Delineating the role of CD4+ T cells in the activation of human cytomegalovirus-specific immune responses following immunization with Ad-gBCMVpoly vaccine: implications for vaccination of immunocompromised individuals

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Reconstitution of the virus-specific CD8+ T-cell response is crucial for the prevention of human cytomegalovirus (CMV)-associated pathogenesis in transplant patients and human immunodeficiency virus-infected individuals. Although adoptive T-cell immunotherapy has been used successfully in various clinical settings, prophylactic vaccination remains the most amenable strategy to prevent CMV disease. However, vaccination in clinical settings where the host is severely immunocompromised due to the loss of CD4+ T cells remains a significant challenge. This study investigated the efficacy of a chimeric CMV vaccine in a model setting that allowed studies on the generation of CD8+ T-cell memory responses in a transient CD4+ T-cell-deficient setting similar to that seen in immunocompromised patients. Immunization with an adenoviral CMV vaccine under transient helpless (complete CD4+ T-cell depletion) or help-deficient (partial CD4+ T-cell depletion) conditions demonstrated that induction of the effector CD8+ T-cell and humoral responses was almost completely eliminated under helpless conditions, and was gradually regained with the recovery of CD4+ T cells. However, this response failed to protect the host from viral infection, suggesting that lack of CD4+ T cells during vaccination can significantly impair the priming and maturation of CMV-specific immune responses. Furthermore, although the induction of CMV-specific immune responses was also significantly reduced in a help-deficient environment, these primed effector cells could mature normally and generate long-term polyfunctional memory responses capable of restricting virus replication in vivo. These results highlight the importance of monitoring CD4+ T-cell numbers before vaccination for the successful implementation of a CMV vaccine in an immunocompromised setting.

INTRODUCTION

Although human cytomegalovirus (CMV), a member of the human herpesvirus family, is found universally throughout all geographical locations and socioeconomic groups and infects 50% of adults in developed countries (Gandhi & Khanna, 2004), it does not cause clinical disease in immunocompetent individuals except as a mononucleosis-like illness in a small number of infected individuals. However, CMV infection does cause serious problems in unborn babies with an immature immune system and in immunocompromised people such as human immunodeficiency virus (HIV)-infected individuals with low levels of CD4+ T cells and organ-transplant patients with drug-induced immunosuppression (Gandhi & Khanna, 2004).

It has been well documented that both humoral and cellular (including CD4+ T cells and CD8+ T cells) immune responses play important roles in the control of CMV infection and disease (Gandhi & Khanna, 2004). Recent studies have demonstrated the importance of CD4+ T-cell help in the generation of fully functional cytotoxic T lymphocytes (CTLs) (Shedlock & Shen, 2003; Sun et al., 2004). Subdominant antigens are particularly dependent upon CD4+ T-cell-mediated help to induce the maturation of dendritic cells, which are required for the priming of naïve CD8+ T cells (Smith et al., 2004). Although other antigens are help-independent in priming CTL responses, the generation of fully functional CTL responses still requires intact CD4+ T-cell help at the time of priming (Shedlock & Shen, 2003; Shedlock et al., 2003; Sun & Bevan, 2003; Sun et al., 2004). These studies further emphasize that T-cell-based vaccines designed to prevent virus-associated diseases in immunocompromised individuals will need to address the potential impact of CD4+ T-cell deficiency on the generation of a long-term effective antiviral CTL response.
Ad-gBCMVPoly is a novel chimeric vaccine based on a replication-deficient adenovirus that encodes a truncated form of CMV-encoded gB antigen and multiple CMV T-cell epitopes from eight different CMV antigens, restricted through multiple human leukocyte antigen (HLA) class I and class II alleles, as a single fusion protein (Rist et al., 2005). Our most recent studies have demonstrated that immunization of an immunocompetent host with this chimeric vaccine consistently generated a pluripotent CMV immunization of an immunocompetent host with this vaccine under transient CD4+ T-cell deficiency. More importantly, severe deficiency of CD4+ T-cell-deficient mice and by 90% in helpless mice, and the anti-gB antibody titre was reduced by 50% in help-deficient mice and by 80% in helpless mice when compared with control mice (mock treated). This reduction in CMV-specific T-cell and antibody responses correlated with the size of CD4+ T-cell population in the animals at the time of immunization.

To investigate further the impact of CD4+ T-cell deficiency, HHD-1 mice were completely depleted of CD4+ T cells (treated with 300 µg anti-CD4 mAb GK1.5) and, at different time points post-depletion (days 5, 25, 45 and 65), the recovery of CD4+ T cells was monitored and mice were immunized with Ad-gBCMVPoly vaccine (Fig. 2a, b). CMV-specific cellular and humoral immune responses were evaluated at day 10 post-immunization at each of these time points. The data presented in Fig. 2(c, d) demonstrated that the recovery of CD4+ T cells (Fig. 2b) was coincident with an increase in CMV-specific cellular and humoral immune responses. Taken together, these results indicated a crucial role of CD4+ T cells in the generation of the effector CMV-specific immune response following immunization with Ad-gBCMVPoly.

**Impact of CD4+ T-cell deficiency on the generation of long-term memory CMV-specific responses primed by Ad-gBCMVPoly**

In the next set of experiments, we assessed the impact of CD4+ T-cell deficiency on the generation of memory CTL responses. HHD-1 mice were treated with 60 µg anti-CD4 mAb GK1.5 to partially deplete CD4+ T cells and then immunized with Ad-gBCMVPoly vaccine. Following immunization, CMV-specific immune responses were evaluated at different time points (Fig. 3a, b). These analyses showed that, at the effector phase (day 10 post-immunization), the number of CMV-specific IFN-γ-producing T cells induced in help-deficient mice was significantly lower than that induced under normal CD4+ T-cell conditions (Fig. 3c). These numbers remained significantly lower during the early memory phase (day 25); however, by day 75 post-immunization, the number of CMV-specific IFN-γ-producing T cells in help-deficient and helped (mock treated) mice was not significantly different (Fig. 3c). A similar pattern in the gB-specific antibody response was observed in help-deficient mice (Fig. 3d). A number of recent studies have demonstrated that the production of tumour necrosis factor (TNF) and granzyme (indicated by the expression of CD107a) in addition to IFN-γ by T cells is a characteristic of greater differentiation and can enhance protection against infectious pathogens (Darrah et al., 2007; McKay et al., 2002).

We next assessed the levels of TNF-α, IFN-γ and CD107a expression by CD8+ T cells using intracellular cytokine staining (ICS) assays. The data presented in Fig. 3(e) showed that, during the early priming phase (day 10), CMV-specific T cells from help-deficient mice displayed limited polyfunctionality compared with mock-treated

**RESULTS**

**Immunogenicity of Ad-gBCMVPoly vaccine under CD4+ T-cell deficiency**

To assess the impact of CD4+ T-cell deficiency on the immunogenicity of Ad-gBCMVPoly vaccine, HLA-A2 transgenic mice (referred to as HHD-1) were treated with 300 or 60 µg anti-CD4 mAb GK1.5, resulting in a complete (>99%) or partial (70%) depletion of CD3+CD4+ cells, respectively, in peripheral blood at 5 days post-injection (Fig. 1a, b). After confirmation of CD4+ T-cell depletion, these animals were then immunized with Ad-gBCMVPoly, and the CMV-specific effector T-cell response and gB-specific antibody response were assessed at 10 days post-immunization using an ELISPOT assay and ELISA, respectively. As expected, strong HLA-A2-restricted epitope-specific T-cell- and gB-specific antibody responses were generated in control mice (mock) (Fig. 1c, d). A significant reduction in the number of gamma interferon (IFN-γ)-producing CMV-specific T cells and serum anti-gB-specific antibody titre was observed in both help-deficient (60 µg per mouse) and helpless (300 µg per mouse) mice (Fig. 1c, d). The numbers of IFN-γ-expressing CMV-specific T cells were reduced by 60% in help-deficient mice and by 90% in helpless mice, and the anti-gB antibody titre was reduced by 50% in help-deficient mice and by 80% in helpless mice when compared with control mice (mock treated). This reduction in CMV-specific T-cell and antibody responses correlated with the size of CD4+ T-cell population in the animals at the time of immunization.

**Impact of CD4+ T-cell deficiency on the generation of long-term memory CMV-specific responses primed by Ad-gBCMVPoly**

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animals. Despite this apparent impairment in T-cell function during the effector phase, the percentage of polyfunctional T cells in help-deficient mice gradually improved and reached the levels seen in mock-treated animals by day 75 post-immunization. These studies demonstrated that, even with partial loss of CD4$^+$ T cells, the priming of virus-specific T cells by CMV vaccine was severely impaired, although a gradual improvement in their functionality was observed during the memory phase when the CD4$^+$ T-cell count recovered to normal levels.

**Impact of CD4$^+$ T-cell deficiency in preventing virus replication in vivo**

In the final set of experiments, we assessed the in vivo protective efficacy of virus-specific T-cell responses induced in helpless, help-deficient and helped mice. As outlined above, HHD-1 mice were treated with 300 or 60 μg anti-CD4 mAb GK1.5 or mock treated and then immunized with Ad-gBCMVPoly vaccine. These animals were rested for 75 days to allow the recovery of CD4$^+$ T cells and development of a CMV-specific memory response. On day 75, these mice were challenged with recombinant vaccinia virus expressing CMV-encoded IE-1 (Vac.IE-1) or gB (Vac.gB) protein. Four days after virus infection, the animals were sacrificed and assessed for CMV-specific T-cell responses and viral load. The data presented in Fig. 4(a and c) showed that mice primed under mock-treatment or help-deficient settings displayed a strong resistance to recombinant vaccinia virus encoding IE-1 (Fig. 4a) or gB (Fig. 4c). This resistance was coincident with strong expression of IFN-γ and/or TNF-α by CMV-specific CD8$^+$ T cells (Fig. 4b), and TNF-α by CMV-specific CD4$^+$ T cells (Fig. 4d). These observations were consistent with our earlier findings that the memory T-cell response in help-deficient mice was comparable to...
that seen in animals with an intact CD4+ T-cell compartment (mock treated) (see Fig. 3c, e). Conversely, animals immunized under helpless conditions (i.e. complete depletion of CD4+ T cells) failed to provide significant resistance against virus infection when compared with unimmunized mice (Fig. 4a, c). Ex vivo analysis of CMV-specific T cells from these animals showed that the majority of virus-specific CD8+ T cells displayed significantly lower levels of IFN-γ and/or TNF-α expression when compared with help-deficient and mock-treated mice (Fig. 4c). Although CMV-specific CD4+ T cells from mice immunized under both helpless and help-deficient conditions displayed minimal differences in the expression of IFN-γ compared with CD4+ T cells in mock-treated mice, a significantly lower level of TNF-α expression was observed in helpless and help-deficient mice (Fig. 4d). Taken together these observations demonstrated that CD4+ T cells not only play a crucial role in the priming of vaccine-induced cellular responses, but, more importantly, severe deficiency of these cells can significantly impair the functional maturation of memory T cells and thus compromise the protective efficacy of a CMV vaccine.

DISCUSSION

The data presented in this study provide an insight into the potential role of CD4+ T cells in the generation of the effector and memory CD8+ T-cell response following immunization with Ad-gBCMVPoly vaccine. These studies have important implications for not only the development of a CMV vaccine but also for other antiviral vaccines that aim to induce long-term memory T-cell responses to prevent virus-associated diseases. Most of the previous studies designed to delineate the role of CD4+ T cells in the generation of CD8+ T-cell responses have been based on either CD4+ T-cell depleted or MHC class II-knockout murine models (Shedlock & Shen, 2003; Shedlock et al., 2003; Sun & Bevan, 2003; Sun et al., 2004). However, it is difficult to extend these findings to immunocompromised
humans where a complete loss of CD4\(^+\) T cells is rarely observed. We have used a novel model system that allows studies on the generation of CD8\(^+\) T-cell memory responses in a transient CD4\(^+\) T-cell deficient (help-deficient) setting similar to that seen in immunocompromised patients. This model allowed us to assess the efficacy of a CMV vaccine formulation in animals carrying varying numbers of CD4\(^+\) T cells. Consistent with our previous findings with

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**Fig. 3.** Long-term memory responses induced by Ad-gBCMVPoly vaccine in a help-deficient setting. (a) Experimental procedure. HHD-1 mice were treated with GK1.5 mAb (60 \(\mu\)g) and then immunized with Ad-gBCMVPoly vaccine. On days 10, 25 and 75 post-immunization, the mice were assessed for CMV-specific humoral and cellular immune response. (b) Representative FACS plots of CD4\(^+\) T-cell staining in splenocytes at the indicated time points post-immunization. (c) CMV-specific memory CD8\(^+\) T-cell response against HLA-A2-restricted peptide epitopes using ELISPOT assays. Data are presented as s.f.c. per 10\(^6\) splenocytes. (d) CMV-specific humoral immune response in serum samples assessed by ELISA. (e) Summary of the polyfunctional analysis of CMV-specific T cells from mock-treated or help-deficient mice immunized with Ad-gBCMVPoly vaccine. Splenocytes from these mice were stimulated with HLA-A2-restricted NLVPATV or VLEETSMVL peptide-pulsed, irradiated HHD-1 splenocytes for 2 weeks. Viable cells were purified and assessed for IFN-\(\gamma\), TNF-\(\alpha\) and CD107a mobilization (as a marker for cytolytic function) using a standard ICS assay. Data from these assays were analysed using FlowJo software. Each pie chart represents the percentage of CMV-specific CD8\(^+\) T cells capable of expressing one, two or three functional markers (IFN-\(\gamma\), TNF-\(\alpha\) and/or CD107a). The results in (a)–(d) are expressed as means ± SD of five mice in each group.
Epstein–Barr virus (EBV) vaccine (Smith et al., 2008), whilst the complete loss of CD4+ T cells (helpless) eliminated the generation of effector and functional memory responses, immunization of help-deficient mice only impacted on the effector T-cell response. In spite of a partial loss of CD4+ T cells in help-deficient mice, immunization with Ad-gBCMVpoly vaccine induced strong memory T-cell and antibody responses. More importantly, these animals showed strong resistance against virus challenge, whilst helpless mice with poor functional memory responses were unable to block virus replication.

The data presented in this study are consistent with recent studies that have also shown a critical role of CD4+ T-cell help during the generation of a functional CD8+ memory response (Janssen et al., 2003, 2005; Sun & Bevan, 2003). Indeed, these studies have shown that CD8+ T cells primed under helpless conditions have severe defects in the expression of crucial genes related to proliferation, survival and cytotoxic functions (Rapetti et al., 2008). We have now extended these observations to demonstrate that T-cell responses generated in helpless mice following vaccination are qualitatively inferior when compared with helped mice and fail to afford resistance against viral challenge. However, a memory T-cell response generated in animals with transient help deficiency are capable of maturing as fully functional effector cells and can block virus replication. Another important aspect of these studies is the impact of CD4+ T-cell deficiency on generation of the antiviral antibody response. Similar to the antiviral CD8+ T-cell response, although the virus-specific antibody response in help-deficient mice was significantly lower during the early priming phase, the memory antibody response was comparable to that seen in control animals (helped mice).

Although it is difficult to identify precisely the role played by CD4+ T cells in the immunogenicity and efficacy of the CMV vaccine, previous studies on viral and bacterial infections have indicated that CD4+ T cells do not control the activation and generation of memory CD8+ T cells in an antigen-specific manner (Sun & Bevan, 2004). There is
now increasing evidence to support the argument that cytokines such as interleukin (IL)-7 and/or IL-15 are secreted either directly by CD4+ T cells or by intermediate cells through CD4+ T-cell help to promote survival and basal homeostatic proliferation of memory CD8+ T cells (Kieper et al., 2002; Schluns et al., 2000, 2004; Schluns & Lefrancois, 2003; Tan et al., 2002). Of particular interest is the expression of IL-7R on memory CD8+ T cells, which is not sustained in the absence of CD4+ T cells (Kaech et al., 2003). These observations are particularly relevant to the current study, where we noticed a contrasting impact of transient helpless and help deficiency on the long-term memory CD8+ T-cell response and the protective efficacy of these cells. Whilst the memory CD8+ T-cell response recovered quite efficiently in help-deficient mice, the loss of function and protective efficacy was significantly more pronounced in helpless mice. It is not clear as yet how the absence of CD4+ T cells contributes towards the loss of IL-7R on memory CD8+ T cells. Furthermore, although IL-15 has also been identified as a crucial cytokine in the generation and maintenance of memory CD8+ T-cell responses (Schluns et al., 2002), its precise role in helpless/help-deficient settings remains to be elucidated. More importantly, these studies provide an important platform for the implementation of a CMV vaccine in immunocompromised individuals with CD4+ T-cell deficiency. In a clinical setting where the reconstitution of CD4+ T cells may be difficult, it may be possible to combine the Ad-gBCMVPoly vaccine with γC-cytokine–antibody complexes (Boymann et al., 2006) to enhance its immunogenicity and protective efficacy.

METHODS

Construction of recombinant adenovirus encoding CMV gB–polyepitope fusion protein. The construction of the recombinant AdS5F35-based adenovirus has been described in our previous work (Rist et al., 2005). This vector expresses the CMV gB sequence from the alanine residue at position 31 to valine at position 700 with deletion of the signal sequence, and 46 contiguous HLA class I- and class II-restricted T-cell epitopes as a fusion protein (Ad-gBCMVPoly).

Synthesis of peptides. Peptides, synthesized by the Merrifield solid-phase method, were purchased from Chiron Mimotopes, dissolved in DMSO and diluted in serum-free Dulbecco’s modified Eagle’s medium for use in standard T-cell assays. The purity of these peptides was tested by mass spectrometry and showed >90% purity.

CD4+ T-cell depletion. Mice were depleted of CD4+ T cells by intraperitoneal injection of anti-CD4 mAb (clone GK1.5). The GK1.5 anti-CD4 mAb was purified from the supernatant of hybridoma GK1.5 culture by a protein G column. The efficiency of depletion was evaluated by peripheral blood CD3+, CD4+ and CD8+ T-cell FACs staining.

Animals and immunization. HLA-A2 transgenic mice (referred to as HHD-1; First et al., 2001; Pascolo et al., 1997) were maintained under conventional conditions at the animal facility at the Queensland Institute of Medical Research. These mice are knocked out for β2-microglobulin and H-2Dβ and are transgenic for a chimeric HLA-A2.1 with the α3 domain derived from H-2Dk to allow interaction with murine CD8 and a covalently attached human β2-microglobulin. These mice were immunized with recombinant Ad-gBCMVPoly virus and CMV-specific humoral and cellular immune responses were evaluated at various time points. All protocols were approved by the Queensland Institute of Medical Research ethics committee.

ELISPOT assay. An ELISPOT assay was used to detect HLA-A2-restricted CMV epitope-specific T cells following stimulation with synthetic peptide(s) as described previously (Zhong et al., 2008). Briefly, 2 × 10⁵ responding cells were incubated in triplicate with six pooled HLA-A2-restricted peptide epitopes (1 μg ml⁻¹ of each epitope: VLEETSVML, NLYPVMAVT, RIFAELGYS, RYTRNHEV, VLAELVKQI and AVGGAVASV) for 18–20 h in 96-well Multiscan HA filtration plates (MAHA S4150; Millipore) coated with anti-IFN-γ mAb (Mabtech AB). Irrelevant EBV latent membrane protein peptide (YLLEMLWRL) was used as a control. After incubation, the plates were washed extensively with PBS with 0.5% Tween 20 and incubated with a secondary biotinylated anti-IFN-γ mAb followed by the addition of streptavidin-conjugated alkaline phosphatase. Cytokine-producing cells were detected as purple spots after a 30 min reaction with BCIP and nitro blue tetrazolium. Spots were counted automatically using image analysis software. T-cell precursor frequencies for each peptide epitope were based on the total number of cells and the number of spot-forming cells (s.f.c.) per well (mean of three wells). Epitope-specific spots were calculated after subtraction of the number of spots in control wells consisting of cells without added peptide (mean of three wells).

Intracellular cytokine staining. Splenocytes from immunized mice were incubated overnight at 37°C with CMV peptide epitopes (1 μg ml⁻¹) or stimulator cells pre-coated with CMV peptide epitopes (1 μg ml⁻¹) in growth medium. Brefeldin A (BD Pharmingen) was added during the last 5 h incubation. For CD107a staining, anti-CD107a antibody was added 1 h before adding the brefeldin A. These cells were then washed and incubated with peridinin-chlorophyll protein complex-conjugated anti-CD8, FITC-conjugated anti-CD4 and allophycocyanin-conjugated anti-CD3 at 4°C for 30 min. Cells were washed and then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) at 4°C for 20 min. Cells were then washed in Perm/Wash (BD Pharmingen), incubated with anti-IFN-γ and anti-TNF-α mAbs (BD Pharmingen) at 4°C for 30 min, washed again with Perm/Wash, resuspended in PBS and analysed on a BD FACS Canto.

ELISA for anti-gB. Serum anti-gB antibody titres were evaluated by ELISA as described previously (Zhong et al., 2008). Briefly, PVC 96-well microplates (MP Biomedicals) pre-coated with recombinant CMV gB protein were incubated with serially diluted serum samples for 2 h at room temperature. After washing with PBS/Tween 20, plates were incubated with HRP-conjugated sheep anti-mouse Ig antibody (for murine samples) or HRP-conjugated sheep anti-human Ig antibody (for human samples) for 1 h. The plates were washed and incubated with 2,3,5,5′-tetramethylbenzidine substrate solution (PanBio) and the absorbance at 450 nm was analysed using a ELISA reader.

Vaccinia virus recombinant. The recombinant vaccinia virus constructs encoding CMV antigens IE-1 (Vacc.IE-1) and gB (Vacc.gB) have been described previously (Elkington et al., 2003).

Virus challenge assay. HHD-1 mice were challenged intraperitoneally with recombinant vaccinia virus expressing different proteins from CMV antigens (Vacc.IE1 or Vacc.gB) at a dose of 10⁵ pfu. per mouse. Mice were sacrificed 4 days later and their spleens collected to evaluate the epitope-specific T-cell response by IFN-γ ICS assay, and their ovaries collected to determine vaccinia virus load by plaque...
assay on monkey fibroblast CV-1 cells. To determine vaccinia virus titres, monolayers of CV-1 cells in a six-well flat-bottomed plate were incubated for 2 h at 37 °C with serially diluted ovary lysates. After incubation, 2 ml RPMI 1640 supplemented with 2 % FCS and 0.75 % methylcellulose was added to each well and incubated for further 3 days. After 3 days, the plates were washed with PBS and stained with crystal violet solution (HT901; Sigma) at a working concentration (0.1 % crystal violet in 15 % ethanol) for 30 min and the number of plaques was counted using standard procedures.

**Statistical analysis.** All statistical analysis was conducted using GraphPad Prism 5 software. The results are expressed as mean ± SD. Differences were considered to be statistically significant where P < 0.05.

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