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Contribution of Monocytes to Viral Replication in Macaques during Acute Infection with Simian Immunodeficiency Virus

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ABSTRACT

Monocytes are known as an alternative target for HIV/SIV infection, but the contribution of monocytes to viral spread in a host is unclear. In this study, CD14 monocytes were monitored in 6 macaques until six weeks postinfection (wpi) with SIVmac239 to evaluate their contribution to viral load. The monocyte count in blood significantly increased with peak viremia at 2 wpi and the expression level of CD14 on monocytes significantly decreased at 1–2 wpi, though the number of CD4 T cells was stable in these macaques. The number of CD14 monocytes and the expression level of CD14 on monocytes at 2 wpi were also significantly related to the extent of viremia in plasma. An increased number of monocytes at 2 wpi was associated with a lower postacute viral load, suggesting that monocytes have a role in suppressing the virus. The lower expression level of CD14 in monocytes at 2 wpi was associated with a higher viral load and greater degree of infection of monocytes. This correlation suggests that monocytes with a low level of CD14 may be more susceptible to SIV and may enhance viral replication. The analysis of monocytes in persistently infected macaques revealed that the expression level of CD14 was also significantly low during persistent infection compared with naïve macaques, though the monocyte count was within the normal range. Monocytes may suppress viruses, perhaps by their immune function, during acute infection. However, infection of monocytes may increase the viral load and spread viruses in a host.

INTRODUCTION

CD4 T CELLS ARE A MAJOR TARGET OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) and simian immunodeficiency virus (SIV). Thus, CD4 T cell counts during infection are used as a marker of disease progression.[1–4] The correlation between CD4 helper responses and disease progression suggests that CD4 T cells are critical for antiviral immunity against HIV/SIV.[5,6] Recent studies revealed that CD4 T cells in mucosal tissues and memory CD4 T cells in blood are selectively depleted during acute infection because of their high level of CCR5 expression.[7–12] The massive depletion of memory CD4 T cells is correlated with rapid progression of disease.[7,11,13] Although countless studies have investigated the roles of CD4 T cells in HIV/SIV infection, few studies have examined the roles of other immune cell populations, such as monocytes, B cells, NK cells, and granulocytes. These cells directly or indirectly interact with T cells, and thus can influence the susceptibility and antiviral activity of CD4 T cells. They may play a major role in antiviral immunity at the very early phase of infection as innate immunity, because adapted immunity, which is controlled by T cells, does not mature until later in the course of infection. Monocytes are known as an alternative target for viral infection[14,15] and differentiate to macrophages, which produce viruses in various tissues.[16,17] Monocytes/macrophages, in addition to their importance as targets of HIV-1, are suggested to be reservoirs in patients who receive highly active antiretroviral therapy (HAART).[18,19] Moreover, they are major

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sources of cytokines and chemokines, which can affect viral replication and even pathogenesis.[20,21] However, the contribution of monocytes to viral replication in a host is still unclear.

Infection of macaques with SIV is a good model with which to study the pathogenicity of AIDS. In SIV infection, as in HIV infection, the plasma viral RNA load and CD4 T cell count are important predictors of disease progression.[3,22,23] Several SIV strains, as well as molecular clones of SIVs, have been well characterized as a result of their use in monkey infection experiments.[24–26] Each SIV strain has its own properties and level of virulence in monkeys. However, host factors affect the progression of disease, and even experimental infection with molecularly cloned SIV can result in a variable disease outcome among monkeys. Susceptibility of CD4 T cells is one of the factors influencing early virus replication.[27,28] However, other factors that potentially contribute to a variable disease outcome have not been examined.

In this study, monocytes were monitored until 6 weeks postinfection (wpi) with SIV to clarify their effect on viral replication *in vivo*. We attempted to determine whether monocyte numbers and CD14 expression levels were related to virus load, and showed that changes of monocytes at 2 wpi were significantly related to the extent of subsequent viremia in plasma.

MATERIALS AND METHODS

Inoculation of monkeys

Six juvenile rhesus macaques (*Macaca mulatta*), MM321, MM322, MM323, MM324, MM325, and MM327, were inoculated with 10^5 $TCID_{50}$ of SIVmac239.[24] Blood samples were periodically collected from all the macaques. After isolating peripheral blood mononuclear cells (PBMC), monocytes were separated by allowing them to adhere to a plastic.[29,30] Nonadherent cells were collected as lymphocytes after 1 h incubation of PBMC in a plastic plate. After washing with phosphate-buffered saline (PBS) three times, adherent cells were removed from the plate by washing with ice-cold PBS, and collected as monocytes.

Eleven naive and 11 infected rhesus macaques were used to analyze monocytes during persistent infection. The infected macaques consisted of one SIVmac239-infected macaque, two SHIV-C2/1[31]-infected macaques, and eight SHIV-C2/1-challenged macaques. Four of the eight SHIV-C2/1-challenged macaques were vaccinated with attenuated SHIV.[32] The other four were vaccinated with DNA vaccine.[33] These macaques were in a stable period after the infection or challenge.

Throughout the experimental period, the monkeys were housed in accordance with regulations approved by the Institutional Animal Care and Use Committee of the Institute for Virus Research, Kyoto University.

Flow cytometry analysis

The following monoclonal antibodies were used for this study: CD3 [FITC, FN-18, Biosource (Camarillo, CA)], CD4 [PE, NU-TH/I, Nichirei (Tokyo, Japan)], CD8 [PerCP, Leu-2a, BD Bioscience (San Jose, CA)], CD14 [PC5, RMO52, Beckman Coulter (Fullerton, CA)], CD16 (FITC, 3G8, BD Bioscience), CD20 (PE, Leu-16, BD Bioscience), and CD56 (PE, B159, BD Bioscience). Aliquots of 50 1 whole blood were incubated with combinations of these antibodies for 1 h at 4°C, lysed with FACS lysing solution (BD Bioscience) for 10 min, washed with 1% bovine serum albumin-phosphate buffered saline (BSA-PBS), and fixed in 3.7% formaldehyde in 0.1% BSA-PBS. Samples were analyzed on FACScan using CellQuest (BD Bioscience). Absolute lymphocyte counts in the blood were determined with an automated blood cell counter (F-820; Sysmex, Kobe, Japan).

The monocyte population was gated on a dot plot of forward and side scatters, and CD14 cells in this gate were counted as monocyte. Mean fluorescence intensity (MFI) of CD14 monocytes is used as the expression level of CD14 in monocytes.

Quantification of viruses in plasma, lymphocytes, and monocytes

The viral RNA loads in plasma were determined by quantitative reverse transcriptase polymerase chain reaction (RT-PCR) using a Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA) as described before.[34] Briefly, total RNAs were prepared from plasma with a QIAamp Viral RNA kit (QIAGEN, Hilden, Germany). RT-PCR was performed with a Taqman EZ RT-PCR kit (Applied Biosystems) using primers: SIV2-696F and SIV2-784R, and a probe, SIV2-731T.

The viral DNA loads in lymphocytes and monocytes were similarly determined by quantitative PCR using the same primers and probe. PCR was performed with a Taqman PCR core reagent kit with controls (Applied Biosystems). Proviruses were detected in monocytes from the three macaques, MM321, MM324, and MM327, but not from the other three. Proviral DNA in monocytes was judged to be positive after the correction by subtracting 10% contamination of lymphocytes from the actual value, because the purity of monocytes was shown to be between 80 and 95% by CD14 staining. Proviruses in monocytes from these three macaques were positive even after the correction by subtracting 30% contamination of lymphocytes.

Statistical analysis

Two-way repeated-measures ANOVA was performed by Prism (GraphPad Software, San Diego, CA) to analyze the effects of infection on cell populations. Data at each time point were also compared with data on day 0 by Dunnett's test. Regression analysis was performed by KaleidaGraph (Synergy Software, Reading, PA) to examine associations of plasma viral load with cell count or fluorescence intensity in each cell population. Coefficients of determination (R^2) were calculated and shown as values that indicate relationships. The statistical significance of any difference between naive and infected macaques was determined by Student's *t*-test.

RESULTS

Changes of monocytes in acute infection with SIVmac239

Six rhesus macaques were intravenously inoculated with SIVmac239. Blood CD14 monocyte count and expression

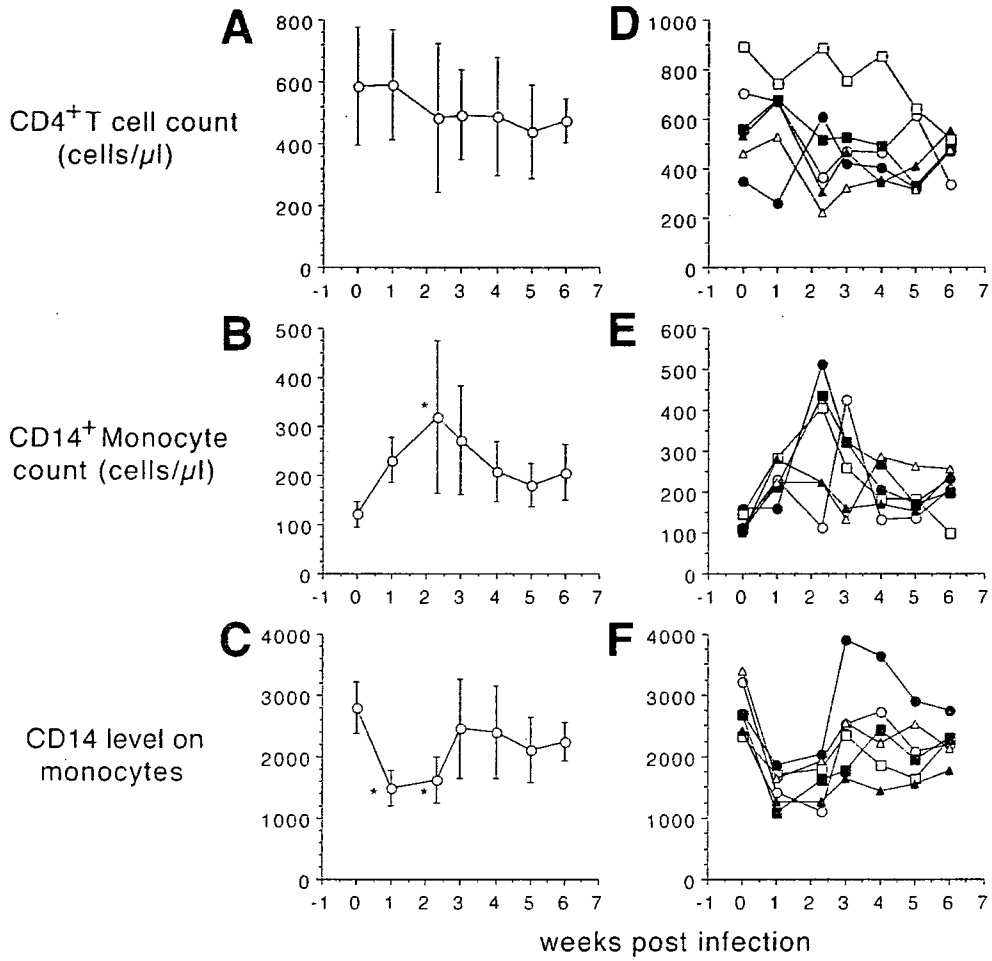

FIG. 1. Average CD4 T cell count (**A**), CD14 monocyte count (**B**), and expression level of CD14 on monocytes (**C**) in six infected macaques up to 6 wpi. (**D–F**) Data for individual macaques. Plasma viral loads in individual macaques up to 6 wpi are also shown (**G**). Dunnett's test was performed, and asterisks show the points that are significantly different from the value at 0 day.

level of CD14 in monocytes, as well as CD4 cell count, were monitored in these macaques up to 6 wpi (Fig. 1). Although the CD16 subpopulation of monocytes was suggested to correlate with AIDS dementia and opportunistic infections,[35–37] expression of CD16 on monocytes was observed in only one of these macaques (data not shown). The average CD4 T cell count in these macaques gradually decreased after infection (Fig. 1A), and some macaques showed a transient decrease at 2 wpi (Fig. 1D). However, this decrease was not statistically significant when analyzed by two-way repeated-measures ANOVA (p 0.258). In contrast to the CD4 T cell count, the monocyte count significantly increased with a peak at 2 wpi (p 0.011, Fig. 1B) and the CD14 expression level on monocytes significantly decreased at 1–2 wpi (p 0.0001, Fig. 1C). The peak height of monocyte count varied among macaques, but all the macaques showed a significant increase (Fig. 1E). The expression level of CD14 decreased in the first week in all the macaques, though the extent of rebound varied among macaques (Fig. 1F). The peak of the increase in monocytes and the decrease of CD14 level were significantly different from those at day 0 by Dunnett's test. These results clearly indicated that infection with SIVmac239 significantly influenced the monocyte count and CD14 expression on monocytes in acute infection. It is noteworthy that the changes in monocyte numbers and CD14 expression level were much greater than the changes in CD4 T cell numbers in blood.

Relationships between plasma viral loads and monocytes

Viral RNA levels in plasma ranged from 10^7 to 10^9 copies/ml at peak viremia in these macaques (Fig. 1G). After the acute viremia, the viral load reached a plateau between 10^6 and 10^7 copies/ml in four of the six monkeys, while the viral load decreased to the undetectable level in two macaques (MM322 and MM323).

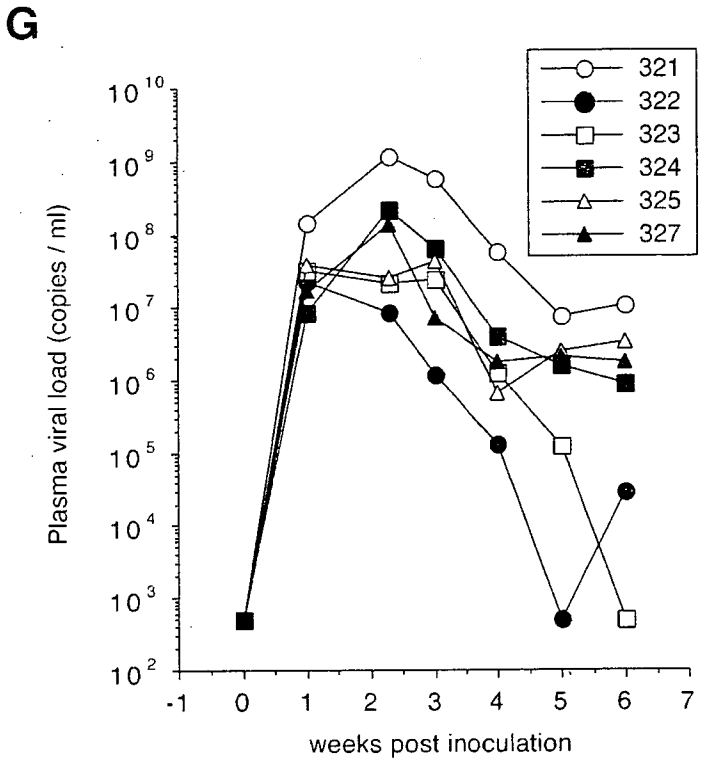

FIG. 1. *(Continued).*

We next evaluated the effects of the monocyte count and CD14 level at each time point on subsequent plasma viral loads (Table 1). Although viral load was unrelated to the CD4 T cell count during the observation period (R^2 0.174), the viral loads at 3, 4, 5, and 6 wpi were strongly correlated with the monocyte count (R^2 0.792) and CD14 expression level (R^2 0.678) at 2 wpi (Table 1). The sporadic relationship was also observed between monocyte count at 3 wpi and the viral load at 4 wpi (R^2 0.848). Although the monocyte count and CD14 level were related to subsequent viral load, viral load did not appear to be related to the subsequent monocyte count or CD14 level except for the relation between viral load at 5 wpi and CD14 level at 6 wpi (R^2 0.641, data not shown).

These results showed that the monocyte count and CD14 level, when they were significantly changed by infection, were closely related to the subsequent viral replication.

Effects of monocytes on viral load

The CD14 monocyte count at 2 wpi, when it was at the peak, was strongly correlated with the viral load at 3–6 wpi, especially at 4–6 wpi (Table 1). The increase of the monocyte count at 2 wpi was negatively correlated with the viral load at 4–6 wpi (Fig. 2A), suggesting that the monocytes suppressed the subsequent viral load. However, the monocyte count at 3 wpi was positively related to the viral load at 4 wpi (Fig. 2B). This suggested an opposite effect of the monocyte count at 3 wpi on the viral load, although this effect was temporary. These results indicate that the early induction of monocytes at the peak of viremia may help to suppress viral replication.

The CD14 level at 2 wpi, when the CD14 level in all the macaques decreased, was negatively related to the viral load at 3–6 wpi, especially at 5 wpi (Fig. 2C and Table 1). In other words, the viral load was lower in macaques in which the decrease of CD14 level was not severe. An increase in CD14 expression has been found to suppress HIV-1 replication.[38,39] From our observation and these reports, we speculated that the decrease of CD14 level may allow SIV to infect monocytes and increase the viral load. Therefore, the infection of monocytes was examined by PCR. As expected, provirus was detected in monocytes in only three monkeys, MM321, MM324, and MM327, which showed lower CD14 expression levels at 1–2 wpi than the other macaques (Table 2 and Fig. 1F). In the three macaques with infected monocytes, viremia peaked at 2 wpi, while in the other three macaques, viremia started to decrease at 2 wpi (Fig. 1G). This additional increase of viremia at 2 wpi may be due to the infection of monocytes.

Low level of CD14 expression in persistently infected macaques

Monocytes from naïve and persistently infected macaques were analyzed to investigate the effect on monocytes in the late phase of infection. Most of the macaques that were persistently infected were at a chronic phase of infection with stable CD4 T cells. The average CD4 T cell count in infected macaques

TABLE 1. COEFFICIENTS OF DETERMINATION[a] (R^2) FOR REGRESSION ANALYSIS[b]

		Plasma Viral Load					
Cell population	*wpi*	*1*	*2*	*3*	*4*	*5w*	*6 pi*
CD4 T cell count	0	0.084	0.024	0.004	0.027	0.005	0.132
	1		0.041	0.019	0.024	0.020	0.043
	2			0.036	0.032	0.086	0.025
	3				0.011	0.058	0.004
	4					0.047	0.005
	5						0.174
CD14 monocyte count	0	0.021	0.008	0.002	0.002	0.021	0.011
	1		0.007	0.018	0.017	0.086	0.030
	2			**0.792**	***0.800***	***0.934***	***0.970***
	3				***0.848***	0.081	0.226
	4					0.167	0.115
	5						0.082
CD14 level on monocytes	0	0.281	0.151	0.145	0.169	0.259	0.149
	1		0.004	0.000	0.001	0.007	0.033
	2			**0.678**	**0.798**	***0.849***	**0.795**
	3				0.036	0.012	0.030
	4					0.020	0.014
	5						0.005

[a]Coefficients of determination (R^2) were calculated from regression analyses that examined the effect of cell count or MFI on plasma viral load. Normal type indicates R^2 0.5; boldface indicates 0.5 R^2 0.8; boldface and italics indicate R^2 0.8.

[b]The relationship of CD4 T cell count, CD14 monocyte count, and CD14 level on monocytes at each time point to the subsequent plasma viral load is shown.

was not significantly lower than that of naive macaques (Fig. 3A). The CD14 monocyte count in infected macaques varied widely, but was not significantly different from that in naive macaques (Fig. 3B). In contrast, the CD14 expression level was significantly decreased in persistently infected macaques, and was half of that in naive macaques (Fig. 3C). The decrease of CD14 level was observed in most of the macaques regardless of disease progression, suggesting that a decrease of CD14 level is a persistent effect of infection. Typical examples of CD14 level in infected and naive macaques are shown in Fig. 3D. The CD14 level in infected macaque #1 was lower than normal as shown in histograms on the left column. The peak CD14 expression level in this macaque was sharp and lower than normal. Most of the persistently infected macaques showed a similar pattern. In another infected macaque, #2, which had the lowest CD14 level, the CD14 expression pattern was diffuse without a sharp peak. $CD14^{low}CD16$ monocytes were clearly observed in infected macaque #2, but were rare in other ma-

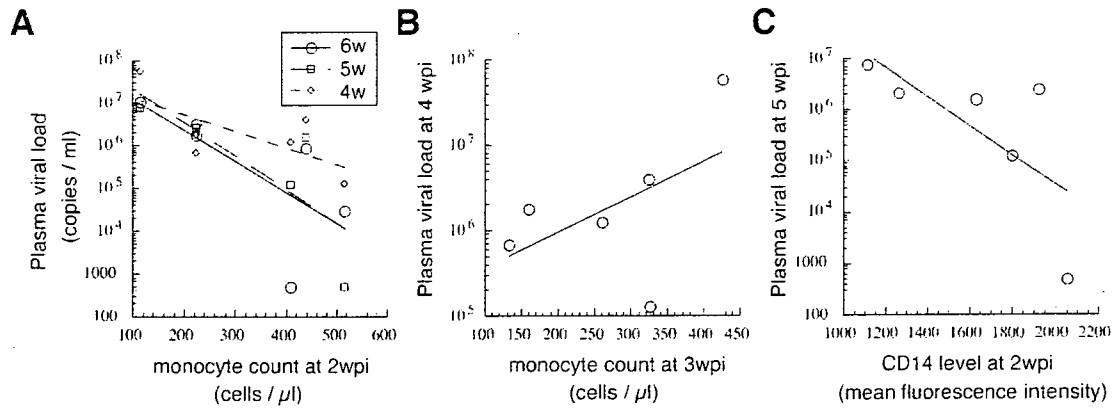

FIG. 2. Relationship of CD14 monocytes to the subsequent viral load. (A) Higher CD14 monocyte count at 2 wpi was significantly related to lower plasma viral load at 4–6 wpi (R^2 0.800, 0.934 and 0.970, respectively). However, (B) higher CD14 monocyte count at 3 wpi was related to higher viral load at 4 wpi (R^2 0.848). (C) Higher CD14 expression level on monocytes at 2 wpi was related to lower viral load at 5 wpi (R^2 0.849).

TABLE 2. PROVIRAL DNA IN MONOCYTES[a]

wpi	*321*	*322*	*323*	*324*	*325*	*327*
1						
2						
3			NA[b]			
4						
6						

[a]Proviral DNA in monocytes was examined by quantitative PCR.
[b]NA, not available.

caques. The CD56 level was also low in the CD14^{low}CD16 subpopulation of macaque #2 (Fig. 3D). This macaque, which was infected with SHIV-89.6p, showed the lowest CD4 T cell count (12 cells/ l) and the highest CD14 monocyte count (1012 cells/ l) of the macaques analyzed. Thus, the presence of CD14^{low}CD16 CD56^{low} monocytes may reflect a disease state.

DISCUSSION

The cell count and CD14 expression level of monocytes drastically changed during acute infection with SIVmac239, while the CD4 T cell count was stable. In some macaques, the CD4 T cell count was decreased at 2 wpi, as we previously reported,[40] but the decrease was not statistically significant because no decrease was observed in the other macaques. Recent studies revealed that memory CD4 T cells, which are a target for SIV infection because of the high level of CCR5 expression, are selectively depleted during acute infection.[7–11] This massive depletion of memory CD4 T cells, as well as depletion of CD4 T cells in mucosal effector sites, is correlated with rapid progression of disease.[7,11,13] The decline of memory CD4 T cells in the acute phase is difficult to speculate from

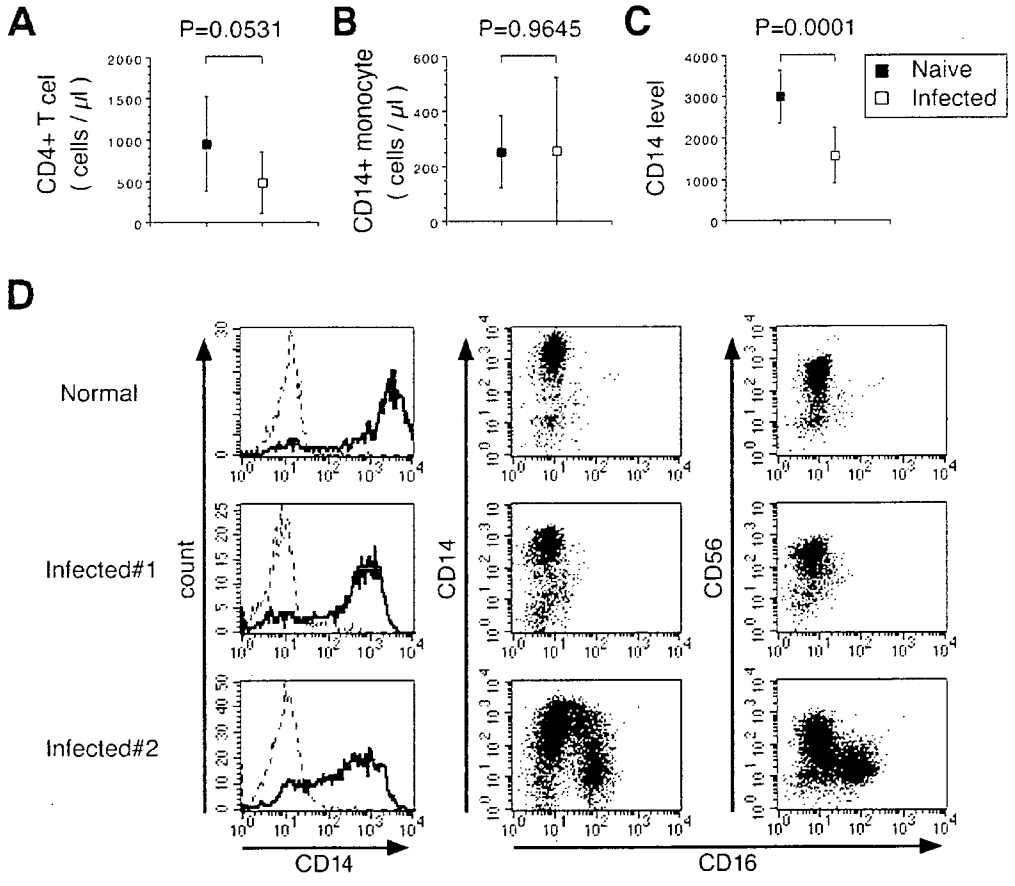

FIG. 3. Level of CD14 expression was significantly lower in persistently infected macques than in naive macaques. Eleven naive and 11 infected rhesus macaques were monitored for CD4 T cell count (**A**), CD14 monocyte count (**B**), and CD14 expression level on monocytes (**C**) by flow cytometry analysis. Average and standard deviation were shown. *p* values were calculated by Student's *t*-test. Expression of CD16 and CD56 was examined in these macaques and representative results are shown (**D**). CD14 level was high in normal macaques, lower in most of the persistently infected macaques such as infected #1, and diffusely low in infected #2. CD14^{low}CD16 CD56^{low} monocytes were observed in infected #2.

total CD4 T cell count in the blood, since naive CD4 T cells predominate in response to the memory CD4 T cell depletion. Unfortunately, memory CD4 T cells were not monitored in this study. However, the total CD4 T cell count is still an essential marker for evaluating disease progression in AIDS diagnosis.[3,22,23] and the changes in monocyte count and CD14 level, which were greater than the changes in the number of CD4 T cells, suggest that monocytes have an important role in pathogenesis.

The drastic changes of monocyte count and CD14 expression level that we observed during acute infection suggest that monocytes are significantly influenced by SIV infection, possibly as a result of the virulence of SIV and the anti-SIV immune response. The changes of monocyte count and CD14 level that we observed are not due to infection of monocytes because the virus was not detected in monocytes of three of the six macaques in this study. Actually, monocytes were reported to be infected with HIV-1,[14,15] but are less susceptible to infection than tissue macrophages.[20,41] Although there is a possibility that attachment of viral particles on monocytes or other indirect mechanisms increase the number of monocytes and decrease the CD14 level on monocytes, these changes may be mainly an immune response to SIV infection. The induction of monocytes and downregulation of CD14 may be the immune activation, which are generally observed during any acute infection. The response to infection is controlled by cytokine/chemokine networks and communications among immune cells, and results in changes in cell counts and activation status of the immune cells. Since the changes of monocytes were associated with the viral load, the changes of monocytes possibly contribute to the dysregulation of immunity that occurs in SIV infection. Further studies will be needed to determine whether monocytes contribute to disease progression, and to clarify the mechanism that changes monocytes during acute infection.

The cell count and CD14 expression level of monocytes were found to be related to viral load, while the CD4 T cell count was not. The close relation of monocytes to viral load suggests that monocytes have significant effects on viral replication *in vivo*. In the early phase of infection, adapted immunity is not mature in hosts, and innate immunity plays an important role. Thus, cell populations other than T cells, which mainly function in adaptive immunity, may play a main role in controlling viral replication in the early phase of infection. The increase of monocyte count and decrease of CD14 level in monocytes may influence the innate immune response against infection with SIV. If monocytes have an important role in innate immunity to control viremia, it is reasonable that the induction of monocytes and the maintenance of a high CD14 level suppress viral replication. In the later phase of infection, monocytes may not have an effect on viral replication or progression of disease because in this phase adaptive immunity plays the main role in controlling viral replication. However, postacute viremia strongly correlates with disease progression in infection with HIV-1[42,43] or SIV.[22,23] This suggests that the suppression of viral load in the early phase of infection by monocytes contributes to disease progression.

The low CD14 expression in monocytes was previously reported in HIV/SIV infection.[35–37,44,45] In these reports, $CD14^{low}$ monocytes also expressed CD16. $CD14^{low}CD16^{-}$ monocytes made a subpopulation that was distinct from normal $CD14^{+}$ monocytes. In this study, the $CD16^{+}$ subpopulation was rare in both acute and persistent infection, and the decrease of CD14 expression level in the early phase of infection did not correlate with CD16 expression. These results indicate that expression of CD16 on monocytes is not a good marker in the early phase of infection. The $CD16^{+}$ subpopulation may be important in persistent infection, because $CD14^{low}CD16^{+}$ monocytes correlated with AIDS dementia and opportunistic infections.[35–37] Actually, a $CD14^{low}CD16^{+}$ subpopulation was clearly observed in one of the persistently infected macaques in this study, and this macaque showed a much lower CD4 count than did the others.

Monocytes are not the main target of viral infection, as mentioned above. However, we detected the virus in monocytes in three of the six macaques. Infection of monocytes with HIV-1 was previously reported,[14,15] and was suggested to play a role as a source of virus in patients on HAART.[18] This indicates a possible direct virulence to monocytes by infection, and the decrease of CD14 level may be due to an infection to monocytes. However, other macaques, in which viruses were not detected, also showed a decrease of CD14 level, though the decrease was severe in macaques with infected monocytes. This suggests that the CD14 level in monocytes is mainly decreased by a mechanism other than infection of monocytes. Infection to monocytes may enhance it, or the lower CD14 level may make monocytes more susceptible to infection. CD14 was reported to inhibit replication of HIV-1,[38] and it was suggested that the higher CD14 level in monocytes correlated with the lower replication of HIV-1.[39] Based on these observations, we propose the following mechanism to enhance viral replication. At first, the level of CD14 expression in monocytes was decreased by an indirect effect by infection with SIV. Then, the low CD14 level in monocytes allowed them to become infected with SIV. The infected monocytes moved into tissues and differentiated to macrophages. These macrophages produced viruses in various tissues and increased the viral load in plasma. This hypothesis explains the correlation between higher viral load and infection to monocytes. Similar hypotheses have been proposed to explain the mechanism of AIDS dementia[46] and the mechanism by which viral reservoirs are maintained.[19] The susceptibility of monocytes/macrophages to infection is dependent on the stage of differentiation, and monocytes are reported to be less susceptible than macrophages.[20,41] Thus, macrophages are considered as a target for infection, and macrophages that are producing viruses are thought to be infected at the same site after the differentiation to macrophages. Our hypothesis proposes that the site and cell population of infection are different from those of viral production.

In summary, the significant relation of monocytes to virus load, which was shown in this study, indicates that monocytes make a considerable contribution to SIV infection. To better understand AIDS pathogenesis, further studies are needed to elucidate how monocytes influence viral replication.

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Th1-type immune responses by Toll-like receptor 4 signaling are required for the development of myocarditis in mice with BCG-induced myocarditis

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Abstract

The immunological aspects of autoimmune myocarditis are difficult to understand because of the existence of many infectious agents and animal models suggesting different mechanisms in autoimmune myocarditis. To overcome these difficulties, two strains of mice, C3H/HeN and C3H/HeJ, showing different immune responses to mycobacteria, were immunized with myosin mixed with BCG. The C3H/HeN mice with a wild-type Toll-like receptor 4 (TLR4) showed severe myocarditis, whereas the C3H/HeJ mice with nonfunctional mutated TLR4 did not. $CD4^+$ cells from both strains of mice exhibited appreciable proliferative responses following myosin stimulation; however, the cytokines from these cells differed between these two strains. The C3H/HeN mice showed T helper (Th)1-type cytokine responses, whereas the expressions of mRNA in C3H/HeJ mice were Th2-type cytokine. When both of these strains of immunized mice were inoculated with a plasmid encoding cDNA of interleukin (IL)-4 or agonistic IL-4, the development of myocarditis was inhibited in C3H/HeN mice. Moreover, C3H/HeJ mice, in which development of myocarditis was not induced by immunization of myosin mixed with BCG, showed myocarditis after injection of IL-4 antagonistic mutant DNA for the induction of Th1-type immune responses. The results suggested that the induction of autoimmune myocarditis by myosin is affected by Th1-type immune responses.

Keywords: Autoimmunity; Bacillus Calmette–Guérin; Myocarditis; Th1/Th2; TLR4

1. Introduction

Myocarditis is a potentially lethal disorder of various etiologies for which no treatment is currently satisfactory [1]. Although the etiology of dilated cardiomyopathy is unknown, more than 10% of cases are associated with a previous virus infection, such as Coxsackievirus B3 [2]. Since heart failure generally occurs long after infection with autoimmune responses, autoimmunity is thought to play an important role in myocarditis as well as contributing to the progression to cardiomyopathy and heart failure [3]. To explore the mechanisms

Abbreviations: DC, dendritic cell; DTH, delayed-type hypersensitivity; EAM, experimental autoimmune myocarditis; TLR, Toll-like receptor.

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of such immune system-mediated damage to the heart in this disease, various animal models have been established by infection of various pathogens and immunization of cardiac myosin (reviewed in [4]). Although animal models of experimental autoimmune myocarditis (EAM) have provided information on pathogenesis that is valuable for the prevention and treatment of myocarditis, an understanding of the pathogenesis of EAM in animal models is difficult to apply to human myocarditis. Animal models of EAM have been established in various species and strains of animals using various types of infectious pathogens and immunization of cardiac myosin, and the pathogenic mechanisms in these models have not shown identical immune responses [5,6]. To overcome these difficulties, animal models of EAM that are established for understanding immune response to myocytes should allow us to identify several factors that induce EAM such as pathogens and the genetic basis of animals.

T helper (Th) cells are thought to have crucial roles in both autoimmune diseases and immunological disorders. Th cells are identified by functions as Th1 or Th2 subsets secreting distinct cytokine patterns that demonstrate effector functions and cross inhibition [7]. Cytokines are important for controlling the response of Th cells to self antigens (Ags) and they play a critical role in shifting the immune response toward a Th1 or Th2 pattern. A Th1 response shifts the cytokine profile toward delayed-type hypersensitivity (DTH), macrophage activation, and proinflammatory T-cell response associated with interferon (IFN)-γ and interleukin (IL)-2 and -12, whereas a Th2 response is associated with B cell activation and humoral immunity and with IL-4, -5, -9, -13 and IgE production. As a result, understanding the Th cell responses to auto-Ags is important for the prevention and treatment of autoimmune diseases such as autoimmune myocarditis in human patients.

Mice with a C3H/He lineage were originally established in 1941, and two laboratories have maintained this strain as C3H/HeN and C3H/HeJ since 1947 and 1951, respectively. These two strains of mice showed different responses to some strains of bacteria, and the differences in the responses to some strains of bacteria have been thought to be caused by Toll-like receptor (TLR) 4 [8]. C3H/HeJ mice have an unfunctional TLR4 [9], and these mice are more susceptible to mycobacteria infection than are TLR4 wild-type mice [10–12]. TLR activation elicits adaptive immune responses with a bias towards Th1 T-cell response. It has also been reported that TLR4 wild-type C3H/HeN mice, but not mutated TLR4 C3H/HeJ mice, showed typical Th1-type immune responses to mycobacteria infection [10,12,13], although both strains of mice have the wild-type Bacillus Calmette–Guérin (BCG) resistant gene *(N-ramp)*.

To elucidate the immunological mechanisms by which cardiac myosin is recognized without various factors, we tried to establish an animal model of EAM by using the responses to mycobacteria in the present study. Two strains of mice, mycobacteria-susceptible C3H/HeJ mice and mycobacteria-resistant C3H/HeN mice, were immunized with porcine cardiac myosin mixed with BCG. Interestingly, mycobacteria-resistant C3H/HeN mice, but not mycobacteria-susceptible C3H/HeJ mice, developed myocarditis. We herein report the differences in immune responses to myosin in the development of an animal model of EAM in mice with a close genetic background.

2. Materials and methods

2.1. Mice

Six- to eight-week-old C3H/HeN (TLR4 wild type) and C3H/HeJ (TLR4 mutated) female mice were purchased from CLEA Japan (Osaka, Japan) and housed in the Laboratory Animal Center of Mie University School of Medicine.

2.2. Immunization of myosin

Each mouse was immunized with 100 μg of porcine cardiac myosin (Sigma) mixed with 1 mg of BCG Tokyo strain (Japan BCG Laboratory, Tokyo, Japan) in IFA into the footpad on day 0 and day 14. The BCG used for immunization was well-ground and killed. This myosin and BCG mixture was completely emulsified with IFA. The injection site (footpad) and degree of emulsification are very important for the development EAM. The control mice were injected with the same amount of BCG alone in emulsified IFA. All mice were sacrificed on day 21 for pathological observations (Fig. 1a).

2.3. Administration of DNA

The plasmids encoding cDNA of antagonistic interleukin (IL)-4 double mutant (Q116D/Y119D) (IL-4DM) and agonistic IL-4 single mutant (Q116D) (IL-4SM) have been described previously [14]. The mice were intraperitoneally administered 100 μg of plasmid DNA encoding IL-4, IL-4SM or IL-4 DM on days −7, 0, 7 and 14 to regulate the Th balances. An empty plasmid (pcDNA 3.1) vector was used as a control (Fig. 1b).

2.4. Proliferative responses of spleen cells to porcine myosin

The responding spleen cells obtained from the immunized mice were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific antibodies (Abs) to confirm the subset of effector cells. The purity of cells (CD4 or CD8 cells) was confirmed by FACS analysis. The proportion of targeted cells did not exceed 0.01%, and dead cells were removed after cell washing (viability >90%). The cells were resuspended in complete medium and cultured at a concentration of 2×10^5 cells per culture well in a total volume of 0.2 ml with 10 μg/ml of myosin. The same amount of OVA was used as a control Ag. Each culture was performed in triplicate in 96-well microculture plates and was then maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The cultures were harvested using a cell harvester at 96 h after a 6-h pulse with 18.5 kBq/well of [^{3}H]thymidine. The results were calculated from the uptake of [^{3}H]thymidine and expressed as the mean uptake in cpm ± SD of triplicate cultures.

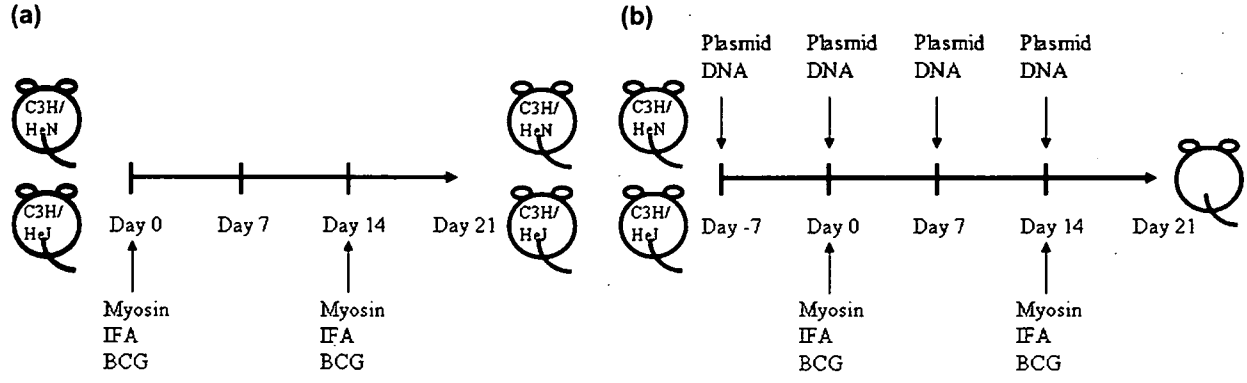

Fig. 1. Experimental design in this study. (a) Each mouse was immunized with 100 μg of porcine cardiac myosin mixed with 1 mg of BCG in IFA into the footpad on day 0 and day 14 (see Section 2). (b) Plasmid DNA of IL-4, IL-4SM, IL-4DM or control was intraperitoneally injected once on days −7, 0, 7 and 14.

2.5. Detection of cytokine mRNA from lymphocytes using RT–PCR

Total RNA was purified from the OVA (control)- or myosin-stimulated spleen cells using Isogen (Nippongene, Japan) following the manufacturer's instructions. For the RT reaction, a reverse transcription system (Promega, WI, USA) was used. PCR was performed in a total volume of 50 μl of 1× PCR buffer (Takara Shuzo, Japan) containing 0.5–1.0 μg of cDNA, 0.25 mM of each dNTP, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo, Japan). The specific primer pairs used were as follows: IL-2, 5′-AAGAT GAACTTGGACCTCTGCGG-3′ (sense) and 5′-CCTTATG TGTTGTAAGCAGGAGG-3′ (antisense); IL-4, 5′-ATGGGT CTCAACCCCCAGCTAGT-3′ (sense) and 5′-GCTCTTTAG GCTTTCCAGGAAGTC-3′ (antisense); IL-12p40, 5′-TCC TGCACTGCTGAAGACATC-3′ (sense) and 5′-TCTCGCCA TTATAGATTCAGAGAC-3′ (antisense); IL-13, 5′-GACCCA GAGGATATTGCATG-3′ (sense) and 5′-CCAGCAAAGTCT GATGTGAG-3′ (antisense); and mouse HPRT, 5′-GATAC AGGCCAGACTTTGTTGG-3′ (sense) and 5′-GAGGGTA GGCTGGCCTATAGG-3′ (antisense). The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 s at 56 °C, extension for 1 min at 73 °C, and denaturation for 30 s at 93 °C. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gel.

2.6. Measurement of interferon-γ (IFN-γ)

Spleen cells from immunized mice (5×10^6) were cultured with 10 μg/ml of myosin in 24-well culture plates at a volume of 2 ml. After incubation at 37 °C in a humidified incubator (5% CO_2) for 96 h, culture supernatants were quantified by using a standard ELISA kit (BioSource International, CA, USA).

2.7. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test and the Kruskal–Wallis test. The values are expressed as means ± SD. A 95% confidence limit was considered to be significant ($p < 0.05$).

3. Results

3.1. Development of EAM

Although rodent models of EAM have been established in various species and strains, it is not easy to understand the mechanisms underlying the development of EAM by recognition of autologous myosin Ags because of the effects of genetic backgrounds. To determine whether cardiac myosin immunization can induce EAM in strains of mice with the same genetic background except for TLR4, C3H/HeN and C3H/HeJ mice were immunized with cardiac myosin mixed with BCG. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. The C3H/HeN mice developed mild (2/20), moderate (14/20) and severe (4/20) myocarditis, whereas only one C3H/HeJ mouse showed mild myocarditis (1/20) by histopathological observations on day 21 (Fig. 2). Two C3H/HeN mice died before sacrifice on day 18 (severe and moderate myocarditis). Moreover, EAM did not develop in either strain of mice immunized with myosin and IFA without BCG, and the control mice immunized with IFA emulsion containing BCG or myosin alone did not show any abnormalities (data not shown). These results demonstrated that immunization of an emulsion containing myosin and BCG induced the development of EAM in wild-type C3H/HeN mice but not in TLR4-mutated C3H/HeJ mice.

3.2. Myosin-specific spleen cell proliferative responses in myosin-immunized mice

We next confirmed the presence of effector cells that recognize the myosin induced by the immunization of myosin mixed with BCG in *in vivo* experiments. Spleen cells from both strains of mice immunized with myosin mixed with BCG were assessed for their proliferative responses after stimulation *in vitro* with myosin. Spleen cells from both C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis exhibited proliferative responses after *in vitro* stimulation with myosin (Fig. 3). These proliferative responses were not observed in the case of stimulation with an irrelevant Ag

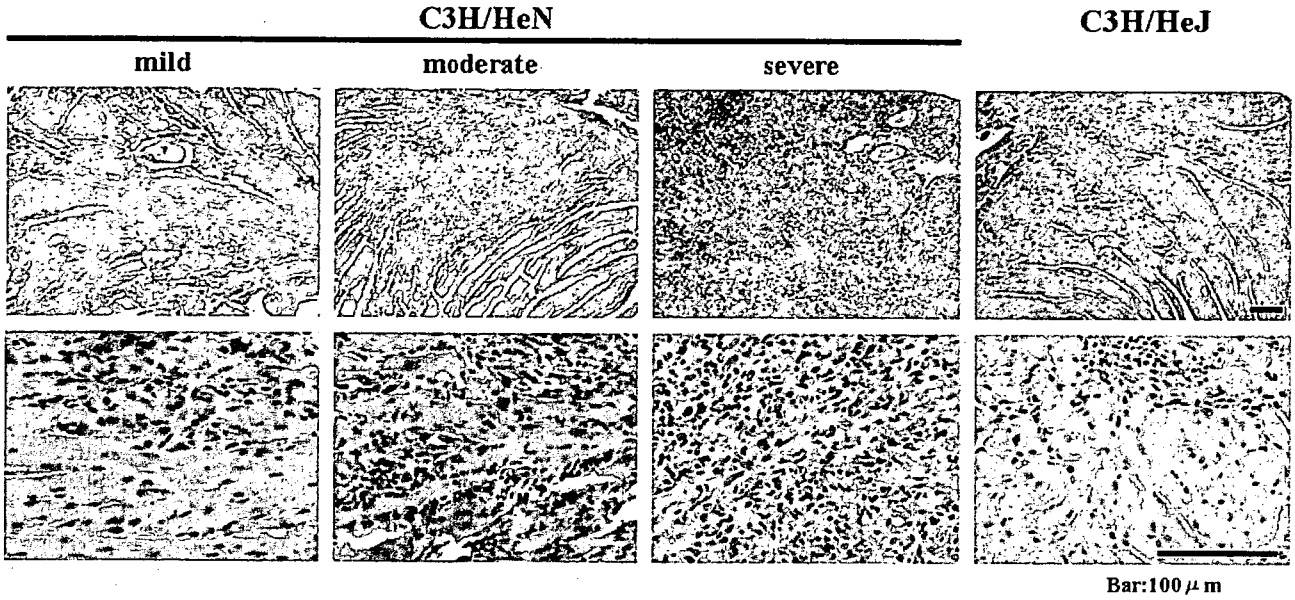

Fig. 2. Results of histopathological examination of hearts from mice that had been immunized with cardiac myosin mixed with BCG in IFA. The histological findings were classified into severe (>50%), moderate (10–49%) and mild (<9%) depending on the ratio of affected area to total myocardium. All tissue specimens were obtained 21 days after the first myosin immunization. The tissue specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. These results are representative of five independent experiments. Bars represent 100 μm.

(OVA). A significant component of this proliferative response was attributed to the presence of $CD4^+$ cells, because $CD4^+$ cell-depleted spleen cells exhibited a substantially reduced myosin-specific proliferative response. Moreover, the C3H/HeN mice did not develop myocarditis when they received a monoclonal Ab to CD4 for depleting $CD4^+$ cells (data not shown). The observation that immunization of myosin mixed with BCG in both strains of mice elicited a $CD4^+$ proliferative T-lymphocyte response suggested that myosin mixed with BCG induces a myosin-specific Th cell response in not only mice with myocarditis but also in mice without myocarditis.

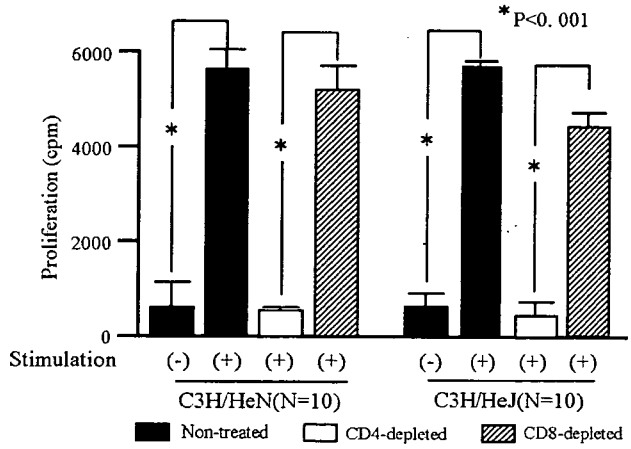

Fig. 3. Myosin-immunized mice develop $CD4^+$, myosin-specific spleen cell proliferative responses. Responding spleen cells were depleted of $CD4^+$ or $CD8^+$ cells using a commercially available system of magnetic bead-coupled specific Abs and co-cultured with myosin. Each value is the mean cpm ± SE of ten mice/group. *$p < 0.001$.

3.3. Myosin-specific cytokine responses of spleen cells from immunized mice

To elucidate the immunological qualities of myosin-specific $CD4^+$ T cells, myosin-specific cytokine responses were analyzed in experimental mice. The responses of myosin-specific cytokines in spleen cells obtained from the experimental mice on day 21 were examined by two different methods. The production of IFN-γ from spleen cells after stimulation *in vitro* with myosin or OVA (control) was assessed by ELISA. Spleen cells from C3H/HeN mice with myocarditis immunized with myosin mixed with BCG produced a significantly larger amount of IFN-γ in the supernatant of the culture than did spleen cells from C3H/HeJ mice without myocarditis after stimulation *in vitro* with myosin (Fig. 4a). We next assessed the mRNA expression levels of Th1-type cytokines (IL-2 and -12) and Th2-type cytokines (IL-4 and -13) in spleen cells after *in vitro* stimulation with myosin or OVA (control). Spleen cells from C3H/HeN mice with myocarditis showed strong IL-2 and -12 expression and weak IL-4 and -13 expression of mRNA, whereas completely opposite results were obtained for spleen cells from C3H/HeJ mice without myocarditis. Spleen cells from C3H/HeJ mice without myocarditis showed strong expression of mRNA of Th2-type cytokines (IL-4 and -13) and weak expression of Th1-type cytokines (IL-2 and -12). Moreover, spleen cells from C3H/HeN mice treated with myosin and IFA without BCG showed Th2-type immune responses (data not shown). Upstream of the release of some Th1-type cytokines are the TLRs, and C3H/HeN mice with wild-type TLR4 developed EAM while also showing a Th1-type immune response to myosin. Although induction of another T-cell linage, Treg, was also assessed by the expression

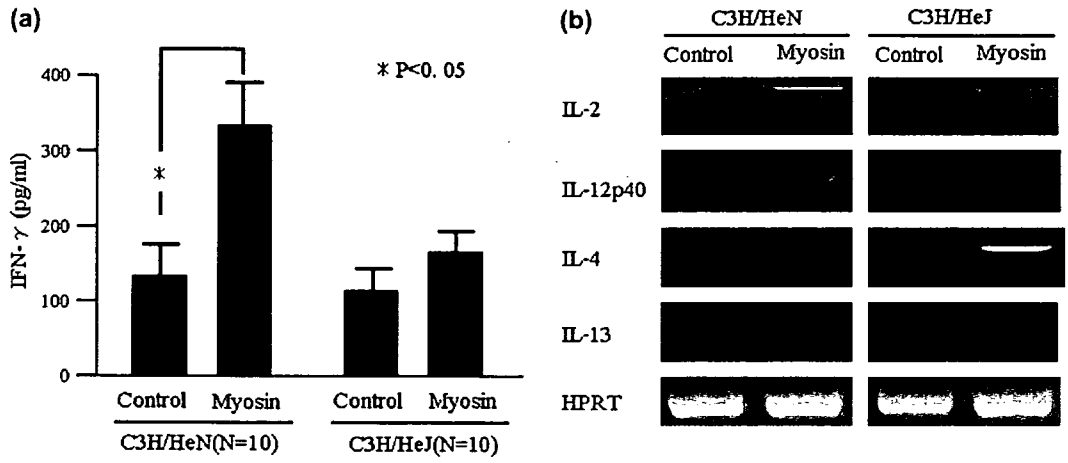

Fig. 4. Cytokine production in culture supernatant and expression of mRNAs of cytokines from spleen cells. (a) The amount of IFN-γ in the culture supernatant was measured by ELISA 21 days after the first myosin immunization. Each value shown is the mean and SD of ten mice per group. (b) Spleen cells were stimulated *in vitro* with myosin for 1 day in culture. Spleen cells stimulated with OVA were used as controls. The reaction products were analyzed on 2% agarose, Tris-buffered EDTA TBE gels. The profiles are representative of three independent experiments.

of mRNA of IL-10 and transforming growth factor-β, these cytokines were not different in C3H/HeN mice with myocarditis and C3H/HeJ mice without myocarditis (data not shown). These results indicated that the $CD4^+$ cells of C3H/HeN mice (TLR4 wild type) and C3H/HeJ mice (TLR4 unfunctional mutated) were polarized toward different Th responses after immunization by an emulsion of myosin mixed with BCG followed by proliferation.

3.4. Effects of IL-4, IL-4SM or IL-4DM DNA administration on the development of myocarditis in mice immunized with myosin mixed with BCG

To examine the effects of regulating Th responses using IL-4, IL-4SM (agonistic IL-4 single mutant) and IL-4DM (antagonistic IL-4 double mutant) DNA on the development of myocarditis in both strains of mice immunized with myosin mixed with BCG, the mice were intraperitoneally administered 100 μg of DNA vaccine or control plasmid on days −7, 0, 7 and 14 (Fig. 1b). The IL-4 mutant Q116D/Y119D, which forms unproductive complexes with the IL-4Rα-chain, acts as an antagonist by inhibiting the formation of heterodimers with other receptors [15]. These IL-4-binding inhibitors act not only by inhibiting IL-4 binding to its receptor but also by preventing IL-13 from eliciting its activity, since the IL-4Rα-chain also forms a functional signaling component of the IL-13R heterodimer [16,17]. We previously reported that such plasmid administration can regulate the systemic Th immune responses in autoimmune and allergic diseases by only a single injection [14]. C3H/HeN mice, in which the development of myocarditis was induced by immunization of myosin mixed with BCG, did not develop myocarditis when they were injected with the IL-4 and IL-4SM (agonistic IL-4 mutant) DNA vaccines to inhibit the Th1-type immune response ($n = 10$ respectively) (Fig. 5). On the other hand, C3H/HeJ mice, in which the development of myocarditis was not induced by the same immunization, were not affected by the injection of IL-4 and IL-4SM DNA vaccines ($n = 10$ respectively). However, surprisingly, C3H/HeJ mice developed myocarditis (mild in 3/10 and moderate in 7/10) when they were injected with antagonistic IL4 mutant, IL-4DM, DNA vaccine for inhibition of Th2-type immune responses by prevention of IL-4 signaling (Fig. 5). The injection of control plasmid did not influence the development of myocarditis in either strain of mice ($n = 10$ respectively) (Fig. 5). Mice did not develop myocarditis without BCG in any plasmid DNA injected (data not shown). These results were derived by four injections of DNA, although the experimental model of allergic inflammation was inhibited by only a single injection of DNA. We previously reported that administration of IL-4DM DNA did not change the Ag-specific Th responses in cytokine production by *in vitro* stimulation of Ag without the presence of IL-4DM protein [14]. In fact, the results for myosin-specific cytokine production from spleen cells of both strains of mice administered IL-4, IL-4SM or IL-4DM DNA vaccines were the same as the results shown in Fig. 4 (without injection of DNA vaccines) after *in vitro* stimulation of myosin (data not shown). The existence of a small amount of IL-4 or IL-4 mutant for a long time *in vivo* might have played a role in the development of EAM. Since pharmacokinetic half-lives of IL-4 and IL-4 mutant proteins are very short *in vivo* ($t_{1/2} = 0.83$ h), a high concentration of these molecules in plasma must be maintained for a long period in order for effects on various phenotypes to be obtained. These effects usually disappeared immediately after discontinuing administration of these proteins. The antagonistic IL-4 is more effective than neutralizing Ab to IL-4, and commercially available Abs to IL-4 do not have sufficient effects to inhibit the activity of IL-4. These results indicated that the regulation of functions of IL-4 played an important role in the development of myocarditis induced by the immunization of myosin mixed with BCG in the strains of C3H/He mice. These results also indicated the possibility

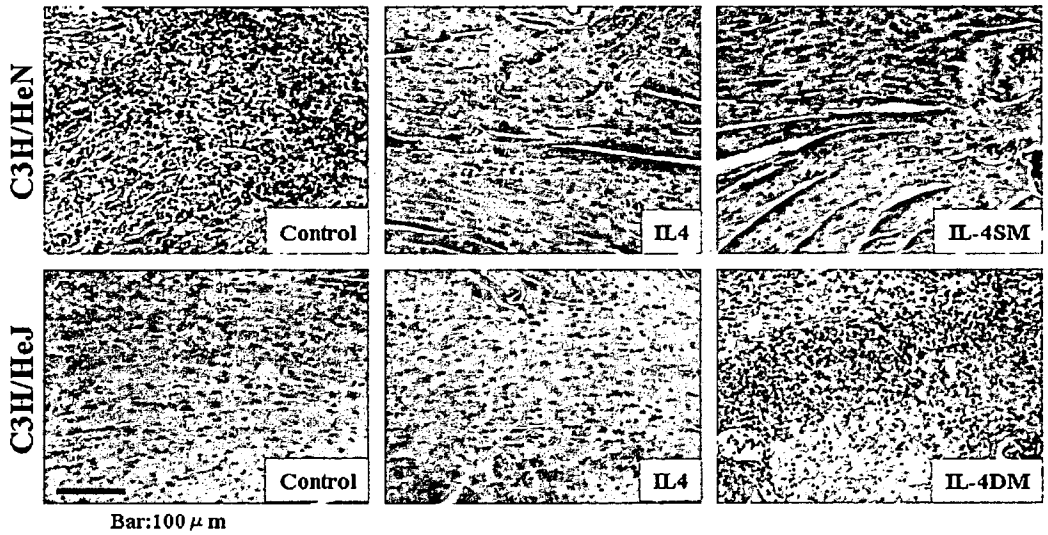

Fig. 5. Results of histopathological examination of hearts from myosin-immunized mice that had been administered IL-4, IL-4SM, IL-4DM or control. All tissue specimens were obtained 21 days after the first myosin immunization. The tissues specimens were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Mice that had been immunized with myosin mixed with BCG in IFA were each injected with 100 μg of IL-4, IL-4SM, IL-4DM or control plasmid DNA four times on days −7, 0, 7 and 14. These results are representative of three independent experiments. Bars represent 100 μm.

that myocarditis is associated with Th1-type $CD4^+$T-cells in these strains of mice.

4. Discussion

$CD4^+$ T cells have been reported to be required for the induction of EAM in mice. The development of EAM in A/J mice was prevented by the depletion of $CD4^+$ T cells, and disease severity was reduced by depleting $CD8^+$T cells (reviewed in [18,19]). A widely held belief is that when the cytokine profile of autoreactive T cells shifts toward an inflammatory Th1 type, the result is pathogenicity and autoimmune diseases (reviewed in [20,21]). Autoimmune myocarditis in the Lewis rat model was promoted by Th1-type immune responses in the same manner as that seen in our experiments [22]. On the other hand, it is difficult to understand the development of myocarditis in the EAM mouse model based on the Th1/Th2 paradigm. Although Th2-type immune responses played a critical role in the development of myocarditis in the mouse model of EAM using A/J and BALB/C strains [23,24], Th1-type immune responses were also suggested to participate in the development of EAM. Moreover, it has been reported that another novel population of T cells, regulatory T cell (Treg), are also important for controlling development of EAM as well as other disease [25–30]. These reports suggested that loss of immune tolerance regulated by Treg cells are one of the mechanisms of development of EAM. It has been reported that EAM did not develop in mice deficient in the Th1-type cytokine IL-12 or its receptor after administration of myosin or myosin Ag peptide, and that the CC-chemokine secreted by Th1-type T cells mediates EAM [31–33]. These differences in immune responses in EAM models are thought to be due to the correlation between mouse strain and mycobacteria species. The EAM model of A/J was established by using a large amount of virulent mycobacteria (*Mycobacterium tuberculosis*), and a small amount of the same bacteria was used for BALB/c mice. We used avirulent *Mycobacteria bovis* BCG (vaccine strain) for establishment of EAM. The A/J mouse strain is susceptible to *Mycobacterium tuberculosis* and resistant to BCG. C3H/HeN mice are resistant to both strains of mycobacteria with the same responses as those in humans, and BALB/c mice show responses opposite to those of A/J mice (resistant to *Mycobacterium tuberculosis* and susceptible to BCG) [34–36]. These differences are dependent on various genetic factors such as *Nramp* gene.

Some studies have suggested a relationship between TLR4 and myocarditis. Infection with a Coxsackievirus, which is a well-known agent of myocarditis, was found to upregulate TLR4 on mast cells and macrophages immediately following infection. TLR4 signaling also increases the occurrence of acute myocarditis and production of proinflammatory cytokines in the heart [37]. Moreover, the critical requirement of TLR4 signaling in dendritic cells (DCs) for myocarditis induction was genetically proven by the fact that myosin Ag peptide (MYHC-α)-pulsed and TLR4/CD40-activated DCs isolated from TLR4-deficient mice did not induce myocarditis in wild-type recipients when DCs isolated by the same procedure from TLR4-wild type transfer elicited myocarditis in a wild-type recipient [38]. In our system, two important points regarding the establishment of EAM were observed. Porcine myosin must be mixed well in IFA, and EAM was only observed by immunization of this emulsion into the footpad. These observations suggest that myosin Ag is incorporated in the same APCs such as macrophages or DCs together with BCG for a long period of time as oil particles, and then myosin-specific immune responses are induced by the influence of the characteristic immune responses of a large quantity of BCG associated with TLR4.

Relationships between TLRs and mycobacteria have been reported. TLR2, TLR4 and TLR1/TLR6 heterodimers with

TLR2 have been implicated in the recognition of mycobacterial Ags [39]. The emerging concept of TLRs as key molecules for shaping the quality of immune responses against microbes is further supported by results of experiments showing that mice lacking MyD88 are incapable of developing Ag-specific Th1 responses after immunization with OVA mixed with CFA (containing dead mycobacteria as an active component) [40]. These results are thought to be due to a mechanism involving both TLR2 and TLR4, since CFA contains a complex mixture of mycobacterial components, some of which are recognized by different members of the Toll family, TLR2 and TLR4. The EAM established in our system utilized these TLRs by immunization with extremely large amounts of BCG and cardiac myosin as a mixed emulsion, thus suggesting the importance of TLR4 for the induction of Th1-type immune responses related to EAM. The C3H/HeJ mouse, which has unfunctional TLR4, showed Th2-type immune responses to myosin after immunization of cardiac myosin mixed with BCG (Fig. 4). Mycobacteria induce Th1-type immune responses through TLR2 and TLR4 stimulation; however, our results showed Th2-type immune responses through TLR2 stimulation without TLR4 by BCG as an adjuvant. Similar results have also been reported by other investigators. Th2-type cytokines were induced from DCs by mycobacteria dependent on TLR2-mediated recognition but not TLR4-mediated recognition [41]. In our experiment, TLR4 mutant C3H/HeJ and wild-type C3H/HeN mice were used for analysis of Th responses in EAM. Unfunctional TLR4 mutations in humans have also been reported (reviewed in [42]). Studies using TLR4 knockout mice are needed to clarify this. Since the commonly used mouse background to generate knockout mice is associated with an increased susceptibility to mycobacteria, extensive backcrossing of such mice is required [43].

Many animal models of myocarditis are available to investigate the optimal therapy for myocarditis. However, the establishment of new animal models of myocarditis is still necessary to better understand myocarditis, because the understanding of myocarditis in humans is still insufficient. In the present study, we utilized two strains of C3H/He mice, which showed different susceptibilities to BCG, for the establishment of EAM involving Th1-type immune responses. The results of this study provide evidence of the potential utility of studying immunological mechanisms in order to both treat and prevent myocarditis.

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Suppression of an Already Established Tumor Growing through Activated Mucosal CTLs Induced by Oral Administration of Tumor Antigen with Cholera Toxin[1]

Ayako Wakabayashi, Yohko Nakagawa, Masumi Shimizu, Keiichi Moriya, Yasuhiro Nishiyama, and Hidemi Takahashi[2]

AQ: A **Priming of CTLs at mucosal sites, where various tumors are originated, seems critical for controlling tumors. In the present study, the effect of the oral administration of OVA plus adjuvant cholera toxin (CT) on the induction of Ag-specific mucosal CTLs as well as their effect on tumor regression was investigated. Although OVA-specific TCRs expressing lymphocytes requiring in vitro** AQ: B **restimulation to gain specific cytotoxicity could be detected by OVA peptide-bearing tetramers in both freshly isolated intraepithelial lymphocytes and spleen cells when OVA was orally administered CT, those showing direct cytotoxic activity without requiring in vitro restimulation were dominantly observed in intraepithelial lymphocytes. The magnitude of such direct cytotoxicity at mucosal sites was drastically enhanced after the second oral administration of OVA with intact whole CT but not with its** AQ: C **subcomponent, an A subunit (CTA) or a B subunit (CTB). When OVA plus CT were orally administrated to C57BL/6 mice bearing OVA-expressing syngeneic tumor cells, E.G7-OVA, in either gastric tissue or the dermis, tumor growth was significantly suppressed after the second oral treatment; however, s.c. or i.p. injection of OVA plus CT did not show any remarkable suppression. Those mucosal OVA-specific CTLs having direct cytotoxicity expressed CD8$\alpha\beta$ but not CD8$\alpha\alpha$, suggesting that they originated from thymus-educated cells. Moreover, the infiltration of such OVA-specific CD8$^+$ CTLs was observed in suppressed tumor tissues. These results indicate that the growth of ongoing tumor cells can be suppressed by activated CD8$\alpha\beta$ CTLs with tumor-specific cytotoxicity via an orally administered tumor Ag with a suitable mucosal adjuvant.** ***The Journal of Immunology*, 2008, 180: 0000–0000.**

Many malignant tumors originate from various epithelial tissues such as the skin or mucosal sites such as the esophagus, stomach, colon, or lung (1). Thus, as a cancer vaccine, it is essential to stimulate mucosal or dermal immune systems, as well as the systemic immune system, with a suitable Ag, adjuvant, and administration route as reviewed by Finn (2). Mucosal immunization using an adjuvant that enables the priming of both mucosal and systemic immunity (3, 4) may be a good way to prevent or treat mucosal tumors. In particular, the induction of mucosal CTLs that can specifically recognize tumor-derived peptide Ags presented by the corresponding class I MHC molecules seems to be one of the most important issues for eliminating tumor cells (5).

In the mucosal compartment, lymphocytes located between the AQ: D intestinal epithelia are almost exclusively T cells called intraepithelial lymphocytes (IELs)[3] (3). Such IELs are mostly CD8$^+$ T cells that are classified into three distinct populations: TCR$\alpha\beta^+$ CD8$\alpha\beta^+$, TCR$\alpha\beta^+$CD8$\alpha\alpha^+$, and TCR$\gamma\delta^+$CD8$\alpha\alpha^+$ (6). IELs contain cytotoxic properties and specifically eliminate virus- or parasite-infected cells (7–9); however, although spontaneous cytotoxicity of human IELs against tumor cells has been reported (10, 11), their actual specificity on tumors is still unknown. Recently, we have reported (12) that a marked increase in the number of HIV-1-specific CD8$\alpha\beta$-positive T cells among IELs was observed in HIV-1-specific TCR transgenic (Tg) mice when they received intrarectal or i.p. administration of the recombinant vaccinia virus (rVV) expressing a known restricted CTL epitope, P18 (rVV-P18), which is restricted by H-2D^d-class I MHC molecules (13). Using H-2D^d/P18 tetramers, we could detect CD8-positive, AQ: E P18-specific TCR-expressing T cells in freshly isolated IELs and splenic T cells of unchallenged naive Tg mice. Although those H-2D^d/P18 tetramer-positive CD8 T cells from naive Tg mice did not show any specific cytotoxicity, freshly isolated mucosal T cells bearing CD8$\alpha\beta$ but not CD8$\alpha\alpha$ from activated Tg mice with rVV-P18 represented P18-specific cytotoxicity against tumor cells expressing the epitope, and the magnitude of cytotoxicity was much stronger than that in activated splenic T cells (12). These results suggest that in vivo activated mucosal CD8$\alpha\beta$ CTLs with tumor-specific cytotoxicity may be critical for controlling tumors expressing the specific epitope in vivo rather than systemic splenic CTLs.

Cholera toxin (CT) derived from *Vibrio cholerae* is known as a potent mucosal adjuvant comprised of one toxic A subunit (CTA) with ADP-ribosyltransferase activity and five nontoxic B subunits (CTB) responsible for binding to monosialoganglioside (GM) 1 on the cell surface (14, 15). CT adjuvant helps to produce both systemic IgG and mucosal IgA (16) as well as to induce Ag-specific

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[3] Abbreviations used in this paper: IEL, intraepithelial lymphocyte; CT, cholera toxin; CTA, CT A subunit (toxic); CTB, CT B subunit (nontoxic); DC, dendritic cell; GM, monosialoganglioside; *Hp*, *Helicobacter pylori*; LPL, lamina propria lymphocyte; MadCAM-1, mucosal addressin cell-adhesion molecule-1; OVA-CT, CT-conjugated OVA; PP, Peyer's patch; rVV, recombinant vaccinia virus; SC, spleen cell; Tg, transgenic; TIL, tumor-infiltrating lymphocyte.

$CD4^+$ T cell responses in the spleen, reflecting the systemic compartment, and in Peyer's patches (PPs), reflecting the mucosal compartment (17). In addition, it has been demonstrated (18) that OVA-specific CTLs could be primed in C57BL/6 mice following oral exposure to a combination of OVA with CT, and specific cytotoxic activity was detected from spleen cells (SCs) only when they were restimulated in vitro with irradiated OVA-expressing syngeneic tumor cells, E.G7-OVA, which are OVA gene-transfected EL4 thymoma cells (19, 20). Also, intranasal preimmunization with OVA peptide (SIINFEKL) plus CT primed similar OVA-specific CTLs in the spleen of C57BL/6 mice, and the immunized mice were protected from the development of transferred E.G7-OVA (21).

Moreover, it has been shown that adoptive transfer of naive $CD8^+$ OVA-specific OT-I T cells into E.G7-OVA tumor-bearing syngeneic mice did not inhibit tumor growth, although adoptive transfer of preactivated OT-I CTL in vitro inhibited tumor growth in a dose-dependent manner (22). Furthermore, it has recently been reported that vaccination with dendritic cells (DCs) prepulsed ex vivo with CT-conjugated OVA (OVA-CT) gave rise to OVA-specific splenic $CD8^+$ T cells that produced IFN-γ, were cytotoxic to E.G7-OVA cells in vivo, and rejected already established in vivo E.G7-OVA tumors associated with high numbers of tumor-infiltrating $CD8^+$ T cells (23), indicating that the elimination of previously established tumor cells might require the infiltration of tumor-specific activated $CD8^+$ CTLs.

In the present study, we found two distinct types of $CD8\alpha\beta$-positive T cells among freshly isolated lymphocytes expressing OVA-specific TCRs, which can be detected by OVA peptide-bearing tetramers. One is in an activated effector state with cytotoxic activity and the other is a resting state and may gain cytotoxicity when stimulated with an OVA epitope peptide in vitro. Based on the observations, we defined direct cytotoxicity as the former state, in which freshly isolated and unstimulated CD8 T cells had specific cytotoxicity. Therefore, by comparing systemic SCs, we asked whether OVA-specific cytotoxic activity could be observed among freshly isolated IELs in mice orally administered OVA plus CT and examined whether those activated CTLs would reject or suppress the growth of already established tumors. Consequently, we observed dominant $TCR\alpha\beta$ and $CD8\alpha\beta$ OVA-specific CTL activities in freshly isolated IELs rather than in SCs after the oral administration of OVA plus CT, and such mucosal CTL activities could be expanded after oral boosting. Moreover, the growth of E.G7-OVA inoculated into the stomach or the epidermis was significantly suppressed, accompanied by the expansion of activated mucosal CTLs, and the infiltration of such OVA-specific $CD8\alpha\beta^+$ CTLs was observed in suppressed dermal tumor tissues. These results indicate that the growth of ongoing tumor cells can be suppressed in vivo by activated $CD8\alpha\beta$ CTLs with tumor-specific cytotoxicity via an orally administered tumor Ag with a suitable mucosal adjuvant.

Materials and Methods

Mice

Six- to 8-wk-old female C57BL/6 ($H\text{-}2^b$) mice were purchased from Charles River Japan, maintained in microisolator cages under pathogen-free conditions, and fed autoclaved laboratory chow and water. All animal experiments were performed according to guidelines for the care and use of laboratory animals set by the National Institutes of Health (NIH; Bethesda, MD) and approved by the Review Board of Nippon Medical School (Tokyo, Japan).

Oral and systemic immunization

Chicken egg OVA, grade V (Sigma Aldrich), was dissolved in sterilized PBS. Mice were orally administered 100 mg of OVA or 10 μg of CT (List Biological Laboratories) alone or 100 mg of OVA plus 10 μg of CT, CTA, or CTB (List Biological Laboratories) in 0.3 ml of PBS. In some experiments, mice were orally administered 10 mg of OVA plus 10 μg of CT. For systemic immunization, mice were i.p. or s.c. injected with 100 mg of OVA or the same dose of OVA plus 10 μg of CT.

Preparation of IELs, lamina propria lymphocytes (LPLs), SCs, and tumor-infiltrating lymphocytes (TILs)

IELs were prepared by the method described previously (12). In brief, after the small intestine, large intestine, or stomach was obtained from mice, fecal materials were flushed from the lumen with HBSS (Invitrogen Life Technologies) and connective tissues were carefully removed. The obtained guts were inverted and cut into several segments that were transferred to a 50-ml conical tube (Becton Dickinson Labware) containing 45 ml of HBSS with 5% FCS, 100 U/ml penicillin (Invitrogen Life Technologies), and 100 μg/ml streptomycin (Invitrogen Life Technologies). The tube was then shaken at 37°C for 45 min (horizontal position; orbital shaker at 150 rpm). Harvested cells from the intestinal epithelium were passed through a 10-ml syringe column containing loosely packed glass wool to remove tissue debris. Subsequently, the cells were suspended in 30% Percoll solution (Amersham Biosciences) and centrifuged for 20 min at 1,800 rpm. Cells at the bottom of the solution were then subjected to Percoll discontinuous gradient centrifugation for 20 min at 1,800 rpm and IELs were recovered at the interphase of 44 and 70% Percoll solutions. LPLs were prepared by the method described previously (24). In brief, after the small intestine, large intestine, or stomach was dissected from mice, fecal material was flushed from the lumen with HBSS and PPs were carefully removed. The obtained guts were inverted and cut into several segments that were transferred to a 50-ml conical tube containing 45 ml of HBSS with 5% FCS and 1 mM EDTA (Wako Pure Chemical Industries). The tube was shaken at 37°C for 45 min (horizontal position; orbital shaker at 150 rpm). The gut segments were then washed with PBS and shaken in 40 ml of HBSS with 5% FCS and 0.1 mg/ml collagenase (Sigma-Aldrich) at 37°C for 45 min (horizontal position; orbital shaker at 60 rpm). Harvested cells were passed through a nylon mesh and suspended in 40% Percoll solution, and then 70% Percoll solution was underlain. The solution was centrifuged for 20 min at 1,800 rpm and LPLs were recovered at the interphase of 40 and 70% Percoll solutions. These procedures provided >95% viable lymphocytes with a cell yield of $5–10 \times 10^6$ of small intestinal IELs, $2–3 \times 10^5$ of large intestinal IELs, $7–12 \times 10^3$ of gastric IELs, $4–9 \times 10^5$ of small intestinal LPLs, $1–3 \times 10^5$ of large intestinal LPLs, or $5–9 \times 10^4$ of gastric LPLs per mouse. The cells were suspended in complete T cell medium (25) composed of RPMI 1640 medium (Sigma-Aldrich) supplemented with 2 mM L-glutamine (ICN Biomedicals), 1 mM sodium pyruvate (Invitrogen Life Technologies), 0.1 mM nonessential amino acid (Invitrogen Life Technologies), a mixture of vitamins (ICN Biomedicals), 1 mM HEPES (Invitrogen Life Technologies), 100 U/ml penicillin (Invitrogen Life Technologies), 100 μg/ml streptomycin (Invitrogen Life Technologies), 50 μM 2-ME (Sigma-Aldrich), and heat-inactivated 10% FCS. For TIL preparation, tumors were removed from mice, incubated in 1 mg/ml collagenase (Wako Pure Chemical Industries) with PBS at 37°C for 1 h, and crushed gently. TILs were prepared using Percoll solutions as described in the previous paragraph regarding IEL preparation. The spleen was aseptically removed and a single cell suspension was prepared. For osmotic hemolysis, single cells were suspended in 0.1× PBS and an equal amount of 2× PBS was added immediately. To enrich IELs, LPLs, and TILs from mice, the interface between the 40 and 70% Percoll solutions (26), in which NK cells and unfractionated SCs, which may also include NK cells, must be included, was collected.

Flow cytometry analysis

Cells were double-stained with PE-labeled $H\text{-}2K^b$/OVA tetramer-SIINFEKL (Beckman Coulter) or $H\text{-}2K^b$/PB1 tetramer-SSYRRPVGI (Medical & Biological Laboratories) and FITC-labeled anti-mouse TCRβ, CD8α (BD Pharmingen), or CD8β (Caltag Laboratories). Peptide PB1 703–711, SSYRRPVGI, for the control tetramer was derived from influenza virus (27). Dead cells were determined using 7-aminoactinomycin D viability dye (Beckman Coulter) and stained cells were analyzed by FACScan using the CellQuest program (BD Biosciences).

In vitro restimulation of SCs or IELs with E.G7-OVA

Lymphocytes were restimulated in vitro by the method described previously (19). Freshly isolated SCs (3×10^7) or IELs (3×10^7) were restimulated with 3×10^6 irradiated (20,000 rad) E.G7-OVA cells (19, 20) ($H\text{-}2^b$; American Type Culture Collection) in 10 ml of complete T cell medium per upright 25-cm^2 flask in 5% CO_2 at 37°C for 6 days. Six days later, the viability of the lymphocytes was 35–51% in SCs and 16–26% in IELs. The