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Induction of Cytomegalovirus-Specific T Cell Responses in Healthy Volunteers and Allogeneic Stem Cell Recipients Using Vaccination With Messenger RNA–Transfected Dendritic Cells

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Background. Infection with human cytomegalovirus (CMV) is a significant cause of morbidity and mortality in solid organ and hematopoietic stem cell transplant (HSCT) recipients. **Methods.** The present study explored the safety, feasibility, and immunogenicity of CMV pp65 messenger RNA–loaded autologous monocyte-derived dendritic cells (DC) as a cellular vaccine for active immunization in healthy volunteers and allogeneic HSCT recipients. Four CMV-seronegative healthy volunteers and three allogeneic HSCT recipients were included in the study. Four clinical-grade autologous monocyte-derived DC vaccines were prepared after a single leukapheresis procedure and administered intradermally at a weekly interval. **Results.** De novo induction of CMV-specific T-cell responses was detected in three of four healthy volunteers without serious adverse events. Of the HSCT recipients, none developed CMV disease and one of two patients displayed a remarkable threefold increase in CMV pp65-specific T cells on completion of the DC vaccination trial. **Conclusion.** In conclusion, our DC vaccination strategy induced or expanded a CMV-specific cellular response in four of six efficacy-evaluable study subjects, providing a base for its further exploration in larger cohorts.

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Infection with human cytomegalovirus (CMV), a member of the β-herpesvirus family, is a significant cause of morbidity and mortality in solid organ and hematopoietic stem cell transplant (HSCT) recipients.[1-5] The virus is present in more than two thirds of donors and recipients before transplantation.[6,7] The overall risk of developing clinically relevant

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CMV disease is mainly determined by baseline CMV-specific serology from donor and recipient as well as the intensity of the immunosuppressive regimen. In CMV-seropositive recipients, CMV infection can be the result of reactivation of latent or persistent virus or superinfection with a different strain of CMV.[8] In CMV-seronegative recipients, CMV disease can result from a primary infection when receiving an allograft from a CMV-seropositive donor.

After primary infection, CMV persists for the lifetime of the infected carrier. In immunocompetent individuals, this state of latency is effectively controlled by the immune system as evidenced by a low viral load as well as a strong CMV-specific T-cell–mediated cellular immune response against certain immunodominant targets, such as the CMV pp65 protein.[9,10] In contrast, given the suppressed T-cell function in immunocompromised patients, there is a significant and unmet need for new immunotherapeutic strategies to reestablish appropriate immune control of CMV. In this perspective, first randomized clinical trials with the Town CMV vaccine, an active vaccination strategy using live-attenuated virus strategies, demonstrated induction of a protective immune response with concomitant protection against CMV disease in renal transplant recipients.[11] Despite encouraging clinical results, this strategy was abandoned because of long-term safety concerns associated with the use of live herpes viruses in the transplant population. Subsequent studies primarily focused on the generation of anti-CMV antibody titers in immunocompromised hosts.[12,13] In a placebo-controlled phase II study, safety and efficacy of a CMV envelope glycoprotein B (gB)-based vaccine supplemented with MF59 adjuvant was demonstrated in seronegative women of child-bearing age.[14] Griffiths and colleagues confirmed that the administration of this vaccine resulted in a significant increase of the gB antibody titer in both CMV-seronegative and CMV-seropositive adults awaiting kidney or liver transplantation.[15] However, this finding only translated in a clinical benefit, that is, reduced duration of viremia, in CMV-seronegative recipients transplanted with grafts from CMV-seropositive donors.

It was suggested that for long-term control of the virus, CMV-specific T cells are also important for immune protection against CMV.[16] Whereas passive immunization by adoptive transfer of CMV-specific T cells has already been successfully applied to HSCT recipients,[17,18] the clinical usefulness of this approach is rather limited because of the cumbersome and time-consuming logistics of CMV-specific T-cell cloning and expansion. Moreover, the technique of adoptive T-cell transfer cannot be applied in the context of solid organ transplantation, in which active immunization protocols may be preferable.[4,19] Others have designed replication-deficient viral vectors encoding CMV antigens to expand T cells directed against viral-encoded antigens. Indeed, in an attempt to address both humoral and cellular immunities a two-component alphavirus replicon particle vaccine expressing CMV gB or a pp65-IE1 fusion protein was shown to induce CMV-specific T cells as well as neutralizing antibodies in seronegative healthy volunteers.[20] However, because this strategy implies the use of virus-like replicon particles based on an attenuated strain of Venezuelan equine encephalitis virus, its use in immunocompromised individuals is limited. Interestingly, in a recent randomized controlled trial with a gB-pp65–based DNA plasmid vaccine in seropositive recipients of an allogeneic HSCT, more time to the first detection of CMV viremia and a shortened duration of viremia was demonstrated in the vaccine group as compared to controls.[21] It remains, however, to be established whether this vaccine is able to induce de novo immune responses in seronegative individuals.

Given the unique capacity of dendritic cells (DC) to initiate primary T-cell responses against pathogens and tumors, DC-based immunotherapy holds promise to trigger CMV-specific immune responses while circumventing the use of viral vectors. Autologous monocyte-derived DC pulsed with CMV protein have been used to ex vivo stimulate T cells from stem cell donors, which in an adoptive setting have been shown to induce an in vivo CMV-specific immune response in HSCT recipients.[22] Feuchtinger et al.[23] reported successful induction of a CMV-specific functional T-cell response by vaccination with protein-loaded DC in an allogeneic HSCT (allo-HSCT) recipient receiving a transplantation from a CMV-seronegative donor. In theory, vaccination with protein-loaded DC has a limited capacity to expand antigen-specific CD8+ T cells because this would require cross-priming of the protein by DC after its processing through the exogenous major histocompatibility complex class II pathway. In contrast, we used messenger RNA (mRNA)-transfected DC, which primarily induce CD8+ T-cell immune responses by means of the endogenous major histocompatibility complex class I pathway. To the best of our knowledge, the present phase I-II study was the first to explore the safety, feasibility, and immunogenicity of CMV pp65 mRNA-loaded autologous monocyte-derived DC from healthy volunteers and allo-HSCT recipients as a cellular vaccine for active immunization against CMV.

TABLE 1.

Yield, purity, and viability of cell products

	Apheresis			DC before freezing		DC after thawing	
Subject no.	WBC ($\times 10^9$)	CD14+ (%)	CD14+ ($\times 10^9$)	Viable DC ($\times 10^6$)	Viability (%)	Viability (%)	Vaccine dose ($\times 10^6$)
HV001	20.3	11	2.2	72.8	69	67	10
HV002	19	11.6	2.2	164	77	72	10
HV003	15.4	23	3.5	130.2	62	60	10
HV004	18.7	25.4	4.7	139	66	66	10
PT001	11.4	37.5	2.3	783	87	87	0.1
PT002	13.4	26.9	3.6	120.5	79	56	0.1
PT003	6.3	23.4	1.5	101.6	77	78	0.1

WBC, white blood cell; DC, dendritic cells; HV, healthy volunteer; PT, allo-HSCT patient.

RESULTS

Feasibility and Safety

There was no statistically significant difference between the viability of mRNA-electroporated monocyte-derived DC before and after cryopreservation, which was 73.9% ± 8.6% and 69.4% ± 10.6%, respectively (P = 0.2; Table 1). All DC vaccines exhibited high expression of CD80, CD83, CD86, and leukocyte antigen (HLA)-DR (Fig. 1A and B). Importantly, the chemokine receptor CCR7, necessary for DC migration to the lymph node, was upregulated on the cell surface, whereas CD14

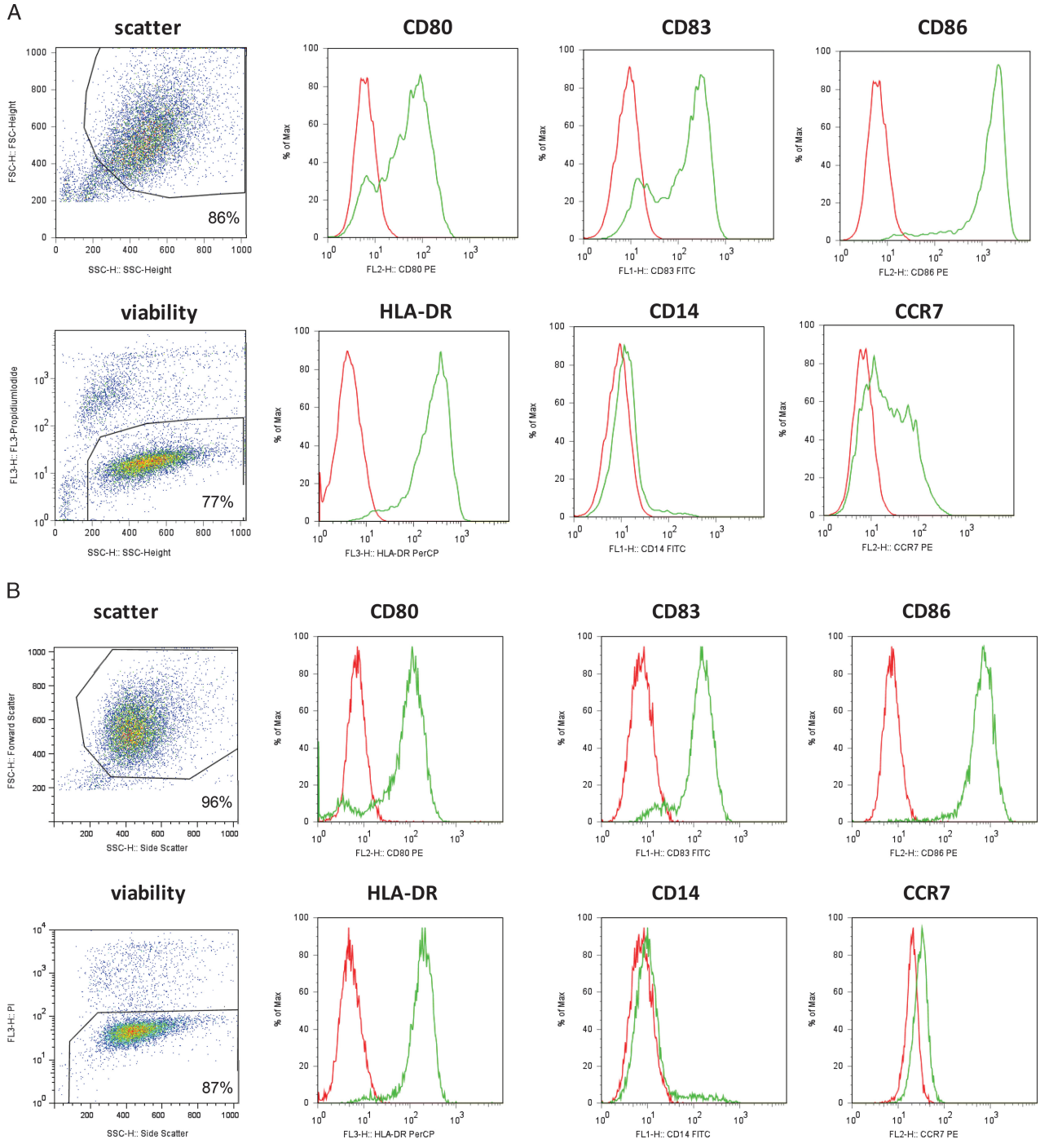

FIGURE 1. Phenotype of mRNA-electroporated DC vaccine. (A) Representative example of healthy volunteers (HV002). (B) Representative example of allo-HSCT recipient (PT001). Upper left: dot plot showing the light scatter profile typical of DC (% of gated cells with high FSC/SSC are indicated). (*lower left*) Dot plot showing the viability as determined by propidium iodide staining (% of viable DC is indicated). (*right*) Histograms showing DC marker expression (*green line*) compared with isotype controls (*red line*). Data shown are representative for all subjects included in the study. HV, healthy volunteer; PT, allo-HSCT patient; DC, dendritic cells; HSCT, hematopoietic stem cell transplantation; mRNA, messenger RNA; FSC, forward scatter; SSC, side scatter.

expression was negative on DC (Fig. 1A and B). Altogether, production of DC vaccines was successful in all subjects from a single leukapheresis procedure.

All study subjects experienced transient local redness and mild swelling of the injection site. Two of four healthy subjects reported of mild headache, and another volunteer reported transient mild myalgia. No serious adverse events were reported in the healthy subjects. One allo-HSCT patient (PT002) developed a moderate grade II graft-versus-host disease (GVHD) of the gastrointestinal tract 6 days after the first DC vaccination; she received immunosuppressive treatment accordingly and was excluded from further participation in the trial. Overall, DC vaccination targeting CMV pp65 was well tolerated in healthy subjects, but requires careful monitoring of GVHD effects in allo-HSCT recipients.

Efficacy

In the healthy volunteers' group and patients' group, CMV pp65 antigenemia remained negative throughout the follow-up period, neither did vaccination result in immunoglobulin (Ig) G nor IgM seroconversion (data not shown), indicative of control of CMV infection.

Dendritic cell vaccination did not affect the relative frequencies of circulating lymphocyte subsets (CD3+, CD4+ and CD8+ T cells, Vdelta2-negative gamma delta T cells, B cells, and natural killer cells), as determined by immunophenotypic analyses (**Table S1, SDC**, http://links.lww.com/TP/B18). Furthermore, in one healthy volunteer (HV004), the relative frequencies of naive (CD45RA+CD62L+), terminally differentiated effector (CD45RA+CD62L–), effector memory (CD45RA–CD62L–), and central memory (CD45RA–CD62L+) subsets within the CD3+CD4+ and CD3+CD8+ T-cell compartments were additionally tested and remained similar post-DC vaccination as compared to pre-DC vaccination (data not shown).

To detect CMV pp65-specific interferon (IFN)-γ–producing T cells, cryopreserved peripheral blood monocyte cells (PBMC) from healthy volunteers and patients were used in an IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay after in vitro rechallenge with CMV pp65 antigens. Three of four healthy volunteers could be defined as CMV pp65-specific immune responders after completion of the DC vaccination cycle, according to the response definition criteria (Fig. 2A–D). Overall, there was a significant increase in the level of expansion of CMV-specific IFN-γ-producing T cells after DC vaccination (Fig. 2E). Furthermore, although both efficacy-evaluable allo-HSCT patients presented a CMV pp65-specific T-cell response already before DC vaccination, one patient (PT001) displayed a threefold increase in CMV pp65-specific spot-forming cells on completion of the DC vaccination trial (Fig. 3A and B).

DISCUSSION

CMV disease is an important risk factor in immunocompromised patients and can result from primary infection (CMV-seronegative recipients) or from reinfection or reactivation (in CMV-seropositive recipients) after solid organ transplantation and allogeneic hematopoietic stem cell transplantation.

We evaluated the use of DC vaccination as a possible strategy to prevent infectious complications of CMV disease. We demonstrated that vaccination with CMV pp65 mRNA-loaded monocyte-derived DC is a safe way to induce or enhance CMV-specific cellular immunity. No acute serious adverse events occurred in any of the four healthy volunteers receiving 4 weekly administered doses of the DC vaccine. Similar observations were made in two of three patients with a hematologic malignancy receiving allo-HSCT. Given the possible risk for development of GVHD after immune-stimulatory therapy, induction or worsening of alloreactivity in the allogeneic HSCT recipients was carefully monitored and the number of injected DC used per vaccine was substantially reduced (100-fold) as compared to "routine" DC injections. One patient (PT002), recipient of a matched unrelated donor graft, developed a moderate grade II GVHD of the gastrointestinal tract. Although the incidence of GVHD is increased in HLA-matched unrelated donors as compared to matched-related donors, with rates of 70% to 90%,[24] we

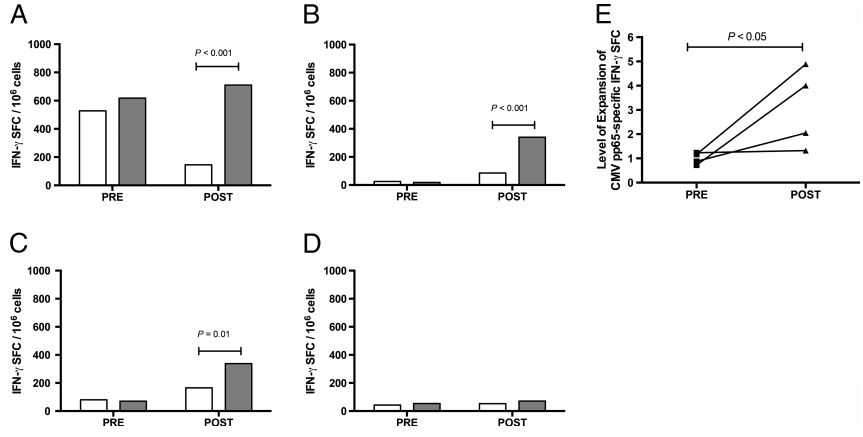

FIGURE 2. CMV pp65 mRNA-electroporated DC induce CMV-specific IFN-γ T-cell responses in CMV-seronegative healthy volunteers. (A–D) PBMC from healthy volunteers, collected before and after the vaccination cycle, were stimulated in vitro by CMV pp65 mRNA-electroporated autologous DC. After 7 days of co-culture, PBMC were harvested and restimulated with a pp65-derived pool of overlapping peptides in an IFN-γ ELISpot assay to evaluate the number of antigen-specific IFN-γ–producing SFC or left untreated as a control. Each bar represents the mean spot number of quadruplicates. (E) Results are shown as the mean level of expansion of quadruplicate analyses as compared to the background reactivity for each donor. ELISpot, enzyme-linked immunosorbent spot; CMV, cytomegalovirus; DC, dendritic cells; IFN, interferon; PBMC, peripheral blood mononuclear cell; mRNA, messenger RNA; SFC, spot-forming cells.

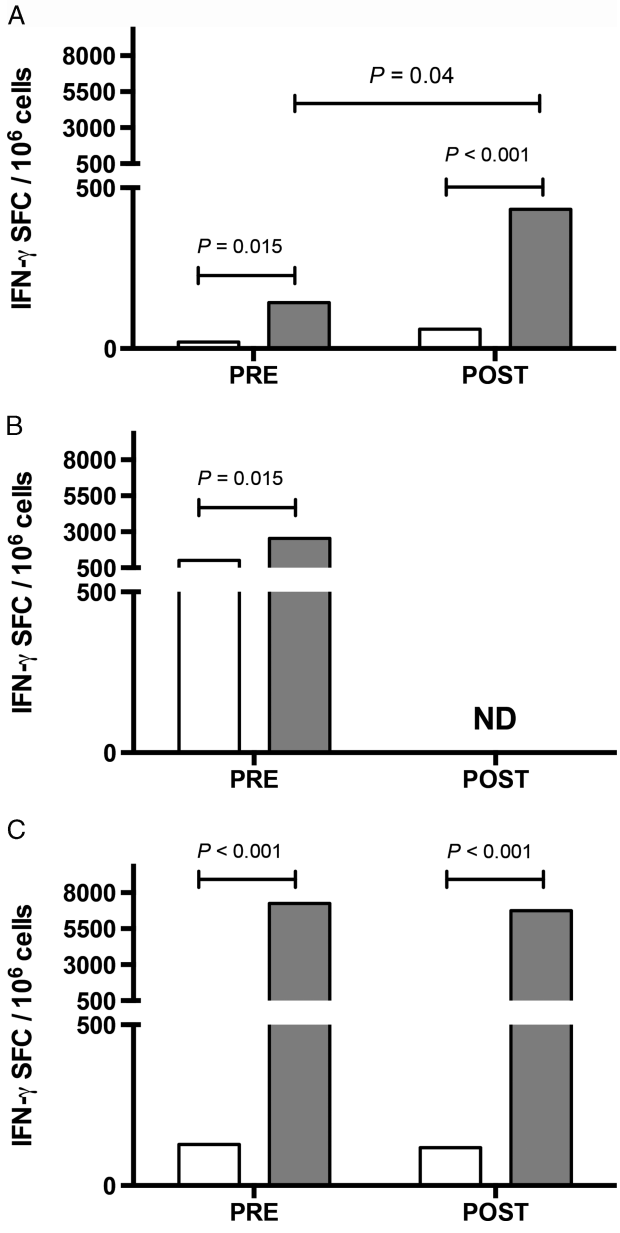

FIGURE 3. CMV pp65 mRNA-electroporated DC induce CMV-specific IFN-γ-producing T cell responses in CMV-seropositive allo-HSCT patients. PBMC of allo-HSCT patients, collected before and after DC vaccination, were thawed and electroporated with CMV pp65-encoding mRNA after an overnight resting period. Using a direct IFN-γ ELISpot, the number of CMV pp65-specific IFN-γ–secreting T cells (SFC) were analyzed 21 hr after electroporation. Analysis of prevaccination and postvaccination samples is shown for (A) CMV PT001, (B) CMV PT002, and (C) CMV PT003. Each bar represents the mean spot number of quadruplicates. HSCT, hematopoietic stem cell transplantation; CMV, cytomegalovirus; DC, dendritic cells; IFN, interferon; PBMC, peripheral blood mononuclear cell; mRNA, messenger RNA; SFC, spot-forming cells; PT, allo-HSCT patient.

cannot rule out a causative relationship between DC vaccination and GVHD. Indeed, because the onset of GVHD occurred 6 days after the first vaccination, one possible explanation might be that donor-derived DC stimulated or expanded alloreactive donor T cells. The patient received appropriate immunosuppressive therapy and was excluded from further participation in the current trial. In agreement with our observations, Grigoleit et al.[25] reported in a previous phase I-II clinical trial that vaccination with donor-derived DC was safe in allo-HSCT recipients (n = 24): no serious adverse events were reported, except for one patient who developed acute grade III GVHD of the skin and gut 2 months after vaccination.

Second, vaccination with CMV pp65 mRNA-loaded monocyte-derived DC is feasible because successful production of four DC vaccines was achieved after one single leukapheresis procedure per subject. Importantly, as little as 0.1×10^6 DC per vaccine seemed to be sufficient to enhance a CMV-specific T-cell response in one patient (Fig. 3A), adding observations to the ongoing debate regarding the number of DC required to initiate an immune response.[26,27]

To assess the efficacy of DC vaccination, we studied the control of CMV infection, as well as the induction and expansion of CMV-specific T-cell responses. The CMV pp65 antigenemia in peripheral blood leukocytes was used to predict the development of CMV disease in transplant patients.[28,29] Transplant recipients are usually at high risk of developing CMV disease. In the context of allo-HSCT, the risk of transmission of CMV by the cell product from a CMV-seropositive donor to a CMV-seronegative recipient is approximately 30%.[30-32] In contrast, without prophylaxis approximately 80% of CMV-seropositive patients experience CMV infection after allo-HSCT. In this study, none of the allo-HSCT recipients, including PT002 who did not complete the entire vaccination cycle, developed primary CMV infection or reactivation, as evidenced by negative CMV pp65 antigenemia in all patients throughout a 6-month follow-up period. Furthermore, at month 6, both patients were negative for CMV DNA by PCR analysis. These results are promising but need further confirmation in larger cohorts with long-term follow-up.

More recently, there has been great interest in using methods to determine CMV-specific immune reconstitution after HSCT as an additional strategy to determine the risk of CMV infection. Indeed, it has been demonstrated that lack of CMV-specific CD8+ cytotoxic T lymphocyte responses predisposes to CMV infection, whereas reconstitution of these responses after HSCT correlates with protection from CMV and improved outcome of CMV disease.[33,34] In this study, DC vaccination resulted in de novo induction of CMV-specific T-cell responses in three of four CMV-seronegative healthy volunteers, and a marked increase (threefold expansion) of IFN-γ-producing spot-forming cells was demonstrated in one of two allo-SCT patients. In the other patient, allo-HSCT PT003, the CMV-specific T-cell response was not boosted on completion of the vaccination cycle, but this subject already showed a high CMV-specific T-cell response at the beginning of the study. Importantly, the observed effects were not dependent on the CMV serostatus. This is in marked contrast to established protocols for the generation of sufficient numbers of donor-derived CMV-specific T cells for adoptive immunotherapy, in which the donor has to be CMV seropositive.[35] Moreover, the use of DC has advantages over adoptive T-cell transfer because DC can amplify virus-specific T cells in vivo. In contrast, up to 8 weeks of in vitro culture are required to obtain enough CMV-specific T cells and to deplete alloreactive T cells, whereas preparation of DC vaccines can be realized after one leukapheresis procedure.

TABLE 2.

Demographic and clinical features of subjects enrolled in a CMV pp65-DC vaccination trial

Subject no.	Sex	Age	Diagnosis	CMV serostatus		Donor	CMV serostatus donor		Day of first DC vaccination after HSCT
				IgG	IgM		IgG	IgM	
HV001	M	32	healthy	–	–	NA	NA	NA	NA
HV002	M	20	healthy	–	–	NA	NA	NA	NA
HV003	M	34	healthy	–	–	NA	NA	NA	NA
HV004	M	30	healthy	–	–	NA	NA	NA	NA
PT001	M	21	ALL	–	–	MRD	+	–	day 56
PT002	F	41	ALL	+	–	MUD	+	–	day 63
PT003	F	59	CLL	+	–	MRD	+	–	day 43

HSCT, hematopoietic stem cell transplantation; HV, healthy volunteer; PT, allo-HSCT patient; M, male; F, female; ALL, acute lymphoblastic leukemia; CLL, chronic lymphoid leukemia; NA, not applicable; MUD, matched unrelated donor; MRD, matched related donor.

In conclusion, our DC vaccination strategy induced or expanded a CMV-specific cellular response in four of six efficacy-evaluable study subjects, providing a base for its further exploration in larger cohorts.

MATERIALS AND METHODS

Subjects

Four CMV-naive healthy volunteers and three patients who underwent allo-HSCT were included in the current study. Detailed medical history, physical examination, and routine laboratory tests revealed no relevant abnormalities in healthy volunteers. Exclusion criteria were (i) difficult peripheral venous access and (ii) medication with an immunomodulatory effect, including administration of vaccines in a period of 100 days before the first DC vaccination and 100 days after the fourth DC vaccination. Cytomegalovirus-naive serostatus was defined as the absence of both CMV-specific IgG and IgM, as determined by enzyme-linked immunosorbent assay (Diasorin, Saluggia, Italy). The leukapheresis procedure for vaccine production in patients who underwent allo-HSCT was performed 4 weeks after transplantation. Two of three patients were CMV-seropositive, as defined by CMV IgG>0.4 IU/mL. All stem cell donors showed positive levels of CMV-specific IgG. Cytomegalovirus antigenemia and CMV-specific IgM was negative at the start of vaccination for all three patients.

The study was approved by the Ethics Committee of the Antwerp University Hospital, according to the principles of the Declaration of Helsinki, and all subjects gave written informed consent. Demographic and clinical data are summarized in Table 2.

Trial Design

Study subjects received a total of four intradermal clinical-grade DC vaccines on a weekly interval (**Figure S1, SDC,** http://links.lww.com/TP/B18) at a dose of 10×10^6 or 0.1×10^6 DC per vaccination for HV and allo-HSCT patients, respectively. DC vaccines were injected at the ventromedial region of the upper arm, approximately 5-10 centimeters from the axillary lymph nodes. Primary endpoints, that is, safety and toxicity, were graded at every visit both clinically as by routine laboratory hematology and biochemistry testing.

Vaccine Preparation

Clinical-grade DC vaccines were prepared after a single leukapheresis of nonmobilized blood and immunomagnetic selection of CD14+ monocytes using a CliniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously.[36-38] Briefly, isolated CD14+ monocytes were cultured in CellGro medium (CellGenix, Freiburg, Germany) supplemented with 1% human AB serum (MP Biomedicals, Belgium), granulocyte-macrophage colony-stimulating factor and interleukin-4 (Gentaur, Brussels, Belgium). Maturation was induced at day 6 through addition of tumor necrosis factor-α and prostaglandin E_2 (Pharmacia Upjohn, Puurs, Belgium) for 48 hr. At day 8, mature DC were harvested, electroporated with CMV pp65 mRNA[19] (Curevac GmbH, Tübingen, Germany) and viably cryopreserved in ready-to-use aliquots at –80°C. After thawing, the vaccine was resuspended in 500 μL 0.9% NaCl solution and delivered to the clinic for intradermal administration. Cell count and viability were analyzed at various time intervals of vaccine preparation (Table 1). For each DC vaccine, the expression of CD80, CD86, CD14, HLA-DR, CD83, and CCR7 was evaluated by multiparametric flow cytometry using the following antibodies (purchased from BD Biosciences, Erembodegem, Belgium; unless stated otherwise): anti-CD80 phycoerythrin (PE), anti-CD86 PE, anti-CD14 fluorescein isothiocyanate (FITC), anti-HLA-DR peridinin chlorophyll protein complex (PerCP), anti-CD83 FITC (Life Technologies, Ghent, Belgium) and anti-CCR7 PE (R&D Systems, Abingdon, UK).

Immunomonitoring

The secondary endpoint was to assess the ability of DC to induce or enhance CMV pp65-specific T-cell responses. To this end, 100 mL of peripheral blood was taken immediately before the first vaccination (PRE) and 1 month after the fourth vaccination (POST) (**Figure S1, SDC,** http://links.lww.com/TP/B18). Peripheral blood mononuclear cells were isolated, cryopreserved viably and thawed for batch analysis.

Lymphocyte Subsets

For immunophenotyping of lymphocyte subsets, the following murine antihuman monoclonal antibodies were used for direct immunofluorescence staining (all antibodies were purchased from BD Biosciences unless stated otherwise): anti-HLA-DR FITC, anti-CD38 PE, anti-CD62 ligand (CD62L) PE coupled to the cyanine dye Cy5 (PE-Cy5), anti-CD16 PE-Cy7, anti-CD56 PE-Cy7, anti-CD3 PerCP coupled to the cyanine dye Cy5.5 (PerCP-Cy5.5), anti-CD8 Pacific Blue (PB) (Dako, Heverlee, Belgium), anti-CD4 Alexa Fluor 700, CD45RA allophycocyanin (APC), anti-CD19 APC-Cy7, anti-V∂2TCR PE, anti-V∂ FITC (R&D Systems, Abingdon, United Kingdom). Labeled cells were analyzed on a CyFlow

ML (Partec, Münster, Germany) flow cytometer. For analytical flow cytometry, at least 10^4 events with forward and side scatter properties of lymphocytes with CD3, CD4, or CD8 staining were measured. All data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

ELISpot Analysis of Antigen-Specific T Cells

Detection of functional CMV pp65-specific T cells was performed in a direct IFN-γ ELISpot assay using CMV pp65 mRNA-electroporated cryopreserved PBMC, as previously described.[39] Alternatively, cryopreserved autologous PBMC were stimulated with CMV pp65 mRNA-electroporated mature DC. After 7 days, PBMC were harvested and rechallenged with a CMV pp65 protein-spanning peptide pool of overlapping peptides for an additional 24 hr or left untreated as a control. Antigen-specific IFN-γ secretion after peptide stimulation was determined by an IFN-γ ELISpot assay (Diaclone, Amsterdam, The Netherlands), according to the manufacturer's instructions. Frequencies of antigen-specific IFN-γ–secreting cells were calculated based on the number of spots counted using an automated iSpot Reader system (AID GmbH, Strassburg, Germany). For the definition of a positive response, guidelines from the Cancer Vaccine Consortium were followed: per 10^6 PBMC, the mean antigen-specific spot count must be greater than or equal to 20 spots and at least two times as high as the background reactivity.[40]

Statistics

Comparisons were validated using a Student's *t* test. A *P* value less than 0.05 was considered as statistically significant.

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