compliance to trial procedures such as chronic illness. At the time of the first vaccine administration (D0), volunteer had to be free of acute illness or infection. All were not allowed to have received or plan receipt of any other vaccine in a period starting 4 weeks before until 3 weeks after trial vaccination. After blood sampling, participants were vaccinated intradermally in the opposite arm with a vaccine containing antigens to A/California/7/2009 (H1N1); A/Perth/16/2009 (H3N2); and B/Brisbane/60/2008 (Barr, Vaccine 2009). According to the marketing authorization, 18–59 yr-olds received 9 μg of each HA strain whereas participants ≥60 yr received 15 μg. A second visit (D21) was scheduled 21 d (+1 d) after the first, for a second blood draw and safety follow-up. D0 and D21 blood samples were used for antibody response assessment only and were centrifuged within 2 h and stored at −20 °C until use.

Participants who agreed to take part in the subsequent study (n = 55 of a total of 66) were invited for a third blood draw within two weeks after D21, for immune phenotyping and CMV serology. Peripheral blood mononuclear cells (PBMC) were isolated within 20 h by ficoll-paque density gradient centrifugation and cryopreserved at −196 °C. A separate sample for CMV antibody assessment was centrifuged within 2 h and frozen at −20 °C until further processing.

2.2. Assessment of humoral response to influenza vaccine

The antibody titers to each vaccine strain were assessed by a standard haemagglutination inhibition assay (HIA) at Sanofi-Pasteur’s laboratory (Global Clinical Immunology [GCI], Swiftwater, PA, USA). The antibody titer was defined as the highest reciprocal dilution inducing complete haemagglutination inhibition (HI) [24]. Each sample was tested in duplicate and the final titer was the geometric mean of the duplicates. Lower and upper limits of quantification were set at 10 and 10,240 respectively. For the B-strain, the HIA used ether-treated split antigen to enhance sensitivity [25].

A positive humoral response was defined as an at least 4-fold antibody titer rise (from D0 to D21) against at least two of the three vaccine strains.

2.3. Assessment of CMV-serostatus

CMV-specific IgG and IgM were measured using Cobas® assays (Roche Diagnostics) [26]. IgM results were qualitative only. All samples were tested at the microbiology laboratory of the University Hospital of Antwerp.

2.4. T-cell analysis

Flow cytometry experiments were performed as described previously [27]. Briefly, PBMC were thawed and treated with human immunoglobulin GAMUNEX (Bayer, Leverkusen, Germany), and ethidium monoazide (EMA) (Invitrogen, Karlsruhe, Germany). Cells were first stained indirectly with anti-KLRG−1 primary antibody (kindly provided by Prof. H-P Pincher, Freiburg, Germany) and Pacific Orange-conjugated anti-mouse IgG (Invitrogen). After blocking with mouse serum (Chemicon/Millipore, Schwabach, Germany) monoclonal antibodies CD3−Alexa Fluor 700, CD4−PerCP, CD8−APC-H7, CCR7−PE-Cy7, CD45RA−V450, CD28−PE (BD Biosciences, Heidelberg, Germany), CD27−APC (BioLegend, San Diego, CA), and CD57−FITC (ImmunoTools, Freiburg, Germany) were added. Cells were washed and analyzed immediately on an LSR II cytometer with FACSDiva software (BD Biosciences). The spectral overlap between all channels was calculated automatically by the BD FACSDiva software, after measuring negative and single-color controls. Data were analyzed using FlowJo software (Tree Star, Portland, OR).

For data analysis, EMA-positive dead cells were excluded. In the viable gate, lymphocytes were gated according to their size and granularity. T-cells within the lymphocyte gate were characterized as CD3+ cells. Differentiation stages of T-cells were characterized according to surface expression of CD45RA, CCR7, CD27 and CD28, according to previously described models [11,28] (Supplementary Fig. 1). Positive and negative populations were gated based on fluorescence−minus-one (FMO) controls for each fluorochrome.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.11.041.

2.5. Intracellular cytokine staining

PBMC were thawed and allowed to rest for 8 h in X-Vivo15 medium (Cambrex) at 1 × 10⁶ cells/ml at 37 °C. The cells were then incubated overnight with 50 ng/ml PMA (Sigma-Aldrich, Munich, Germany), 750 ng/ml ionomycin (Merk, Darmstadt, Germany) and 1 μl/ml Golgi-Plug (BD Biosciences). Following fixation and permeabilisation using Cytofix–Cytopert solution (BD Biosciences), the cells were stained with CD3−Qdot 655 (Invitrogen), CD4−Pacific Blue, IL-2-Alexa Fluor 700, IL-5−PE, TNF-FITC (BioLegend), CD8−APC-H7, IFN−γ−PE-Cy7 (BD Biosciences), IL-10−APC (Miltenyi Biotec, Bergisch Gladbach, Germany), and IL-17−PerCP-Cy5.5 (eBioscience, Frankfurt, Germany). EMA was used to exclude dead cells. Samples were measured directly using the BD LSR-II as described above. Production of different cytokines by T-cells in a
representative donor is shown in Supplementary Fig. 2. An unstimulated control stained in the same way as the sample was used to determine cytokine-producing populations in each donor.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.11.041.

2.6. Statistical analyses

For comparison of two independent groups, Mann–Whitney testing was used. Differences between groups for the categorical variables were assessed with the Chi-square test. Correlation between two continuous variables was analyzed using Spearman’s correlation analysis. All statistical analyses were performed using Graphpad Prism v5.

3. Results

3.1. CMV serostatus

All subjects were CMV IgM-negative, whereas 26 were CMV IgG-positive. Prevalence of CMV-seropositivity was higher in >60 year-olds than in younger participants (Table 1), but not related to age within the former group (Table 2).

3.2. Anti-vaccine humoral responses in vivo

Three weeks after vaccination, anti-influenza humoral responses were detected in 41/54 of study subjects (76%). The response rate was significantly higher in individuals <60 yr of age (96%) compared to >60 yr (60.0%, p = 0.003, Table 1). This was true for responsiveness to H1N1/California and B/Brisbane strains (p = 0.049 and 0.0023 respectively), but not H3N2/Perth (p = 0.14, Table 1). Considering the higher prevalence of CMV-seropositivity in the older group (60%-vs-33.3%), we sought to determine if the difference observed between the two groups correlated with CMV infection. Stratifying the subjects by age into groups < or >60 yr revealed no influence of CMV infection in the younger group. Thus, CMV+ and CMV- subjects <60 yr showed very similar humoral responses to the vaccine. In contrast, in people >60 yr, CMV-seropositivity was associated with a significantly lower response rate to the vaccine (p = 0.033, Table 2). Responsiveness to single virus strains was also generally lower in the CMV+ group, but only reached statistical significance for Perth (p = 0.045, Table 2).

3.3. Correlation between T-cell phenotypes and humoral responses to vaccination

Next, we sought to determine whether the differentiation status of the CD4+ and/or CD8+ T-cell compartments was different in people mounting a humoral response to the vaccine (responders, R) and those not responding (non-responders, NR). Considering the known large impact of CMV on T-cell phenotypes to be analyzed here, only groups matched for CMV-serostatus were compared. Furthermore, because almost all of the subjects aged <60 yr were vaccine responders, the following analyses were limited to those >60 yr. We found that the frequency of T-cells carrying the costimulatory receptors CD27 and CD28, or putative markers of senescence CD57 and KLRG-1, did not differ significantly between responders and non-responders. This was the case for both CD4+ and CD8+ T-cells (Fig. 1). However, within the responders we observed a significant difference in the expression of all of these markers depending on their CMV-serostatus (Fig. 1A–D). Furthermore, no difference was documented between R and NR in the frequency of naive and different memory T-cell phenotypes within the CD8 subset (Fig. 2). The only significant difference was observed for just one phenotype in the CD4 subset: CMV+ elderly responders had significantly lower frequencies of the most late-differentiated effector cells (CD45RA+CCR7−CD27−CD28−) (Fig. 2D). Except for one individual, this particular phenotype was completely absent in all responders regardless of their CMV-serostatus (median 0.05%, IQR 0.32), but present at almost 40-fold higher frequencies (median 1.96%, IQR 3.75) in non-responders.

3.4. Correlation between the proportion of late-differentiated CD4+ T-cells and cytokine repertoire

In order to begin dissect the mechanism(s) behind the negative impact of CD45RA+ late-differentiated CD4+ T-cells on vaccination outcome, we correlated the proportion of these cells with the cytokine production repertoire of CD4+ and CD8+ T-cells in response to PMA/Ionomycin, a non-specific stimulus. This analysis was performed in CMV-seropositive individuals only (n = 26), as this phenotype was absent in CMV-seronegative donors. A higher proportion of these cells was significantly associated with lower production of IL-2, IL-17 and TNF in CD4+, as well as IL-2, IFN-γ and TNF in the CD8+ subset (Table 3).

4. Discussion

Influenza vaccination is less effective in the elderly compared to the young. Identifying immune parameters that differ between responders and non-responders will assist in development of better, more effective vaccines in the elderly or define screening

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>&lt;60 yr (n = 24)</th>
<th>&gt;60 yr (n = 30)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>37.5 (19–56)</td>
<td>68.4 (60–81)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CMV seropositive, n (%)</td>
<td>8 (33.3)</td>
<td>18 (60.0)</td>
<td>0.05</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>14 (58.3)</td>
<td>13 (43.3)</td>
<td>0.27</td>
</tr>
<tr>
<td>Humoral response to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2 antigens, n (%)</td>
<td>23 (95.8)</td>
<td>18 (60.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>A/California, n (%)</td>
<td>22 (91.6)</td>
<td>21 (70.0)</td>
<td>0.049</td>
</tr>
<tr>
<td>A/Perth, n (%)</td>
<td>23 (95.8)</td>
<td>25 (83.3)</td>
<td>0.14</td>
</tr>
<tr>
<td>B/Brisbane, n (%)</td>
<td>18 (75.0)</td>
<td>10 (33.3)</td>
<td>0.0023</td>
</tr>
</tbody>
</table>

The p-value for age was calculated by Mann–Whitney testing. Remaining p-values were calculated by Chi-square test.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>&lt;60 yr (n = 16)</th>
<th>&gt;60 yr (n = 12)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (range)</td>
<td>34.7 (19–53)</td>
<td>70.5 (61–76)</td>
<td>0.036</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>11 (68.7)</td>
<td>5 (33.3)</td>
<td>0.88</td>
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<tr>
<td>Humoral response to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2 antigens, n (%)</td>
<td>15 (93.7)</td>
<td>10 (83.3)</td>
<td>0.52</td>
</tr>
<tr>
<td>A/California, n (%)</td>
<td>15 (93.7)</td>
<td>10 (83.3)</td>
<td>0.60</td>
</tr>
<tr>
<td>A/Perth, n (%)</td>
<td>15 (93.7)</td>
<td>12 (100)</td>
<td>0.47</td>
</tr>
<tr>
<td>B/Brisbane, n (%)</td>
<td>12 (75.0)</td>
<td>6 (50.0)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

The p-value for age was calculated by Mann–Whitney testing. Remaining p-values were calculated by Chi-square test.
policies for adapted immunization. CD28−CD8+ T-cells have been reported to correlate with poor vaccination outcome in the elderly in three studies [7–9]. Despite the fact that the frequency of these cells is directly associated with CMV-serostatus of the donor, this parameter was not taken into account in any of these studies. Our data presented here demonstrate that seropositivity for CMV, reflected in accumulation of CD28−CD8+ T-cells, and not these cells per se, is associated with poor responsiveness in the elderly. Indeed, there was a large, statistically significant difference for the frequency of these cells within the responders when they were stratified according to CMV-serostatus, whereas CMV-matched responders and non-responders had equal levels of these T-cells.

Therefore, a higher frequency of CD28−CD8+ T-cells is a surrogate marker for seropositivity for CMV, which might be associated with poor responsiveness to vaccination [20].

In the cohort studied here there was a strong negative association between CMV-seropositivity and the humoral response to the vaccine in the elderly. This finding is in line with earlier data [20,21], but contrary to a more recent study [22]. In the latter, responsiveness to the vaccine was characterized as a serological response to only one particular strain in the vaccine, whereas in the study reported by Trzonkowski et al. response to all three strains included in the vaccine was used to stratify responders and non-responders, an approach that we used as well [21,22]. The intradermal route of vaccine administration in our cohort is different from that in the study by Trzonkowski et al., suggesting that our data may also apply to at least the intramuscular delivery route and may not be limited to the intradermal vaccination setting.

There was no negative impact of CMV latency on responsiveness to influenza vaccine in the younger group. Furthermore, CMV-individuals >60 yr responded in much the same way as the younger donors to the vaccine. This might be explained by an increased CMV-associated pro-inflammatory status in the elderly carrying this virus [29–31]. Treatment with acetylsalicylic acid has been shown to result in increased serological responses to influenza vaccine, particularly in individuals >75 yr of age [32]. Thus, treatment with anti-inflammatory might be a possible approach for improved vaccination outcome in CMV+ elderly.

We identified late-stage differentiated CD4+ T-cells, lacking CCR7, CD27 and CD28 and re-expressing CD45RA as the
and T-cells CD4+ have these cells in vitro in the presence of CMV+ cells. Correlating only immune correlate to be associated with poor vaccination outcome in individuals >60 yr. We and others have documented the presence of rare CD45RA+ re-expressing late-differentiated CD4 T-cells in different populations and shown that their accumulation is directly associated with CMV-seropositivity, independently of age (Fig. 2D left panel [18,33,34]). Accordingly, the majority of them are specific for CMV [33]. In the earlier literature CD45RA+CD4+ T-cells cells have often been termed suppressor-inducer T-cells, due to their ability to induce suppression of IgG synthesis in B-cells in response to mitogens [35–38]. Furthermore, data from in vitro cultures of CD4+ T-cell clones as a model of T-cell aging have demonstrated a suppressive function of CD4+ T-cells at the very late-stage of their differentiation [39]. Consistent with this, more recent studies have identified a subset of suppressor regulatory Foxp3+ T-cells that express CD45RA [40,41]. These “resting” Tregs are suppressive themselves and can give rise to CD45RA−Tregs with higher suppressive activity [40]. Our data on a negative correlation between the frequency of CD45RA+ late-differentiated CD4+ T-cells and cytokine production capacity in both CD4+ and CD8+ subsets, support the hypothesis of a suppressive function of these CMV-induced cells. Considering the fact that regulatory T-cells express various skin-homing chemokine receptors [42] and if the late-differentiated CD4 T-cells identified in our study as correlating with poor humoral response are indeed regulatory T-cells, it will be interesting to determine whether these cells seen here in the intradermal vaccination setting are also observed when the vaccine is delivered via the intramuscular or intranasal routes.

Our data are thus consistent with a negative impact of persistent infection with CMV on the ability to mount a broad humoral response to influenza vaccination in the elderly, which might be mediated through CD45RA+CCR7−CD27−CD28−CD4+. Further understanding of these responses and the mechanisms of action of the late-differentiated CD4 T-cells, as well as parameters involved in their induction (as they do not accumulate in all, but only some CMV+ individuals) may provide a means for targeting them, hence leading to improved vaccination outcome in the majority of the elderly, who are CMV seropositive.

**Role of the funding source**

This work was supported by the European Commission [FP7 259679 “IDEAL”]; German Research Foundation [DFG-PA 361/14-1]; German Federal Ministry of Education and Research [BMF 0315890F, “Gerontoshield”] and the Methusalem funding program of the University of Antwerp (22858). NC and HD are postdoctoral fellows of the Fund for Scientific Research (FWO)-Flanders. The funding sources were not involved in study design; in the collection,
analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

Author’s contributions
ED contributed to study design, analysis and interpretation of the data. HD, PVD and NC were involved in the conceptual design of the study, carried out the clinical trial and generated the serological data. KH contributed to generation and analysis of the flow cytometry data. GP contributed to study design and interpretation of the results. All authors were involved in drafting the manuscript and have approved the submitted version.

Conflict of interest
The authors declare no conflict of interest.

Acknowledgements
We would like to thank Prof. Hans-Peter Pircher (University of Freiburg) for providing the anti-KLRG-1 primary antibody, Lilly Oettinger for antibody titration and flow cytometry quality control and Kevin Lenders for processing the blood samples.

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