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ORIGINAL ARTICLE

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Increased liver temperature efficiently augments human cellular immune response: T-cell activation and possible monocyte translocation

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Abstract Hyperthermia (HT), in combination with other conventional therapeutic modalities, has become a promising approach in cancer therapy. In addition to heat-induced apoptosis, an augmented immunological effect is considered to be a benefit of hyperthermic treatment over chemo- or radiotherapy. Here, we investigated the effect of regional HT targeting the liver on immune cells, especially T cells and antigen-presenting cells, which are important in recognizing and eliminating tumor cells and pathogens such as viruses. In healthy volunteers exposed to such regional HT, both $CD4^+$ and $CD8^+$ T cells that express an activation marker CD69 increased transiently at 1 h post-treatment, with a subsequent decrease to base levels at 6 h after the treatment. At 24 h post-treatment, the percentage of CD69-positive cells significantly increased again but only among $CD8^+$ T cells. IFN-γ production from PHA-stimulated peripheral blood mononuclear cells was gradually and significantly increased in the 2 days following the heating procedure, peaking at 36 h post-treatment. Furthermore, we found marked increases in plasma levels of IL-1β and IL-6 starting at 24 h post-treatment. With regard to the number of each leukocyte subpopulation, a transient and dramatic decrease in the number of a subset of monocytes, $CD14^+$ $CD16^-$ cells, was observed at 1 h after the hyperthermic treatment, suggesting that the regional HT aimed at the liver may have influenced the extravasation of blood monocytes. No significant changes in T-cell activities or monocyte counts were observed in the volunteers exposed to heating of the lungs or the legs. These results suggest that heating of the liver may efficiently induce cellular immune responses to liver cancers.

Keywords T cell · Monocyte · Cytokines · Cellular immunity · Hyperthermia · Liver cancer

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Introduction

Hyperthermia (HT) has been used in combination with chemotherapy and/or radiation therapy of human malignant disorders and is considered to be a promising adjuvant therapeutic strategy for the treatment of certain types of tumors [9, 31, 33]. In parallel to the encouraging clinical observations, a large number of investigations have been performed to evaluate the ability of heat in modulating cell death, tumor blood flow, and actions of radiation therapy and antineoplastic drugs (reviewed in [12]). Furthermore, increased body temperature has been shown to stimulate the immune system through augmentation of (a) activities of T cells and NK cells, (b) production of cytokines, (c) immune responses to viral infection, and (d) mobility of leukocytes [4, 8, 12, 21, 24, 27].

The number of patients with malignant liver tumors is increasing in Japan, the vast majority of which are associated with Hepatitis C virus (HCV)/Hepatitis B virus (HBV) infection. Regional HT aimed at the liver in combination with other modalities has produced promising results in the treatment of hepatocellular carcinomas [16, 29, 35]. We have recently demonstrated that, in patients with hepatocellular carcinoma, their $CD4^+/CD8^+$ ratio of T cells and the NK-cell activity increased following the regional HT treatment [20]. Stimulation of host immune responses is considered to

be a possible mechanism by which regional HT treatment, as well as systemic HT, exerts its antitumor and antiviral activities, although clear evidence is still lacking. To maximize the potential clinical benefits of the HT treatment, it is now necessary to understand thoroughly the presumed immunological effects of the regional HT. Therefore, we have focused our attention here on the effects of heating of the liver on T cells and monocytes that are involved in cellular immune responses. Our findings include a drastic decrease in blood $CD14^+$ $CD16^-$ inflammatory monocytes soon after the HT treatment, along with the activation of T cells. This is the first report indicating the effect of regional HT on changes in the populations of peripheral monocytes.

Materials and methods

Volunteer blood donors

Eleven healthy volunteers, five males and six females aged between 29 and 65 (average: 34.2 ± 3.4) were enrolled in the regional HT treatment or the heating of the legs and feet. All of the volunteers had no signs or symptoms of fever, infectious diseases, or immune disorders, and their leukocyte counts in peripheral blood ranged from 4,000 to 8,000 per microliter of blood. Among our subjects, one had undergone splenectomy 22 years prior to the enrollment. Some volunteers were treated with both the regional HT procedures aimed at the liver and at the lungs with an adequate interval in between. All the volunteers were given thorough explanation of the purpose, procedures, and possible risks of the experiment through written information, and have given their consent. The entire experimental procedure has been examined and approved by the Research Evaluation Committee of the Wakayama Medical College.

Hyperthermic treatment

A radio-frequency capacitive heating device Thermotron RF-8 (Yamamoto Vinita, Yao, Japan) was used to achieve regional capacitive heating of the right upper abdominal region across the liver or of the chest across the lungs as described [20]. The device has been approved as a therapeutic instrument for non-invasive HT treatment of deep-seated malignant tumors by the Ministry of Health, Labor and Welfare of Japan (Medical Device Approval No. 59B1728). Applications of this device are covered by the government's health insurance system, being regarded as a routine clinical modality [28]. Each volunteer's body was wrapped with a cooling jacket and placed between two electrodes. The effective diameter of the electrodes was 18 cm, and each enrollee received an average continuous radio-frequency irradiation of 750 W over a duration of 1 h. The estimated temperature of the liver achieved by this heating procedure was 40°C, based on the data collected by the actual measurements of tissue temperature during HT treatment of liver tumors [16]. As an additional control group, legs and feet of four healthy volunteers were placed in a water bath and kept at 41°C for 1 h. Axillary or sublingual temperatures were measured at the end of each heating procedure in the volunteers of each group: 38.5 ± 0.5°C in the liver-targeted HT, 38.9 ± 0.3°C in the lung-targeted HT, and 37.8 ± 0.3°C in the leg-heating group, without significant differences in the body temperature between the groups. Vital signs including blood pressure and pulse rate were monitored during and after the HT treatment. No adverse effect due to the above heating procedures was reported, except regional irritation of the skin adjacent to the electrodes and flushing of the face, which were well tolerated.

Preparation of peripheral blood mononuclear cells (PBMC)

Blood samples were drawn from each volunteer through a subcutaneous vein in the forearm just before and soon after a 1 h HT treatment, and 1, 2, 6, 24, 36, 48, and 72 h after the end of the heating procedure. Twenty microliters of heparinized peripheral blood was collected at each time point and kept at room temperature. Within 1 h after blood collection, each peripheral blood sample was diluted with an equal volume of phosphate-buffered balanced salt solution (PBBS), and mononuclear cells were separated by density gradient centrifugation using Ficoll-Paque PLUS (Amersham Bioscience, Piscateway, New Jersey). Mononuclear cells were washed twice with PBBS, counted in a hemocytometer, and resuspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 10^7 cells/ml.

Analyses of cell-surface markers and cell numbers

A total of 10^6 PBMC were incubated with the following combinations of fluorescent-labeled antibodies. For analyses of lymphocyte subsets, we used fluorescein isothiocyanate (FITC)-labeled anti-human CD4, FITC-labeled anti-human CD8, phycoerythrin-cyanin 5.1 (PC5)-labeled anti-human CD3, PC5-labeled anti-human CD16, FITC-labeled anti-human HLA-DR reactive to a monomorphic α-chain epitope, and PC5-labeled anti-human CD19. For staining of monocyte subsets and dendritic cells (DCs), we utilized phycoerythrin (PE)-labeled anti-human CD14, PC5-labeled anti-human CD16, FITC or PC5-labeled anti-HLA-DR, and FITC-labeled mixture of multiple lineage-specific antibodies (Lin 1; Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) that contains antibodies reactive to human CD3, CD14, CD16, CD19, CD20, and CD56 to distinguish peripheral blood DCs and basophils from other leucocytes [19, 30]. To examine the levels of T-cell activation, PE-labeled anti-human CD69 was used in combination with FITC-labeled anti-CD4 or

anti-CD8, and PC5-labeled anti-CD3. Isotype-matched mouse myeloma proteins labeled with the above fluorescent dyes were used as negative control antibodies in all analyses. All antibodies except Lin 1 cocktail were purchased from Immunotech Coulter, Marseille, France. A number of 0.3–3.5×10^5 cells stained with the antibodies were analyzed with a Becton-Dickinson FACSCalibur (Becton-Dickinson) and CellQuest software. Dead cells and debris were excluded based on their forward and side scatter profiles, and percentages of each leukocyte subpopulation among viable mononuclear cells were determined by multi-color staining with appropriate antibodies. Absolute numbers of each cell subset were then calculated by multiplying the number of total mononuclear cells with the corresponding cell percentage determined. Geometric mean fluorescence intensity for CD14 expression was obtained from the appropriate histograms for the $CD14^+$ $CD16^-$ subset of monocytes.

In vitro stimulation of isolated PBMC and measurements of cytokine production

A number of 8×10^5 PBMC were cultured in each well of 96-well microculture plates in RPMI-1640 medium supplemented with 10% FBS and 10 μg/ml (final) PHA (PHA-P, Sigma Chemical Co., St. Louis, MO). After 48 h of culture at 37°C, supernates were collected, and the amounts of IFN-γ and IL-4 were measured in duplicate by an enzyme-linked immunosorbent assay using OptEIA human kits (Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Measurements of plasma IL-1β and IL-6

Plasma were separated from a small aliquot of blood drawn at the same time when peripheral blood was collected for the isolation of PBMC. The concentrations of IL-1β and IL-6 in each plasma sample were measured by using the Opt EIA human kits as described above.

Statistical analyses

The paired t test for samples exhibiting Gaussian distribution and the Wilcoxon's test for samples exhibiting non-Gaussian distribution were employed to compare the values at each time-point after the HT treatment with the pre-treatment values in the same volunteer group by using the online tools archived in the Multifunctional Web Calculator established and maintained by Prof. S. Aoki, Faculty of Social and Information Studies, Gunma University, Maebashi, Japan (http://www.aoki2.si.gunma-u.ac.jp/calculator/index.html), and a software for statistical analysis, JSTAT, which was developed by M. Sato and downloadable from the following website: http://www.vector.co.jp. P values less than 0.05 were considered to represent statistically significant differences.

Results

Changes in the number of blood mononuclear cells following the regional HT treatment

To investigate the effects of the regional HT aimed at the liver on immune cells, we first assessed possible changes in the numbers of peripheral mononuclear cells that consist of lymphocytes, monocytes, and DCs in healthy volunteers exposed to the regional HT treatment. Following a 1 h HT treatment aimed at the liver, the absolute numbers of mononuclear cells in the peripheral blood slightly decreased throughout the 48 h of observation, although significant differences were not found in comparison with the pre-treatment value (Table 1). This change in the number of mononuclear cells was mainly due to the decrease in lymphocytes. On the other hand, there was a drastic decrease in the number of monocytes soon after the treatment in four of the six subjects who received the HT aimed at the liver. The average number of monocytes per 1 ml of blood significantly decreased from 8.5×10^4 at pre-treatment to 4.5×10^4 at 1 h post-treatment. Due to the decreased number of monocytes, a reduction in the monocyte/lymphocyte ratio was also found at 1 h post-treatment.

To evaluate whether the above changes were distinctive to the heating of the liver, the same parameters were analyzed in healthy volunteers exposed to a regional HT treatment aimed at the chest, in which the lungs were the primary target of heating, or in those received heating of the legs. No significant changes in the

Table 1 Absolute numbers and the percentages of mononuclear cells in volunteers exposed to the regional hyperthermic treatment aimed at the liver ($n=6$)

	Cell numbers (×10^4/ml blood)			Percent among mononuclear cells		Ratio
	Total mononuclear cells	Lymphocytes	Monocytes	Lymphocytes	Monocytes	Monocyte/lymphocyte
Pre-HT	184±31	165.1±32.1	8.5±0.8	87.2±2.3	5.4±1.1	6.4±1.4
1 h after HT	158±14	142.5±13.5	4.5±1.4*	89.2±1.3	2.8±0.8	3.2±1.0
6 h	148±15	131.5±13.9	7.6±1.7	88.6±0.5	4.8±0.6	5.4±0.7
24 h	173±29	155.2±27.3	7.9±2.0	89.4±1.2	4.6±1.0	5.2±1.2
48 h	147±9	130.4±7.7	7.2±2.1	89.0±1.6	4.7±1.2	5.4±1.3

*Significant difference ($P<0.05$) in comparison with the pre-treatment value

number of monocytes at 1 h post-treatment were observed in any of the subjects who underwent the lung-targeted HT or the heating of the legs (data not shown), indicating a specific role of the liver in the HT-induced decrease in the monocyte numbers.

Changes in the percentages of lymphocyte subpopulations

We further investigated whether the regional HT treatment affected the percentages of lymphocyte subpopulations. In both groups of the volunteers exposed to the HT treatment aimed at the liver and that aimed at the lungs, the number of $CD4^+$ T cells decreased, while that of $CD8^+$ T cells was unchanged (Table 2). The $CD4^+/CD8^+$ ratio at 1 h post-treatment was reduced in five of the six subjects who received the liver-targeted HT and in all who received the lung-targeted HT treatment; however, significant differences were observed only in those whose chest was heated. Only in the volunteers exposed to the regional HT aimed at the liver, the numbers of B cells were slightly decreased at all time-points following the treatment, although the changes in comparison with the pre-treatment value were not statistically significant, and no significant changes in NK cell numbers were observed. No significant changes were observed in the subjects whose legs were heated (data not shown).

Enhancement in the activation of peripheral T cells

To examine whether T-cell functions were influenced by the regional HT treatment, we next analyzed the expression of the early activation antigen CD69 on blood T cells. Figure 1a shows representative patterns of CD69 expression in T-cell subsets before and at different time-points after the HT treatment aimed at the liver. The frequency of CD69-expressing cells in both $CD4^+$ and $CD8^+$ populations of T cells was significantly elevated at 1 h after the treatment in all subjects: the mean percentages of CD69-expressing cells among $CD4^+$ and $CD8^+$ T cells increased by 1.7- and 1.5-fold, respectively, compared to the corresponding percentages at pre-treatment (Fig. 1b). They returned to basal levels at 6 h after the treatment, but again increased in both T-cell subsets at 24 h post-treatment, although only the changes in $CD8^+$ T cells were significant. The CD69-expressing cells in both T-cell subsets showed a tendency to increase in the subjects whose lungs were heated, although there were no significant differences in comparison with the pre-treatment values. No changes in CD69 expression were found in the subjects who received the heating of the legs.

Changes in IFN-γ production from PBMC

To further evaluate the possible effect of the regional HT treatment on T-cell activation, cytokine productions from PBMC were also analyzed. PBMC collected before and at different time-points after the treatment were stimulated with PHA, and the accumulation of interferon (IFN)-γ and interleukin (IL)-4 in the medium during 48 h of cell culturing was measured. In the volunteers who received the liver-targeted HT, the amount of IFN-γ produced gradually and significantly increased in the 2 days following the heating procedure, peaking at 36 h after the end of the HT treatment (Fig. 2a). In the subjects exposed to heating of the lungs or the legs, there were no significant changes in the PHA-induced production of IFN-γ. On the other hand, the amount of IL-4 produced after PHA stimulation showed no change at all during the 3 days of observation in all groups (Fig. 2b).

Elevation of plasma cytokine levels

Plasma levels of proinflammatory cytokines, IL-1β and IL-6 were measured before and following the regional HT. In subjects who underwent HT aimed at the liver, both cytokines showed no significant change immedi-

Table 2 Absolute numbers ($\times 10^4$/ml blood) of lymphocyte subsets in volunteers exposed to the regional hyperthermic treatment

	$CD4^+$T	$CD8^+$T	$CD4^+/CD8^+$	B ($CD19^+$)	NK ($CD16^+$ HLA-DR^-)
Liver-targeted HT ($n=6$)					
Pre-HT	71.8±20.0	28.3±3.8	2.4±0.4	20.6±6.8	16.5±4.0
1 h after HT	55.6±6.8	28.8±4.9	2.1±0.4	14.9±2.2	17.2±3.0
6 h	52.0±6.8	24.3±3.0	2.2±0.3	15.1±2.6	16.8±4.4
24 h	65.9±17.3	28.5±4.2	2.3±0.4	15.6±2.5	17.8±3.7
48 h	49.7±5.0	24.4±3.9	2.4±0.5	16.4±2.0	16.9±3.2
Lung-targeted HT ($n=5$)					
Pre-HT	63.5±9.8	27.8±5.3	2.6±0.6	15.4±3.2	16.1±1.2
1 h after HT	54.6±8.8	29.9±7.4	2.1±0.4*	15.9±3.7	15.5±2.3
6 h	60.2±12.2	24.9±6.1	2.7±0.6	14.7±4.9	11.6±2.4
24 h	58.0±11.4	26.2±4.6	2.4±0.5	18.6±5.6	16.4±1.7
48 h	50.4±7.9	24.4±3.8	2.2±0.4	09.1±2.8	13.8±2.9

*Significant difference ($P<0.05$) in comparison with the pre-treatment value

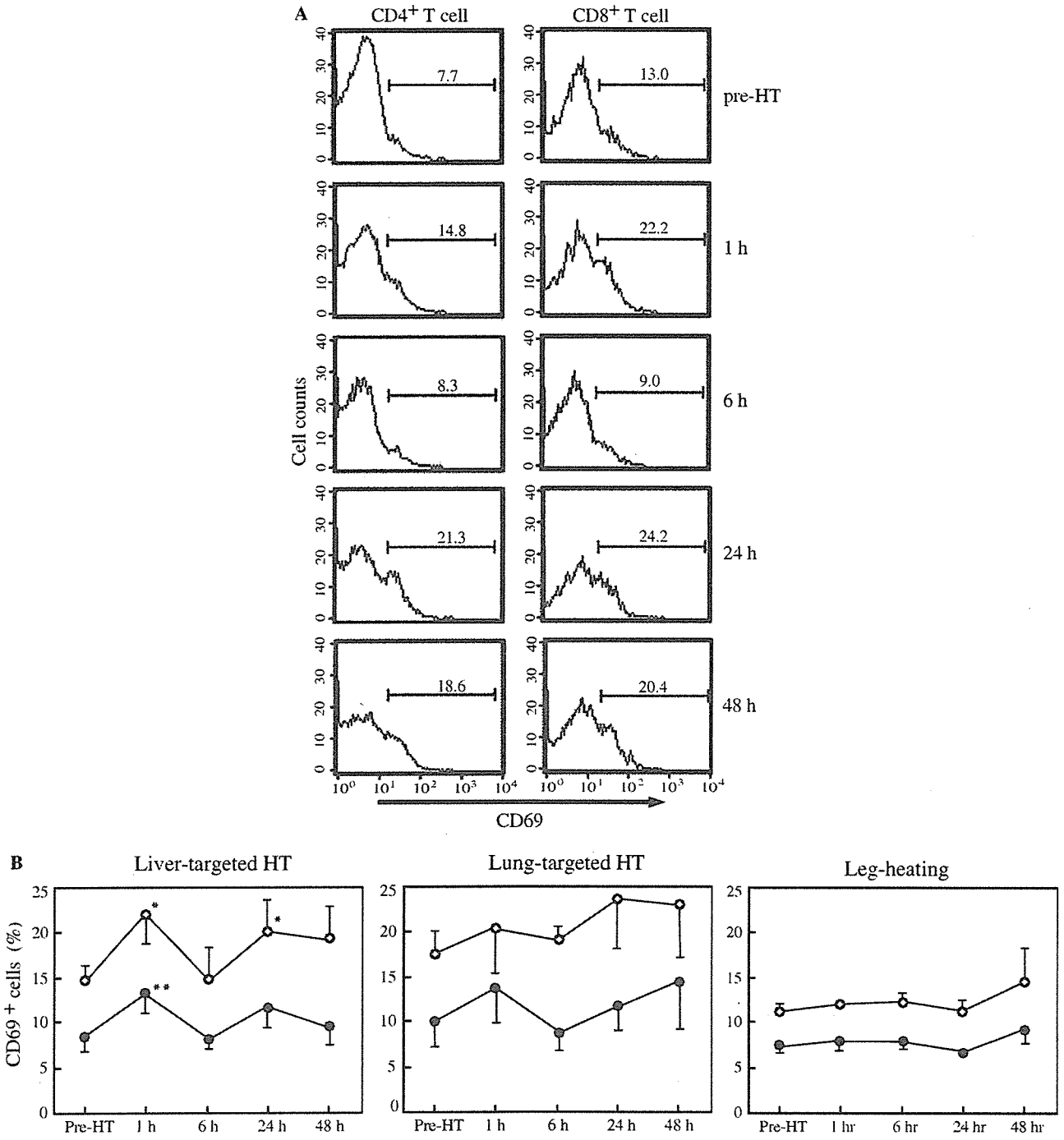

Fig. 1 Effects of regional hyperthermic treatment aimed at the liver on peripheral T-cell activation. PBMC were prepared before and at the indicated time-points after the 1 h HT treatment aimed either at the liver or at the lungs, or after 1 h heating of the legs, and CD69 expression on $CD4^+$ and $CD8^+$ T cells was monitored by flow cytometry. **a** Histograms showing CD69 expression on each T-cell subset in a representative volunteer who received the liver-targeted HT treatment. *Numbers* indicate the percentage of CD69-positive cells. **b** Time course of CD69 induction on $CD4^+$ (*closed circles*), and $CD8^+$ (*open circles*) T cells after the HT treatment aimed at the liver (*left panel*) or at the lungs (*middle panel*), or heating of the legs (*right panel*). Each *circle* and *bar* show a mean ± SEM calculated from the data obtained from four to six volunteers. *Significantly different from the mean value observed before the HT treatment, $P < 0.05$; **significantly different from the same pre-treatment value, $P < 0.01$

ately after the treatment, but continued to increase gradually towards the end of the observation period, exhibiting significant differences at 24 and 48 h post-treatment (Fig. 3). Again, no changes in the plasma cytokine levels were found in the volunteers who underwent heating of the lungs or the legs.

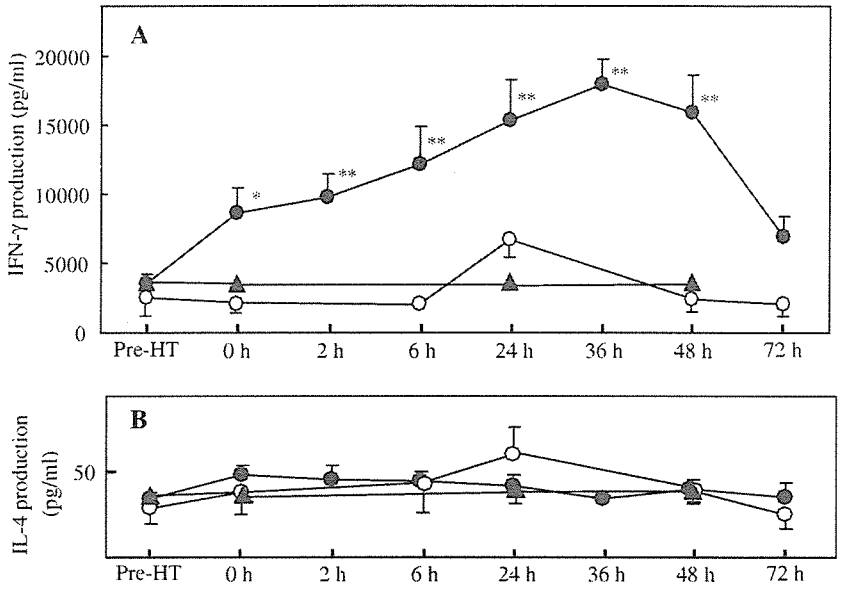

Fig. 2 Changes in PHA-induced cytokine production from cultured PBMC after the 1 h regional HT aimed either at the liver (*closed circles*) or at the lungs (*open circles*), or after 1 h heating of the legs (*closed triangles*). PBMC prepared from each subject at each indicated time-point were stimulated with PHA, and the concentrations of IFN-γ (**a**) and IL-4 (**b**) produced into culture medium were measured after 2 days of incubation. Each *symbol* and *bar* show mean ± SEM calculated from the data obtained from 4 to 11 volunteers. * Significantly different from the mean value observed before the HT treatment, $P < 0.02$; **significantly different from the same pre-treatment value, $P < 0.01$

Changes in the numbers of monocyte subsets

Recently, functional heterogeneity of human blood monocytes has been demonstrated based on the expression levels of the lipopolysaccharide (LPS) receptor CD14 and the Fcγ receptor III (CD16) [10]: $CD14^+$ $CD16^-$ cells that correspond to classical inflammatory monocytes and $CD14^{lo}$ $CD16^+$ cells that have been characterized as resident monocytes (Fig. 4a). As shown in Table 1, a marked reduction in the number of the whole monocyte population in the peripheral blood was observed following the HT treatment aimed at the liver. In order to examine which subset among the monocytes was affected by the treatment, we further analyzed both frequencies and absolute numbers of the above monocyte subsets as well as those of DCs which also exist within the blood monocyte population defined by conventional gating based on the forward and side scatter profiles (Fig. 4b). The DC population was distinguished in the present study by their expression of HLA-DR and the lack of the expression of CD3, CD14, CD16, CD19, CD20, and CD56. At 1 h after the liver-targeted HT, the frequency of $CD14^+$ $CD16^-$ cells among the gated monocyte population was markedly reduced in five of the six volunteers, the mean values of which were $87.0 \pm 1.4\%$ at pre-treatment and significantly lower $76.2 \pm 4.2\%$ at 1 h after the HT treatment. A remarkable reduction in the absolute number of $CD14^+$ $CD16^-$ monocytes was also observed at 1 h post-treatment. In contrast to the sharp decrease in the percentage of $CD14^+$ $CD16^-$ monocytes, $CD14^{lo}$ $CD16^+$ monocytes increased significantly in their percentage at 1 h after the liver-targeted HT treatment. However, due to the decrease in the total number of peripheral monocytes (Table 1), their absolute numbers were unchanged. With respect to DCs, their number tended to decrease soon after the HT treatment, and remained low throughout the 48 h of observation following the heating of the liver. There were no significant changes in the frequencies and absolute numbers of monocyte subsets in volunteers exposed to the lung-targeted HT. In addition, cell surface expression of CD14 molecules on the $CD14^+$ $CD16^-$ monocytes increased starting from 6 h following the heating procedure, exhibiting significant differences ($P < 0.05$) at 6 and 24 h post-treatment in the liver-targeted HT, and at 6 h post-treatment in the lung-targeted HT group (Fig. 4c). No significant changes in the monocyte-counts or the CD14 expression were observed in the volunteers exposed to the heating of the legs (data not shown).

Discussion

The findings described herein provide evidence that the regional HT treatment aimed at the liver induces activation of peripheral T cells, production of proinflammatory cytokines, and changes in the number of blood monocyte subpopulations and their CD14 expression. To heat the liver selectively in the present study, we placed a pair of electrodes on the front and back of the subject's right upper abdominal region; however, it is also conceivable that other lymphoid organs that lie adjacent to the liver might have been heated. With

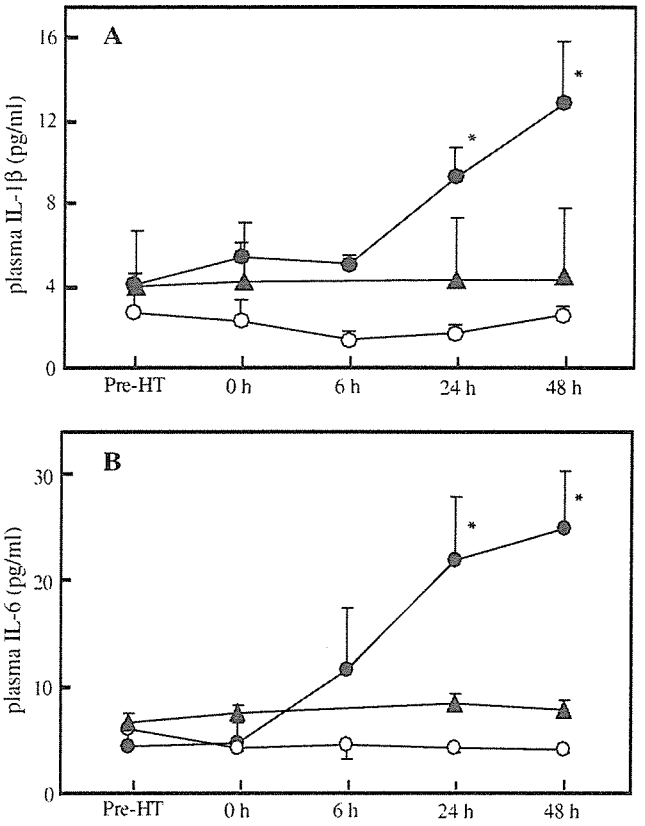

Fig. 3 Average plasma concentrations of IL-1β (**a**) and IL-6 (**b**) after the 1 h regional HT aimed either at the liver (*closed circles*) or the lungs (*open circles*), or after 1 h heating of the legs (*closed triangles*). Each *symbol* and *bar* represent mean ± SEM calculated from the data obtained from 4 to 11 volunteers. *Significantly different from the mean value observed before the HT treatment, $P < 0.01$

respect to the spleen, however, there happened to be a volunteer who had undergone splenectomy among our subjects. Importantly, we observed the same changes in the above immunological parameters in the splenectomized volunteer following the HT aimed at the liver, ruling out the possibility that the effects of the right upper abdominal heating depend on the presence of the spleen. When administering HT to the chest, in which the lungs were supposed to be the primary target organs, the subject's body temperatures reached levels as high as those measured in the volunteers treated with the liver-targeted HT; however, the lung-targeted treatment resulted in no significant activation of T cells (Figs. 1 and 2) or changes in the numbers of blood monocyte subsets (Fig. 4). Thus, it is highly conceivable that the changes observed following the heating of the right upper abdominal region can be mainly attributed to the heating of the liver.

Immunological changes in humans who received systemic hyperthermic treatment have been described, most of which refer to its effect on the composition of lymphocyte subpopulations, the concentration of serum cytokines, and the NK cell activity (reviewed in [12]). On the other hand, there are very few reports describing the effect of regional HT on the host's immune system. Regarding the changes in lymphocyte subpopulations, we observed a slight decrease in the peripheral $CD4^+/CD8^+$ ratio following the HT treatment aimed at the liver and lungs, which was statistically significant in the latter case. The decrease in $CD4^+/CD8^+$ ratio is mainly accounted for by the reduction in the number of $CD4^+$ T cells, rather than an increase in $CD8^+$ T-cell counts, in the both experimental groups. In line with this result, a decrease in $CD4^+/CD8^+$ ratio has been shown in patients with hepatocellular carcinoma immediately after the regional HT at the upper abdominal region [20], as well as in the persons who underwent whole-body hyperthermia (WBH) [1, 7]. By way of experiment with mice, WBH has been shown to enhance the homing of antigen-specific T cells to the inflammatory site; however, differences in sensitivity to this treatment of each T-cell subset have not been described [21]. As a possibility, chemotaxis of $CD4^+$ T cells into tissues might be more quickly and markedly affected by heat than that of $CD8^+$ T cells, thus resulting in the sharp decrease in $CD4^+/CD8^+$ ratio we observed.

In the present study, T-cell activation was assessed by the expression of the activation marker CD69 and by the generation of cytokines after the stimulation with PHA. CD69 molecule is a phosphorylated and disulfide-linked 27/33 kD homodimeric protein [26], and signals triggered by anti-CD69 antibodies result in the synthesis of different cytokines and their receptors, and the enhancement of T cell proliferation [5, 17]. The expression of CD69 on human T cells is induced in vitro by a wide variety of stimuli: an increase in CD69 expression is observed at as early as 1–4 h after stimulation with anti-CD3 antibody or polyclonal mitogens including phorbol ester 12-myristate 13-acetate (PMA), an activator of intracellular protein kinase C (PKC), whereas it takes 24–72 h after an in vitro stimulation through antigen–T-cell receptor interactions for the induction of CD69 expression [5, 11]. In the volunteers exposed to the HT treatment aimed at the liver, we observed the increase in the fraction of CD69-expressing cells in both $CD4^+$ and $CD8^+$ populations of T cells in two waves (Fig. 1). The first elevation, accompanied by the nearly twofold increase in PHA-induced IFN-γ production (Fig. 2a), was found at 1 h after the liver-targeted HT treatment. Based on the above knowledge, this "early phase" of T-cell activation, as demonstrated by the CD69 expression, is not attributable to an antigen-dependent reaction. Our observation is rather consistent with a report demonstrating that the expression of CD69 molecule on the surface of resting human T cells was induced at as early as 1 h after an incubation at 44°C for 30 min, which subsided 3 h later, due to a rapid translocation of the molecules already present in the cell cytoplasm [23]. In addition, fever-range (39.5°C) WBH has been shown to result in a rapid increase in PKC activity within T cells [32]. Thus, the liver-targeted HT treatment may have caused the very early induction of CD69 molecules to

the cell surface via yet undescribed PKC signal transduction pathways, which in turn lead to the observed T-cell activation and the resultant increase in IFN-γ production. Of note, an extreme WBH of 41.8–42.2°C has led to a reversible and transient immune impairment in the patients with metastatic cancers during or immediately after the heating procedure [1, 4]. The regional heating aimed at the liver may have an advantage in that no intense immune impairment is induced during the treatment.

On the other hand, the "late phase" of CD69 induction was observed at 24 h post-HT treatment aimed at the liver. At the same time-point, a more than threefold increase in IFN-γ production from the PHA-stimulated PBMC was observed as compared to the pre-treatment value, suggesting that the peripheral T cells were fully activated at 24 h after the HT treatment of the liver. Furthermore, the plasma concentrations of IL-1 and IL-6, which are known to be secreted predominantly from vascular endothelial cells and professional phagocytes and act on the activation of lymphocytes [2, 6], were also elevated in our volunteers at this time-point, while there were no significant elevations in the early phase. In contrast, the plasma IL-6 increased during or just after the heating treatment in patients treated with an extreme WBH of 41.8°C, and returned to basal levels at 24-h post-treatment [4, 25]. The rapid increase in plasma IL-6 may be attributable to an augmentation in IL-6 release from the circulating monocytes or T cells. In fact, the TNF-α release from PBMC has been shown to increase immediately after the WBH [36]. On the other hand, the increase in plasma IL-6 was observed only in the subject who received the liver-targeted HT, but not in those receiving the heating of the lungs or legs, at the late phase. Liver cells can secrete a variety of cytokines including IL-1, IL-6 and TNF-α [2]. Among these, TNF-α is a potent inducer of IL-1 and IL-6 expression [2, 14]. Interestingly, the HT treatment has been shown to enhance TNF-α release from Kupffer cells in LPS-challenged mice [13]. It is possible, therefore, that the TNF-α locally produced by the HT-treated hepatic tissue may have, in turn, stimulated IL-1 and IL-6 release from the liver cells, which resulted in the observed late-phase increases in the plasma cytokines.

Unprecedented heterogeneity in their surface marker phenotypes and functions of blood monocytes has been demonstrated only recently: classical $CD14^+$ $CD16^-$ monocytes that migrate into the sites of inflammation, where they differentiate into macrophages and DCs, and $CD14^{lo}$ $CD16^+$ monocytes that extravasate into tissues, where they serve as specific resident myeloid cells such as Kupffer cells, alveolar macrophages, or Langerhans cells [10, 37]. Interestingly, our study revealed a remarkable decrease in only the $CD14^+$ $CD16^-$ monocyte subset in the peripheral blood, but not in the $CD14^{lo}$ $CD16^+$ monocytes and circulating DCs, at 1 h after the HT treatment. So far, there have been no reports demonstrating the decrease in the number of monocytes by HT treatment, whereas an increased number of total monocytes has been shown in the peripheral blood of healthy volunteers at 6 h after a fever-range WBH treatment [36]. Our observation raises two possibilities for the action of the heating of the liver: (1) apoptotic loss of the peripheral $CD14^+$ $CD16^-$ monocytes may have been selectively induced, or (2) the entrance of $CD14^+$ $CD16^-$ monocytes into tissues such as the liver may have been facilitated, resulting in their disappearance from the peripheral blood. Regarding the heat-induced apoptosis, a number of experiments both in vitro and in vivo have shown that HT treatments influence cell viability in various types of tumors [3, 34]. Exposing monocytes to 41°C for 1 h, however, had only a minor effect on their viability [22]. We also observed no effect of an in vitro treatment at 40°C for 1 h on the viability of blood monocytes (S. Kinoshita et al. unpublished observation). Taken together, it is not likely that the decrease in blood monocytes at 1 h post-HT treatment is due to a heat-induced apoptosis. With respect to the other hypothesis concerning the trafficking of $CD14^+$ $CD16^-$ monocytes, chemokines produced locally in the liver should be taken into consideration. It has recently been reported in fulminant hepatic failure that the expression of intrahepatic chemokines, MCP-1, MIP-1α, MIP-1β, and RANTES, which are produced from Kupffer cells, sinusoidal endothelial cells, and hepatocytes, was closely correlated with the extent of infiltration by macrophages or monocytes and T cells into the liver [15]. Moreover, mRNAs of these intrahepatic chemokines have started to be expressed at as early as 1 h after an administration of toxins which elicit liver damage in mouse models of fulminant hepatic failure [15]. Of note, inflammatory chemokine receptors, CCR1, CCR2, and CCR5, which bind to MCP-1, MIP-1α, MIP-1β, and RANTES, are expressed on human $CD14^+$ $CD16^-$ monocytes at much higher levels than on $CD14^{lo}$ $CD16^+$ monocytes [10]. Collectively, the heat-exposed liver may produce the above-described inflammatory chemokines immediately after an HT treatment, resulting in the entrance into the liver of blood $CD14^+$ $CD16^-$ monocytes. Further, upregulation of the CD14 expression levels on the $CD14^+$ $CD16^-$ monocytes in the blood was found at as early as 6 h following the liver-targeted HT treatment, and this increase lasted at least until 24 h post-HT (Fig. 4). In contrast to the above result, a short-lasting increase in the CD14 expression on monocytes was observed in healthy volunteers exposed to fever-range WBH [36] and in our volunteers treated with the lung-targeted HT. The CD14 expression level on the monocytes was associated with an ability to release TNF-α following the stimulation with LPS [36]. Taken together, the possible early migration of $CD14^+$ $CD16^-$ monocytes into the heated liver may have contributed to the observed long-lasting activation of peripheral monocytes after the HT treatment and the observed increase in plasma levels of IL-1β and IL-6.

As there is still no enough evidence demonstrating substantial direct anticancer effect of regional HT when

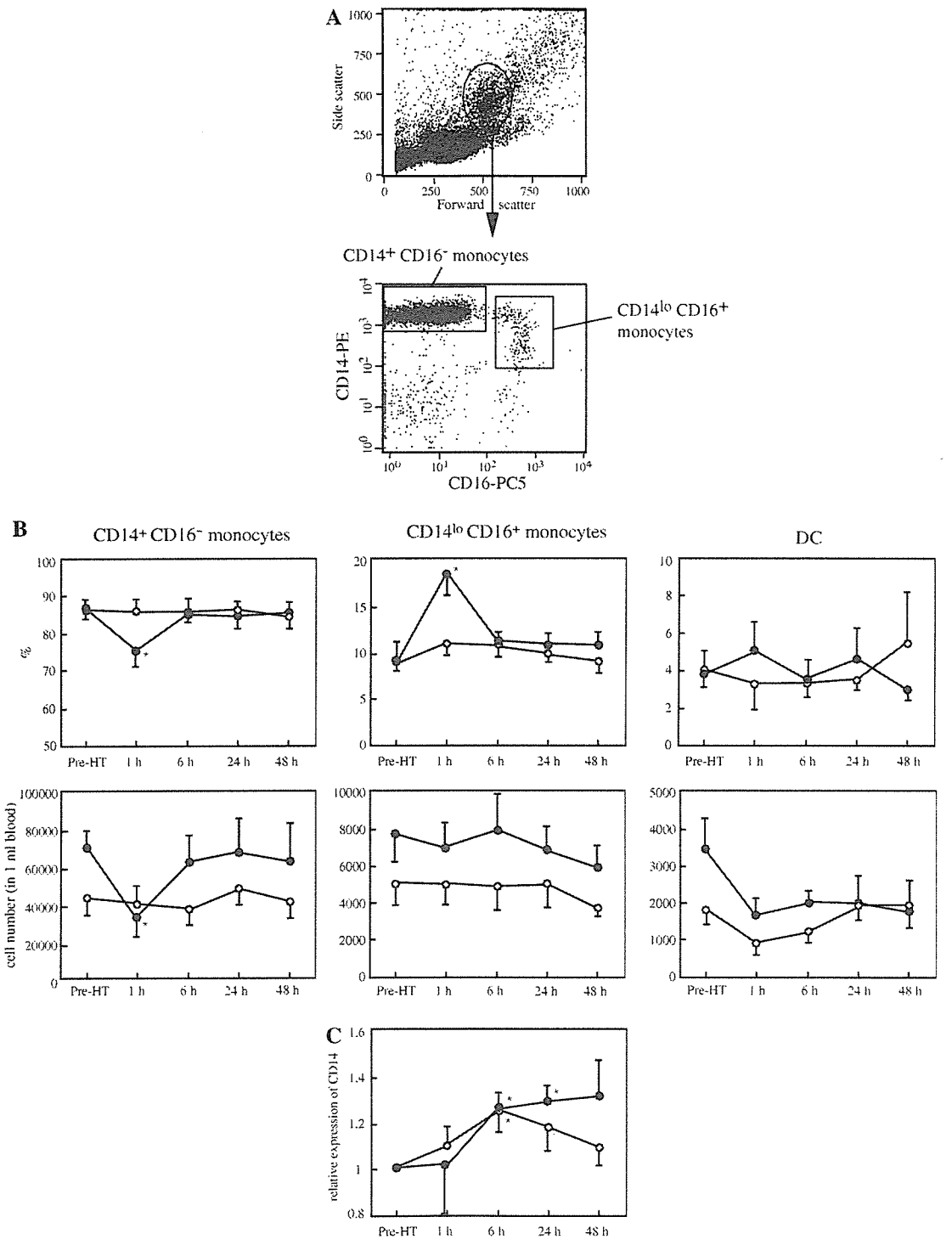

Fig. 4 Effects of the 1 h regional HT on cell numbers of monocyte subsets and their CD14 expression. **a** Monocytes and DCs in PBMC prepared fell into an oval gate in the dot plot of forward and side scatters (*the upper panel*). The monocyte subsets were distinguished by multi-color staining (*lower panels*). **b** Percentages of the above-defined monocyte populations and DCs (*upper panels*) among the whole peripheral monocytes and their absolute numbers (*lower panels*) were analyzed in both groups of volunteers that were exposed to either the liver-targeted (*closed circles*) or lung-targeted (*open circles*) HT treatment. The cell numbers of each subset were calculated by the product of numbers of the entire mononuclear cells counted and the corresponding cell percentages among the mononuclear cells. **c** Cell surface CD14 expression on the $CD14^+$ $CD16^-$ monocytes obtained from the volunteers exposed to either liver-targeted (*closed circles*) or lung-targeted (*open circles*) HT treatment was evaluated by measuring the geometric mean of the fluorescent intensities. The results are represented relative to the expression of CD14 molecules at pre-HT. Each *circle* and *bar* represent mean ± SEM determined from data obtained from five or six volunteers. *Significantly different from the mean value observed before the HT treatment, $P < 0.05$

used as a single treatment modality, we believe that it is currently applicable only in combination with other modalities, as a way to improve the efficacy of chemo-, radio- and, possibly other immunotherapies. The chemo- and radiotherapies often inevitably cause the suppression of host immune responses. Reduced immune responses have also been observed in patients with liver tumors after transcatheter arterial embolization [18]. In contrast, various regimens of HT are known to cause an augmentation in immune responses [4, 12, 24, 25]. This study has provided the evidence that the liver-targeted regional HT treatment induces the full activation of peripheral T cells and suggests the possible enhancement in chemotaxis of monocytes into the liver without severe stress reactions, indicating that this HT treatment may be a promising modality for liver tumors in combination with other anticancer treatments.

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Induction of Gag-specific T-cell responses by therapeutic immunization with a Gag-expressing Sendai virus vector in macaques chronically infected with simian-human immunodeficiency virus

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Abstract

Recent prophylactic vaccine trials inducing virus-specific $CD8^+$ T-cell responses have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) in macaques. In the chronic phase, therapeutic immunization replenishing virus-specific $CD8^+$ T-cells is likely to contribute to sustained control of virus replication. In this study, we have administered a recombinant Sendai virus (SeV) vector into five rhesus macaques that had received prophylactic vaccinations and had controlled SHIV replication for more than 1 year after challenge. Our results indicate that virus-specific $CD8^+$ T-cell responses can be expanded and broadened by therapeutic immunization with SeV vectors in the chronic phase after prophylactic vaccine-based control of primary immunodeficiency virus infections.

Keywords: AIDS; Sendai virus; Therapeutic vaccine

1. Introduction

Virus-specific $CD8^+$ cytotoxic T lymphocyte (CTL) responses play a central role in the control of immunodeficiency virus infections. The importance of CTL in the control has been indicated not only in the acute phase but also in the chronic phase of infections by several clinical correlations in human immunodeficiency virus type 1 (HIV-1)-infected humans [1–3] and $CD8^+$ T-cell-depletion experiments in macaque AIDS models [4–6]. Therefore, AIDS vaccine studies have been making efforts to develop methods efficiently inducing virus-specific $CD8^+$ T-cell responses.

Recombinant viral vectors can be a promising tool for AIDS vaccines because of their potential for inducing virus-specific $CD8^+$ T-cell responses. Recently, preclinical trials of prophylactic vaccines using recombinant viral vectors have shown control of primary infections of a pathogenic simian-human immunodeficiency virus (SHIV) that induces acute $CD4^+$ T-cell depletion in macaques [7–10]. These vaccinated macaques have contained the challenge virus leading to reduction in plasma viral loads to be undetectable at the setpoint and maintained peripheral $CD4^+$ T-cell counts, although they have failed to eliminate the virus and shown detectable levels of proviral DNA in lymphocytes in the chronic phase

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[11]. $CD8^+$ T-cell depletion by anti-CD8 monoclonal antibody treatment in these macaques in the chronic phase after prophylactic vaccine-based control of primary SHIV infection has shown a rise in plasma viral loads [12]. Additionally, loss of the control due to appearance of a CTL escape mutant has been observed in the chronic phase in a macaque that had controlled primary SHIV infection [13]. Thus, virus-specific $CD8^+$ T-cell responses have been indicated to play a central role in maintaining the control of virus replication in the chronic phase and therapeutic immunization replenishing virus-specific $CD8^+$ T-cell responses are likely to contribute to the sustained control.

We previously developed a prophylactic DNA vaccine system that uses FMSIV [14], which is a chimeric SHIV with ecotropic Friend murine leukemia virus (FMLV) *env* in place of SHIV *env*, in combination with the FMLV receptor, mCAT1 [15], which is not normally expressed in primate cells. Vaccination of macaques with both of the FMSIV proviral DNA and an mCAT1-expression plasmid DNA induced mCAT1-dependent FMSIV replication leading to efficient elicitation of virus-specific $CD8^+$ T-cell responses. We also established a prophylactic Sendai virus (SeV) vector-based vaccine system [16–19]. Not only the replication-competent (transmissible) but also the replication-defective (non-transmissible) SeV vector showed the potential for efficiently inducing virus-specific $CD8^+$ T-cell responses [20,21]. Additionally, combination of the DNA vaccine and the SeV vector vaccine, DNA-prime/SeV-boost, elicited extremely high levels of virus-specific $CD8^+$ T-cell responses [8]. Preclinical trials of these prophylactic vaccine systems showed control of replication of a pathogenic SHIV89.6PD and prevented macaques from acute AIDS progression [8,21].

In this study, we have examined if the SeV vector can be used for therapeutic immunization to induce virus-specific $CD8^+$ T-cell responses in the chronic phase. We considered Gag as a promising vaccine-antigen candidate to avoid CTL escape because it has been indicated that Gag CTL escape variants mostly diminish viral fitness and require multiple additional compensatory mutations to restore their replicative competence [22]. We administered a Gag-expressing SeV (SeV-Gag) vector into those macaques that had controlled SHIV replication for more than 1 year after challenge and analyzed Gag-specific T-cell responses.

2. Materials and methods

2.1. Animals

Male rhesus macaques (*Macaca mulatta*) were maintained in accordance with the Guideline for Laboratory Animals of the National Institute of Infectious Diseases. These macaques were tested negative for SeV, simian immunodeficiency virus (SIV), and simian retrovirus type D before use. Blood collection, vaccination, and virus challenge were performed under ketamine anesthesia.

Macaques used in this study were previously subjected to prophylactic vaccination and challenge experiments (Table 1) [8,23]. In brief, macaques R011 and R012 received four times FMSIV plus mCAT1 DNA vaccinations and single intranasal SeV-Gag booster, whereas macaques R003 and R006 were boosted intranasally with a recombinant SeV expressing HIV-1 Tat (SeV-Tat) after the DNA vaccinations. Macaque R022 received the DNA vaccinations only. An infectious FMSIV clone DNA obtained by replacing the gene fragment encoding Env surface protein of $SHIV_{MD14YE}$ [24] with an FMLV *env* fragment [25] has simian immunodeficiency virus-derived long terminal repeat, *gag*, *pol*, *vif*, *vpx*, and partial *vpr* sequences, HIV-1-derived partial *vpr*, *tat*, *rev*, and partial env (containing the second exon of *tat*, the second exon of *rev*, and RRE) sequences, and FMLV-derived *env* sequences [14]. At each DNA vaccination, animals received 800 μg of individual DNA intramuscularly and 10 μg of individual DNA by gene gun. At the booster, animals received 1×10^8 cell infectious units (CIU) of replication-competent F(+)SeV-Tat or F(+)SeV-Gag. Animals were challenged intravenously with 10 TCID50 (50% tissue culture infective doses) of SHIV89.6PD [26].

2.2. Therapeutic immunization

We used two kinds of SeV vectors expressing SIVmac239 gag, a replication-competent one (F[+]SeV-gag) and a replication-defective F-deleted one (F[−]SeV-gag), for therapeutic immunization. Recombinant F(+)SeV-Gag and F(−)SeV-Gag were prepared as described previously [16,19,20]. Animals received 1×10^8 CIU of F(+)SeV-Gag (macaques R003 and R006) or 6×10^9 CIU of F(−)SeV-

Table 1
Vaccination and challenge protocol in macaques

Macaques	Prophylactic vaccination	Challenge	Therapeutic vaccination
R003	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R006	DNA and F(+)SeV-Tat	SHIV89.6PD	F(+)SeV-Gag at week 56
R011	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R012	DNA and F(+)SeV-Gag	SHIV89.6PD	F(−)SeV-Gag at week 176
R022	DNA	SHIV89.6PD	F(−)SeV-Gag at weeks 139 and 146

DNA vaccinations were performed four times at weeks 0, 0.5, 1, and 6 after the initial vaccination. SeV-Tat or SeV-Gag vaccination for booster was performed once at week 12 after the initial vaccination. Macaques R003, R006, R011, and R012 were challenged with SHIV89.6PD at week 26 after the initial vaccination, whereas macaque R022 at week 14. Therapeutic SeV-Gag vaccination was performed at indicated time points after challenge. F(+)SeV-Tat and F(+)SeV-Gag are replication-competent and F(−)SeV-Gag is replication-defective.

Gag (macaques R011, R012, and R022) intranasally for the immunization.

2.3. Detection of SeV RNA in lymph nodes (LN)

Lymphocytes were prepared from minced lymph nodes (LN) by using Ficoll-Paque Plus (Amersham Biosciences). RNA was isolated from 1×10^6 lymphocytes by using RNeasy Mini kit (Qiagen K.K.) and eluted by 50 μl of water. Ten microliters of RNA was subjected to reverse transcription and nested PCR (RT-PCR) using SeV NP-specific primers (ATGGCCGGGTTGTTGAG and GGGCTCTTGTTGACCATAGG for the first RT-PCR, and AGTCGGGAAGAGGTGCTG and CGTCTTCACAATGAATCCGTC for the second DNA PCR) for detection of SeV RNA.

2.4. Quantitation of plasma viral loads

Plasma RNA was extracted using high pure viral RNA kit (Roche Diagnostics). Serial 5-fold dilutions of RNA samples were amplified in quadruplicate by nested RT-PCR using SIV *gag*-specific primers (AGAAACTCCGTCTTGTCAGG and TGATAATCTGCATAGCCGC for the first RT-PCR, and GATTAGCAGAAAGCCTGTTGG and TGCAACCTTCTGACAGTGC for the second DNA PCR) to determine the end-point. Plasma SIV RNA levels were calculated according to the Reed–Muench method as described [24,27]. The lower limit of detection in this assay is about 4×10^2 copies/ml.

2.5. Quantitation of proviral DNA levels in peripheral blood mononuclear cells (PBMC)

Genomic DNA was extracted from PBMC by using DNeasy kit (Qiagen K.K.). For quantitation of proviral SIV DNA copy numbers in cell lysates, serial 5-fold dilutions of cell lysates were amplified in quadruplicate by nested DNA-PCR using SIV *gag*-specific primers to determine the end-point as described [24]. The lower limit of detection in this assay is about 5 copies/μg DNA.

2.6. Measurement of antigen-specific T-cell frequencies

We measured antigen-specific T-cell frequencies by flow-cytometric analysis of interferon-γ (IFN-γ) induction after specific stimulation as described previously [8]. In brief, PBMC were cocultured with autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCL) [28] infected with a vaccinia virus (Vv) vector [29] for non-specific Vv-control-stimulation, B-LCL infected with a Vv vector expressing SIVmac239 Gag for Gag-specific Vv-Gag-stimulation, and B-LCL infected with SeV for SeV-specific stimulation, respectively. Intracellular IFN-γ staining was performed by using Cytofix-Cytoperm kit (BD Biosciences). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD4, peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8, allophycocyanin (APC)-conjugated anti-human CD3, and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (BD Biosciences) were used. Gag-specific T-cell frequencies and SeV-specific T-cell frequencies were calculated by subtracting the IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation from those after Gag-specific Vv-Gag-stimulation and those after SeV-specific stimulation, respectively. The background IFN-γ^+ T-cell frequencies after non-specific Vv-control-stimulation were less than 200 cells per million PBMC. Gag-specific T-cell frequencies (and SeV-specific T-cell frequencies) less than 100 cells per million PBMC were considered negative, those between 100 and 200 borderline, and those greater than 200 positive.

In case of examining peptide-specific T-cell frequencies, B-LCL were pulsed with peptide mixture (final concentration of each peptide, 1–10 μM) for peptide-specific stimulation or incubated without peptide for non-specific stimulation. A panel of 117 overlapping peptides (15–17 amino acid (aa) in length and overlapping by 10–12 aa) spanning the entire SIVmac239 Gag aa sequence were purchased from Sigma Genosys, Japan, and divided into 10 pools, each consisting of 11 or 12 peptides. The background IFN-γ^+ T-cell frequencies were less than 100 cells per million PBMC. Peptide-specific T-cell levels less than 100 cells per million PBMC were considered negative, and those greater than 100 positive.

3. Results

3.1. SeV-Gag immunization into macaques chronically infected with SHIV

In this study, we used five rhesus macaques that had received prophylactic vaccines and had controlled SHIV replication for more than 1 year after challenge (Table 1). All five macaques (R003, R006, R011, R012, and R022) had received DNA vaccinations; additionally, macaques R003 and R006 had been boosted with SeV-Tat whereas macaques R011 and R012 boosted with SeV-Gag as described previously [8,23]. They had been challenged with SHIV89.6PD 8 weeks (in R022) or 14 weeks (in R003, R006, R011, and R012) after the last vaccination. Plasma viral loads had been below the detectable levels after the setpoint and peripheral CD4$^+$ T-cell counts had been maintained until therapeutic immunization in all five macaques (data not shown). Macaques R003 and R006 received therapeutic immunization with replication-competent F(+)SeV-Gag at week 56 post-challenge, whereas macaques R011 and R012 with replication-defective F(−)SeV-Gag at week 176. Macaque R022 was immunized with F(−)SeV-Gag twice at weeks 139 and 146.

No macaques displayed apparent clinical symptoms after the therapeutic SeV-Gag immunization. No apparent pathological signs were observed by histological analysis of tissues obtained at autopsy from macaque R003 euthanized 1 week post-immunization (p.i.) (at week 57 after challenge),

Table 2
Detection of SeV RNA by nested RT-PCR

Macaques	Therapeutic vaccination	Autopsy	SeV RNA[a]		
			SM-LN	MC-LN	IG-LN
R003	At week 56	At week 57	Positive	Negative	Negative
R006	At week 56	At week 58	Negative	Negative	Negative
R011	At week 176	At week 181	Negative	Negative	Negative
R012	At week 176	At week 181	Positive	Negative	Negative
R022	At weeks 139 and 146	At week 147	Positive	Negative	Negative

[a] RNA was extracted from LN-derived lymphocytes and nested RT-PCR was performed for detection of SeV RNA. SM-LN, submandibular LN; MC-LN, mesenchymal LN; IG-LN, inguinal LN.

macaque R006 euthanized 2 weeks p.i. (at week 58), R011 or R012 euthanized 5 weeks p.i. (at week 181) or R022 euthanized 1 week after the second SeV-Gag immunization (at week 147). SeV RNA was detected by nested RT-PCR in the submandibular lymph node in three macaques (R003, R012, and R022) but undetectable in other two (R006 and R011) (Table 2). In the mesenchymal LN and the inguinal LN, however, SeV RNA was undetectable in all five macaques.

3.2. *Gag-specific T-cell responses after therapeutic SeV-Gag immunization*

To see the effect of therapeutic SeV-Gag immunization on Gag-specific T-cell responses, we measured Gag-specific T-cell frequencies in PBMC before and after the immunization by detection of Gag-specific IFN-γ induction. In all five macaques, Gag-specific $CD8^+$ T-cell levels were increased after SeV-Gag immunization (Fig. 1). The second SeV-Gag immunization at week 146, 7 weeks after the first immunization, also increased the levels in macaque R022.

In macaques R012 and R022, Gag-specific $CD8^+$ T-cell responses were not clearly detected before SeV-Gag immunization but appeared after that, indicating that new epitope-specific $CD8^+$ T-cell responses were induced by the immunization. In contrast, Gag-specific $CD8^+$ T-cells were detectable even before immunization and their levels were largely increased after that in macaques R003, R006, and R011. We then examined whether increases in their levels were only due to expansion of epitope-specific $CD8^+$ T-cells that had been detectable before immunization or new-epitope specific $CD8^+$ T-cells were induced by the immunization. Using a panel of overlapping peptides spanning the entire SIV Gag aa sequence, IFN-γ induction was assessed after stimulation with pools of peptides (Fig. 2). In macaque R003, analysis of PBMC at week 56 (just before immunization) detected $CD8^+$ T-cells specific for three pools of peptides, #4 (corresponding to the 155th–213th aa in SIV Gag), #5 (202nd–265th aa), and #10 (453rd–510th aa). At week 57, 1 week p.i., no significant changes were observed in #4-specific $CD8^+$ T-cell or #10-specific $CD8^+$ T-cell levels, but #5-specific $CD8^+$ T-cells expanded efficiently. Additionally, the immunization induced #7 (306th–364th aa)-specific $CD8^+$ T-cells that had been undetectable before immunization. In

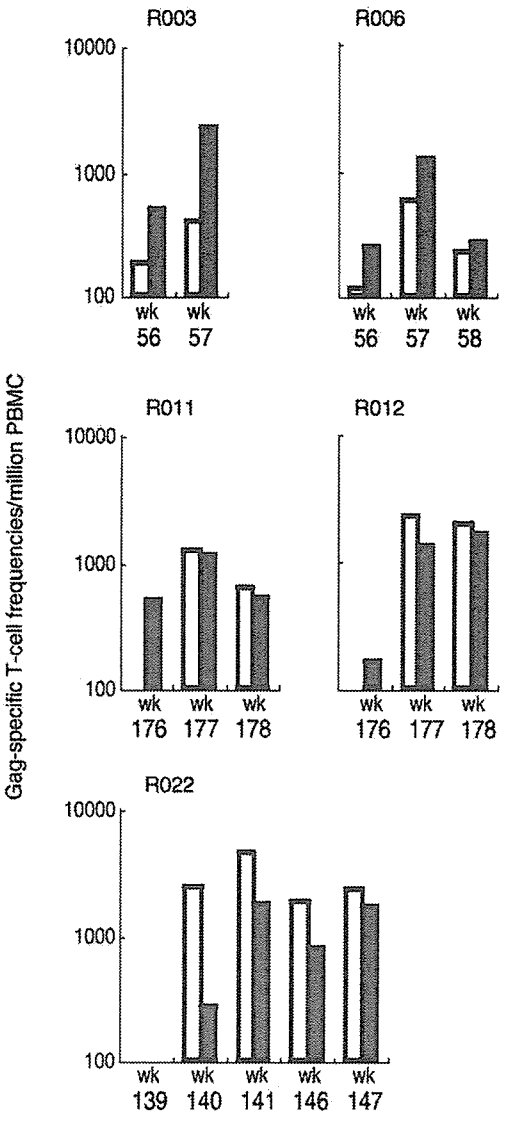

Fig. 1. Frequencies of Gag-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization. Macaques R003 and R006 were immunized at week 56 post-challenge, macaques R011 and R012 at week 176, and macaque R022 at weeks 139 and 146.

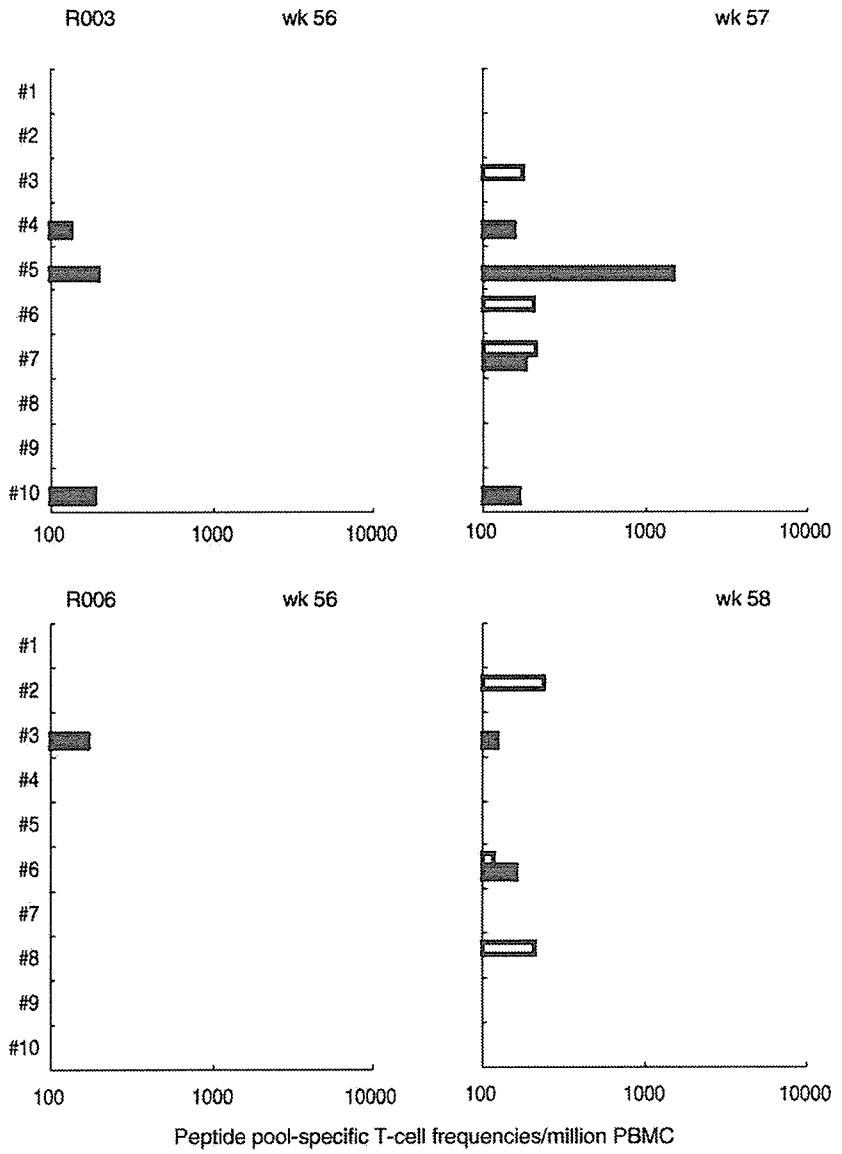

Fig. 2. Frequencies of CD4+ T-cells (open bar) and CD8+ T-cells (shaded bar) specific for pools of SIV Gag peptides in PBMC. A panel of overlapping peptides spanning the entire SIV Gag aa sequence were divided into 10 pools (each consisting of 11 or 12 peptides), #1 (corresponding to the 1st–65th aa in SIV Gag), #2 (55th–114th aa), #3 (104th–165th aa), #4 (155th–213th aa), #5 (202nd–265th aa), #6 (255th–316th aa), #7 (306th–364th aa), #8 (354th–416th aa), #9 (406th–464th aa), and #10 (453rd–510th aa), and used for the stimulation to detect peptide pool-specific T-cells.

macaque R006, #3 (104th–165th aa)-specific CD8+ T-cell levels remained unchanged at week 58, 2 weeks p.i. The immunization, however, induced #6 (255th–316th aa)-specific CD8+ T-cells that had been undetectable before immunization. Thus, the immunization induced new epitope-specific CD8+ T-cells that had been undetectable before immunization in macaques R003 and R006 as well as in macaques R012 and R022, indicating that therapeutic SeV-Gag immunization not only expanded but also broadened Gag-specific CD8+ T-cell responses. We failed to obtain enough PBMC samples for analysis of peptide-specific responses in macaque R011.

Therapeutic SeV-Gag immunization efficiently induced Gag-specific CD4+ T-cell responses also although the responses were undetectable before immunization in all five macaques (Fig. 1). In macaques R003 and R006, several epitope-specific CD4+ T-cells became detectable after immunization (Fig. 2).

3.3. SeV-specific T-cell responses after therapeutic SeV-Gag immunization

We also examined SeV-specific T-cell responses in macaques (Fig. 3). SeV-specific CD8+ T-cells and CD4+ T-cells both were undetectable just before immunization not only in macaque R022 that had been naive to SeV but also in other four macaques that had received a prophylactic vaccination with SeV vectors before challenge. In the latter, efficient induction of SeV-specific CD8+ T-cell and CD4+ T-

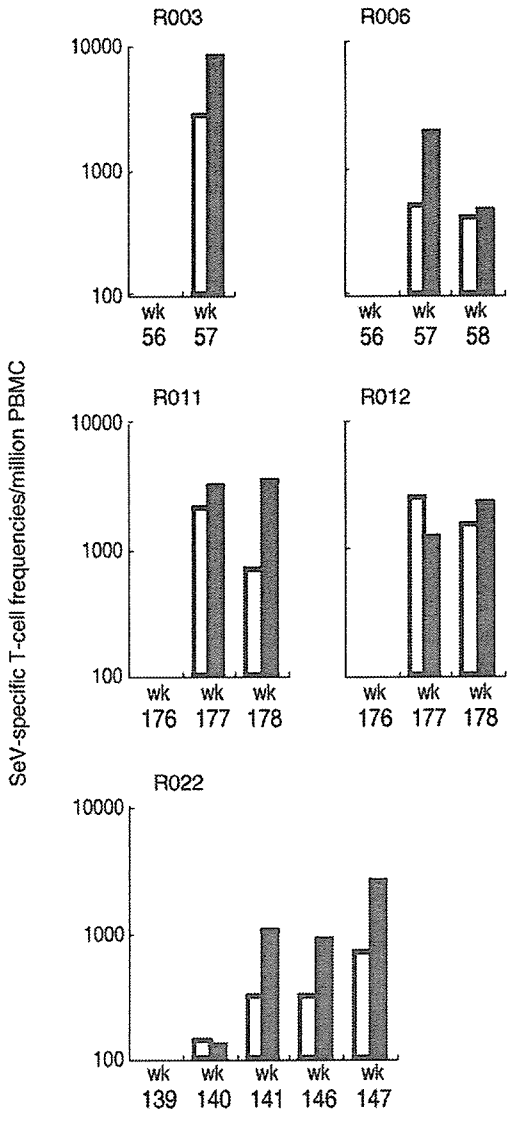

Fig. 3. Frequencies of SeV-specific $CD4^+$ T-cells (open bar) and $CD8^+$ T-cells (shaded bar) in PBMC before and after therapeutic SeV-Gag immunization.

cell responses both was observed 1 week p.i. In the former (R022), SeV-specific T-cell responses appeared with some delay and became apparent 2 weeks after the first immunization, whereas the second SeV-Gag immunization resulted in efficient expansion of SeV-specific T-cells in a week.

3.4. Viral loads after therapeutic SeV-Gag immunization

In all five macaques, plasma viremia remained undetectable after therapeutic SeV-Gag immunization. We then examined proviral DNA levels in PBMC in macaques R011, R012, and R022 (Fig. 4). These macaques kept proviral loads at low levels and we found no significant changes in their levels after immunization.

4. Discussion

The purpose of current therapeutic AIDS vaccines is to maintain HIV-1-specific CTL in the HIV-1-infected individuals who control viral replication and show little CTL responses, because CTL is crucial for sustained control in the chronic phase. The first object is HIV-1-infected individuals who control HIV-1 replication due to antiretroviral therapy, and therapeutic vaccines with recombinant viral vectors for replenishing CTL responses have been studied in HIV-1-infected individuals and SIV-infected macaques during antiretroviral treatment [30–32]. Further, HIV-1-infected individuals who show prophylactic vaccine-based control of HIV-1 replication can be an object of therapeutic vaccines, if an effective prophylactic AIDS vaccine is developed. Indeed, advances in recombinant viral vector technologies have contributed to progress in the development of CTL-based prophylactic AIDS vaccines, and recent studies in macaques have shown the importance of CTL maintenance for keeping prophylactic vaccine-based control of SHIV replication in the chronic phase [12]. Our study presents the first trial of therapeutic immunization into macaques that

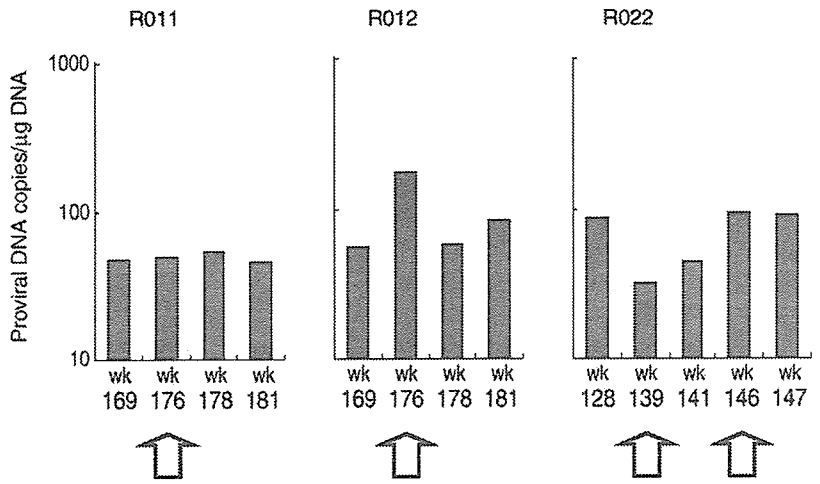

Fig. 4. SHIV proviral DNA copy numbers in PBMC before and after therapeutic SeV-Gag immunization. Arrows indicate the time-points of immunization.

have maintained prophylactic vaccine-based control of viral replication without antiretroviral help and suggests that the therapeutic SeV vector immunization can contribute to the maintenance of virus-specific CTL responses in the chronic phase.

The potential of the SeV vector as a prophylactic AIDS vaccine for inducing virus-specific T-cell responses have been studied, but its potential as a therapeutic vaccine has not yet been examined. The present study is the first report on its therapeutic administration in the chronic phase of immunodeficiency virus infections. Histological analysis revealed no pathological signs after immunization. The SeV vector distribution after therapeutic immunization was consistent with the previous analysis of prophylactic vaccination indicating that the vector was not disseminated but localized in the nasal mucosa and its primary LN [19]. These results support the notion that this vector can be safely used as a therapeutic vaccine.

Four of five macaques in this study had received a prophylactic SeV vaccination before SHIV challenge. SeV-specific T-cell responses that can interfere with the vector expression were undetectable before therapeutic SeV-Gag immunization but appeared rapidly after that. However, Gag-specific T-cell responses were efficiently induced in the presence of SeV-specific T-cell responses. Notably, SeV-Gag re-immunization only 7 weeks after the first immunization showed rapid expansion of SeV-specific T-cell responses but was able to augment Gag-specific T-cell responses although not so efficiently in macaque R022. These results suggest feasibility of SeV vector re-administration for induction of virus-specific T-cell responses.

In this study, Gag-specific $CD8^+$ T-cell responses were diminished in the chronic phase but augmented by therapeutic SeV-Gag immunization. Importantly, the immunization not only expanded but also broadened CTL responses. Broader responses may be advantageous for avoiding appearance of CTL escape mutants. A long-term follow-up study would be required to see if such CTL expansion and broadening by therapeutic immunization can contribute to sustained control of immunodeficiency virus replication.

Therapeutic SeV-Gag immunization elicited Gag-specific $CD4^+$ T-cell as well as Gag-specific $CD8^+$ T-cell responses in this study. It has been indicated that virus-specific $CD4^+$ T-cell as well as $CD8^+$ T-cell responses play an important role in the control of immunodeficiency virus infections [33–35]. Recent studies, however, have reported that HIV-1-infected patients with viremia frequently keep HIV-1-specific $CD4^+$ T-cells able to produce IFN-γ but do not have those able to proliferate and produce interleukin-2 in response to HIV-1 antigens, suggesting that the HIV-1-specific $CD4^+$ T-cell subpopulation able to produce IFN-γ may not contribute to the proliferative responses for the $CD4^+$ T-cell helper function [36,37]. Therefore, it has remained unclear if Gag-specific $CD4^+$ T-cell responses induced by SeV-Gag immunization can contribute to sustained control of virus replication. On the other hand, virus-specific $CD4^+$ T-cell induction may result in augmentation of virus replication because HIV-1 has been reported to preferentially infect HIV-1-specific specific $CD4^+$ T-cells [38]. However, no significant changes were observed in proviral loads after therapeutic SeV-Gag immunization in the present study.

In conclusion, we administered SeV-Gag as a therapeutic immunization into macaques that had maintained prophylactic vaccine-based control of SHIV replication for more than 1 year. Our results indicate that the therapeutic immunization can induce higher and broader virus-specific T-cell responses that may contribute to sustained control of immunodeficiency virus replication.

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Reversion In Vivo after Inoculation of a Molecular Proviral DNA Clone of Simian Immunodeficiency Virus with a Cytotoxic-T-Lymphocyte Escape Mutation

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Vaccine-based control of the replication of a simian immunodeficiency virus (SIV), SIVmac239, in macaques has recently been shown. In the process of the control, a mutant virus escaping from epitope-specific cytotoxic-T-lymphocyte (CTL) responses was rapidly selected and contained. In this study, we show that the wild-type virus appeared and became predominant in the absence of the epitope-specific CTL after inoculation of naive macaques with a molecular clone DNA of the CTL escape mutant SIV. This is the first report describing reversion in vivo from an inoculated, molecular proviral DNA clone of immunodeficiency virus with a CTL escape mutation.

Virus-specific $CD8^+$ cytotoxic-T-lymphocyte (CTL) responses are crucial for the control of immunodeficiency virus infection. The importance of CTL has been indicated by temporal association of CTL appearance with the resolution of primary viremia in human immunodeficiency virus type 1-infected humans (4, 13) and by monoclonal anti-CD8 antibody-mediated CD8 depletion experiments with macaque AIDS models (10, 15, 23). Therefore, AIDS vaccine studies have been making efforts to develop methods efficiently eliciting virus-specific CTL responses (18). However, viral escape from CTL recognition can lead to viral evasion from immune control and has frequently been observed in immunodeficiency virus infection (1, 5, 8, 19, 21, 22). Under strong immune pressure exerted by CTL, viruses are often forced to mutate, with viral fitness costs, to escape from the CTL responses (7, 9, 11, 17, 20, 24). Some CTL escape mutant viruses with lower viral fitness require additional compensatory mutations to restore their replicative competence (6, 11, 20). It is important to evaluate replicative ability of CTL escape mutants in vivo.

Recently, CTL-based control of replication of a pathogenic simian immunodeficiency virus (SIV), SIVmac239 (12), has been shown in a preclinical vaccine trial using non-Indian rhesus macaques (17). In that study, macaques vaccinated with a DNA priming followed by a Gag-expressing Sendai virus vector-booster were challenged intravenously with SIVmac239. Five of eight vaccinees controlled viral replication and had undetectable levels of plasma viremia after 5 weeks of infection. All of the five macaques showed rapid selection of CTL escape mutations in *gag*, indicating that vaccine-induced CTL contained replication of the wild-type challenge virus. Among the five, three vaccinees that share a major histocompatibility complex class I (MHC-I) haplotype, *90-120-Ia*, showed high levels of $Gag_{206\text{-}216}$ (IINEEAADWDL) epitope-specific CTL responses and rapid selection of a mutant escaping from the CTL. The replicative ability of the virus with the CTL escape mutation, Gag216S, leading to a substitution from leucine (L) to serine (S) at the 216th amino acid (aa) in Gag was diminished compared to the wild type. In the present study, we have observed replication of this mutant SIV, SIVmac239Gag216S, in the absence of $Gag_{206\text{-}216}$-specific CTL responses after its inoculation into naive macaques. All the animal experiments in this study were performed in accordance with the guidelines for laboratory animals of the National Institute of Infectious Diseases.

First, two cynomolgus macaques (*Macaca fascicularis*), C99049 and C99058, were coinoculated intramuscularly with 5 mg of the wild-type SIVmac239 molecular clone DNA (pBRmac239) and 5 mg of the mutant SIVmac239Gag216S molecular clone DNA (pBRmac239Gag216S) (17). We extracted RNA from plasma and quantitated plasma SIV RNA levels as described previously (17); both that of the wild type and that of the mutant are detectable in this assay. In both of the animals, plasma viremia was observed after the inoculation (Fig. 1A). Both the wild type and mutant viral genomes were detected at comparable levels by sequencing of a *gag* gene fragment amplified by reverse transcription and nested PCR from plasma RNA at week 1, but the mutant was poorly detected and the wild type was dominant at weeks 2 and 3. We then subcloned the amplified fragments into plasmids for sequencing and counted the numbers of clones carrying the wild-type (Gag216L) or the mutant (Gag216S) sequence at the region encoding the 216th aa in Gag. It revealed that the wild-type SIV became dominant 2 or 3 weeks after the inoculation (Fig. 1B). This result indicates that the replicative ability of this CTL escape mutant virus was diminished compared to

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