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The route of immunization with adenoviral vaccine influences the recruitment of cytotoxic T lymphocytes in the lung that provide potent protection from influenza A virus

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ABSTRACT

Virus-specific cytotoxic T lymphocytes (CTLs) in the lung are considered to confer protection from respiratory viruses. Several groups demonstrated that the route of priming was likely to have an implication for the trafficking of antigen-specific CTLs. Therefore, we investigated whether the route of immunization with adenoviral vaccine influenced the recruitment of virus-specific CTLs in the lung that should provide potent protection from influenza A virus. Mice were immunized with recombinant adenovirus expressing the matrix (M1) protein of influenza A virus via various immunization routes involving intraperitoneal, intranasal, intramuscular, or intravenous administration as well as subcutaneous administration in the hind hock. We found that the immunization route dramatically impacted the recruitment of M1-specific IFN-γ^+ $CD8^+$ T cells both in the lung and the spleen. Surprisingly, hock immunization was most effective for the accumulation in the lung of IFN-γ-producing $CD8^+$ T cells that possessed M1-specific cytolytic activity. Further, antigen-driven IFN-γ^+ $CD8^+$ T cells in the lung, but not in the spleen, were likely to be correlated with the resistance to challenge with influenza A virus. These results may improve our ability to design vaccines that target virus-specific CTL responses to respiratory viruses such as influenza A virus.

1. Introduction

It is well established that $CD8^+$ cytotoxic T lymphocytes (CTLs) play a major role in the specific clearance of viruses. CTLs recognize and kill virus-infected cells based on the presentation of peptides bound to major histocompatibility complex (MHC) class I molecules on the cell surface. These peptides are generated from endogenous antigens derived from virus through the classical MHC class I-restricted, antigen processing pathway. While neutralizing antibodies are mostly sufficient for clearing cell-free virus, CTLs are principal players in the immune response to intracellular infections. Furthermore, unlike neutralizing antibodies, CTLs can recognize conserved peptides derived from internal proteins of viruses, which allows CTLs to generate heterosubtypic immunity against influenza A virus (Brown and Kelso, 2009; Matsui et al., 2010).

Since mucosal immune responses are an early and important defense against a wide variety of pathogens, a critical goal of vaccine development would be the induction of potent and long-lasting mucosal immunity rather than systemic immunity (Belyakov and Ahlers, 2009; Woodland and Kohlmeier, 2009). In the case of respiratory virus infection such as influenza, particular attention should be paid to the lung as a target for mucosal immunity because the lung is an entrance for respiratory viruses (Kohlmeier and Woodland, 2009). Virus-specific CTLs in the lung are considered to confer protection against respiratory viruses that initiate infections at mucosal surfaces (Kohlmeier and Woodland, 2009; Lin et al., 2010). However, the requirement for the efficient induction of potent CTLs in the lung is still unclear. The route of priming is likely to influence the trafficking of antigen-specific CTLs to the lung and the generation of protective immunity against mucosal pathogens (Belyakov and Ahlers, 2009). It was shown that intranasal (i.n.) immunization with recombinant adenovirus-based vaccine (Kaufman et al., 2010; Santosuosso et al., 2005; Wang et al., 2004) or DNA vaccine (Bivas-Benita et al., 2010) provides a large number of vaccine-elicited CTLs in the lung and remarkably better immune protection against pulmonary pathogens than intramuscular (i.m.) immunization. Conversely, it was demonstrated that i.m. vaccination with recombinant adenovirus could overcome immune compartmentalization and generate remarkably potent and durable CTLs at multiple mucosal surfaces (Kaufman et al., 2008).

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In the current study, we investigated the influence of the immunization route on the recruitment of pulmonary CTLs and protective immunity against influenza A virus using adenoviral vector expressing the matrix (M1) protein of influenza A virus. We here employed various immunization routes including a new immunization protocol, hock immunization (Kamala, 2007). We conclude that the route of vaccination is important in influencing immune responses in the lung where protection against influenza A virus is effective.

2. Materials and methods

2.1. Mice

Mice express a transgenic HLA-A*0201 monochain, designated as HHD, in which human β2-microglobulin (β2m) is covalently linked to a chimeric heavy chain composed of HLA-A*0201 (α1 and α2 domains) and H-2D^b (α3, transmembrane, and cytoplasmic domains) (Matsui et al., 2004; Pascolo et al., 1997). Eight- to twelve-week-old mice were used for all experiments. Eight-week-old, C57BL/6 (H-2^b) female mice were purchased from Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Mice were housed in appropriate animal care facilities at Saitama Medical University, Saitama, Japan, and handled according to international guidelines for experiments with animals.

2.2. Cell lines

A mouse lymphoma cell line transfected with the HHD gene, RMA-HHD (H-2^b) was previously described (Pascolo et al., 1997). C1R-A2 is a human B cell line, HMy2.C1R that was transfected with an HLA-A*0201 gene (Kohyama et al., 2009). RMA-HHD and C1R-A2 cells were maintained in RPMI-1640 medium (Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) containing 500 μg/ml G418 (Sigma–Aldrich). The Madin-Darby canine kidney (MDCK) cell line and a human kidney cell line, 293 were obtained from the American Type Culture Collection (Rockville, MD). The AD-293 cell line which is derived from the 293 cell line but has improved cell adherence and plaque formation properties was provided with the AdEasy adenoviral vector system (Agilent Technologies, La Jolla, CA). MDCK, 293, AD-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) supplemented with 10% FCS (JRH Biosciences).

2.3. Construction of recombinant adenovirus

Recombinant adenovirus expressing the M1 protein of influenza A virus (Ad-M1) was generated using the AdEasy Adenoviral Vector System (Agilent Technologies). Briefly, the M1 gene of influenza A virus (A/PR8/34) (H1N1) was synthesized by Operon Biotechnologies, Tokyo, Japan, and was inserted into the multiple cloning site of the pShuttle-IRES-hrGFP-1 vector (Agilent Technologies) that allows the M1 protein to be fused to the 3× FLAG tag sequence at C-terminus. The M1 gene-inserted shuttle vector was linearized with Pme I (New England Biolabs Ltd., Herts, UK) and electroporated into the BJ5183-Ad-1 (Agilent Technologies) electrocompetent cells. Potential recombinant plasmids were then recovered, amplified and digested with Pac I (New England Biolabs Ltd.). The Pac I-digested gene was transfected into AD-293 cells (Agilent Technologies) by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfected cells were collected 10–14 days after transfection by scraping cells off flasks. Three cycles of freezing and rapid thawing were performed to release adenoviruses from cells. Virus was amplified in 293 cells and virus titers were determined by calculating the 50% cell culture infectious dose ($CCID_{50}$) on 293 cells. Wild-type adenovirus (Ad-WT) was used as a control.

To detect expression of the M1 protein, Western blotting was performed as described previously (Ohno et al., 2009). Briefly, C1R-A2 cells were infected with either Ad-WT or Ad-M1 at a multiplicity of infection (MOI) of 30 for 1.5 h. After 2 days' incubation, cells were lysed and the solubilized proteins were separated by electrophoresis on a 10% SDS–PAGE under reducing condition, and blotted onto a nitrocellulose membrane. The blot was stained with 5 μg/ml of the anti-FLAG M2 monoclonal antibody (mAb) (Sigma–Aldrich), followed by secondary antibody.

2.4. Immunization

Mice were immunized with the indicated doses (mostly 1×10^8 $CCID_{50}$) of either Ad-WT or Ad-M1 via various immunization routes, including i.n., intraperitoneal (i.p.), i.m., intravenous (i.v.), and subcutaneous (s.c.) administration. For i.n. immunization, mice were anesthetized by administration of ketamine (175 mg/g weight) (Sigma–Aldrich) and xylazine (3.5 mg/g weight) (Bayer Holding Ltd., Tokyo, Japan), and inoculated intranasally with 40 μl of virus solution. For s.c. delivery, each mouse was injected subcutaneously in the both hind hocks with total 100 μl of virus solution (Kamala, 2007).

2.5. Synthetic peptides

Two M1-derived peptides, M1 58–66 (GILFGVFTL) (Gotch et al., 1987) and M1 128–135 (MGLIYNRM) (Vitiello et al., 1996), which were restricted by HLA-A*0201 and H-2K^b, respectively, were synthesized by Operon Biotechnologies (Tokyo, Japan).

2.6. Preparation of lymphocytes in lung tissues

Isolation of lymphocytes from lungs was performed as described previously (Lin et al., 2010). Briefly, lung tissues were cut into small pieces and incubated with 50 U/ml collagenase (Invitrogen) at 37 °C for 1 h. The digested tissue suspension was then passed through a 100 μm nylon mesh filter (Cell Strainer, BD Falcon, Bedford, MA) to remove cell clumps and non-dissociated tissue. Cells were washed once with medium, layered onto a density separation medium, Lympholyte-M (Cedarlane, Ontario, Canada), and centrifuged for 20 min at 2000 rpm. The lymphocytes recovered were washed three times in PBS prior to use.

2.7. Intracellular cytokine staining

To detect interferon-gamma (IFN-γ)$^+$ CD8$^+$ T cells, intracellular cytokine staining was performed as described (Matsui et al., 2005). Briefly, after two weeks following immunization, 2×10^6 lymphocytes derived from lungs or spleens of immunized mice were incubated with 10 μM of a relevant peptide for 5 h at 37 °C in the presence of brefeldin A (GolgiPlug™, BD Biosciences, San Jose, CA). After blocking Fc receptors with the rat anti-mouse CD16/CD32 mAb (Fc Block™, BD Biosciences), cells were stained with FITC-conjugated rat anti-mouse CD8α mAb (BD Biosciences) for 30 min at 4 °C. Cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse IFN-γ mAb (BD Biosciences). After washing cells, flow cytometric analyses were performed.

The CD107 mobilization assay detects the transient surface expression of CD107a and CD107b that occurs during T cell degranulation following an antigenic stimulation. It has been shown that the presence of CD107$^+$ T cells correlated well with cytotoxic activity for antigen-stimulated CD8$^+$ T cells (Betts et al., 2003). For this assay, 2×10^6 lymphocytes derived from lungs or

spleens of immunized mice were stained with FITC-conjugated rat anti-mouse CD107a and CD107b mAbs (BD Biosciences) during the stimulation with 10 μM of a relevant peptide for 6 h at 37 °C in the presence of monensin (GolgiStop™, BD Biosciences) and brefeldin A (GolgiPlug™, BD Biosciences). Cells were washed and stained with PE-Cy5-conjugated rat anti-mouse CD8α mAb (BD Biosciences), and then with PE-conjugated rat anti-mouse IFN-γ mAb (BD Biosciences) as described above. The stained cells were analyzed by flow cytometry.

2.8. Influenza A viruses

Two influenza A virus strains, H3N2 (A/Aichi/2/68) and H1N1 (A/Brisbane/59/2007) were propagated in 10-day-old embryonated hen's eggs at 35 °C for 3 days. Egg allantoic fluid containing virus was then harvested and stored at −80 °C. Viral titers were determined by calculating $CCID_{50}$ using MDCK cells (Matsui et al., 2010). The H3N2 (A/Aichi/2/68) and H1N1 (A/Brisbane/59/2007) viruses were kindly provided by Dr. H. Kida at Hokkaido University, Sapporo, Japan, and Dr. T. Odagiri at the National Institute of Infectious Diseases, Tokyo, Japan.

2.9. Viral challenge

After 2 weeks following immunization, mice were anesthetized by i.p. injection of ketamine and xylazine, and were challenged intranasally with 1×10^4 $CCID_{50}$ of influenza virus resuspended in 40 μl of PBS per animal. Mice were sacrificed on day 5 after the virus challenge, and the virus titers in their lungs were determined by calculating $CCID_{50}$ using MDCK cells as described previously (Matsui et al., 2010). Briefly, lungs were homogenized in 1 ml of PBS and the homogenate was clarified by centrifugation at 2000 rpm for 10 min. The lung homogenates were then serially 10-fold diluted in 96-well U-bottomed plates, 5 wells per dilution, starting from 10^1 to 10^7 in DMEM with 5% FCS (D-5). MDCK cells in D-5 were added to all wells and incubated at 35 °C in 5% CO_2. One day later, the culture medium in each well was replaced by DMEM containing 2 μg/ml acetylated trypsin (Sigma–Aldrich), and the plates were incubated in a CO_2 incubator at 35 °C for 4 more days. After the addition of 50 μl of 0.5% chicken red blood cell suspension in PBS, the agglutination pattern for each sample was observed and virus titers were determined by calculating the $CCID_{50}$. Three to six mice were used in each experimental group.

2.10. ^{51}Cr-release assay

^{51}Cr-release assays were carried out as described before (Matsui et al., 2004). In brief, after 2 weeks following immunization, lymphocytes prepared from lung tissues were cultured for 1 week with gamma-irradiated (30 Gy), syngeneic spleen cells pulsed with 10 μM of a relevant peptide, and used as effector cells in standard ^{51}Cr-release assays. RMA-HHD cells pulsed with or without a relevant peptide were labeled with 100 μCi of $Na_2{}^{51}CrO_4$, and used for target cells. After a 4-h incubation, supernatant of each well was harvested and the radioactivity was counted. Results were calculated as the means of triplicate assays. Percent specific lysis was calculated according to the formula: % specific lysis = $[(cpm_{sample} - cpm_{spontaneous})/(cpm_{maximum} - cpm_{spontaneous})] \times 100$. Spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100.

2.11. Statistical analyses

Statistical analyses between two groups were performed with Student's *t*-test. One-way ANOVA followed by post hoc tests were carried out for statistical analyses between multiple groups using GraphPad Prism 5 software. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of M1 protein in cells infected with Ad-M1

To investigate whether Ad-M1 can express the M1 protein of influenza A virus in cells, Western blot analysis was performed. C1R-A2 cells were infected with either Ad-M1 or Ad-WT, and lysed after a 48 h incubation at 37 °C. The cell lysates were then subjected to Western blot analysis with anti-FLAG mAb. As shown in Fig. 1, a single band of 35 kDa corresponding to the M1 protein was clearly detected in the lane for Ad-M1-infected cells, but not in the lane for Ad-WT-infected cells. This result indicates that Ad-M1 can efficiently express the M1 protein of influenza A virus in Ad-M1-infected cells.

3.2. Influence of the immunization route on the recruitment of pulmonary CTLs

Because the lung is the local place where primary immune protection takes place against the respiratory virus infection, it is supposed to be crucial to recruit virus-specific CTLs in the lung for the protection from influenza A virus. Hence, it was tested whether the induction of pulmonary CTLs was influenced by the route of adenoviral immunization. HHD mice were immunized once with 1×10^8 $CCID_{50}$ of either Ad-WT or Ad-M1 via various immunization routes (Fig. 2A and B). Two weeks after the immunization, lymphocytes were prepared from the spleen and the lung, and stimulated with the M1 58–66 peptide. Cells were then stained

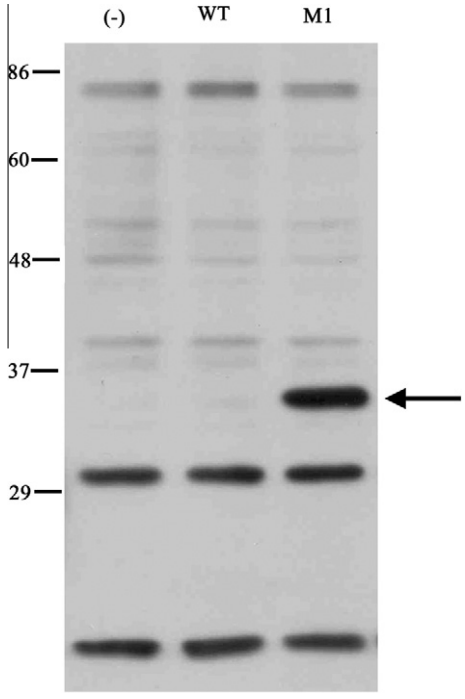

Fig. 1. Expression of influenza virus M1 protein in C1R-A2 cells infected with Ad-M1. C1R-A2 cells were infected with either Ad-WT (WT) or Ad-M1 (M1) at an MOI of 30. After 2 days' incubation, cells were lysed, separated by electrophoresis on a 10% SDS–PAGE under reducing condition, and subjected to Western blotting analysis with anti-FLAG mAb. Non-infected C1R-A2 cells (−) were used as a negative control. The positions of protein molecular mass markers (in kDa) are shown in the figure, and an arrow indicates the band of M1 protein.

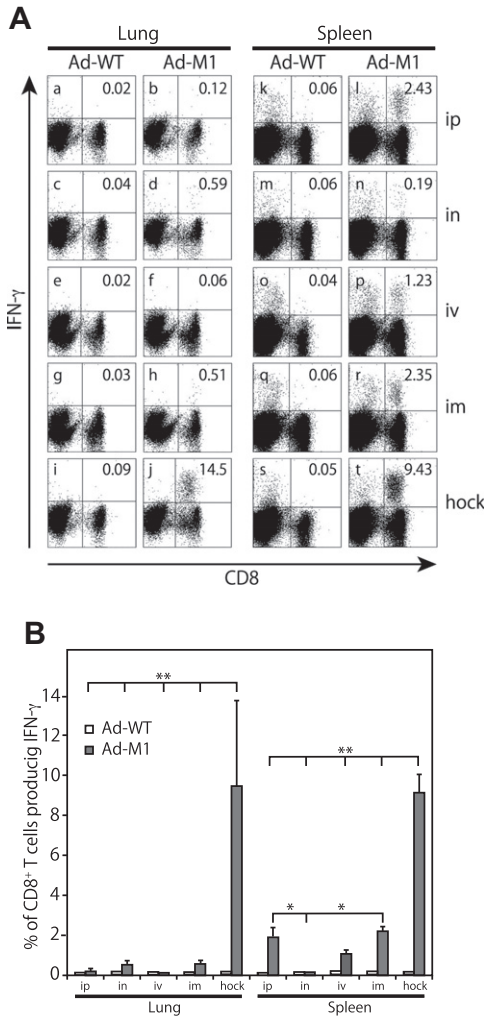

Fig. 2. Intracellular IFN-γ staining of $CD8^+$ T cells specific for the M1 58–66 peptide in the lungs and the spleen of HHD mice infected with Ad-M1. (A) HHD mice were infected with 1×10^8 $CCID_{50}$ of either Ad-WT (a, c, e, g, i, k, m, o, q, and s) or Ad-M1 (b, d, f, h, j, l, n, p, r, and t) via various immunization routes including i.p. (a, b, k, and l), i.n. (c, d, m, and n), i.v. (e, f, o, and p), i.m. (g, h, q, and r), and s.c. hock (i, j, s, and t) administration. Two weeks after immunization, lymphocytes were prepared from lungs (a–j) or spleens (k–t), and stimulated with or without (data not shown) the M1 58–66 peptide for 5 h. Cells were then stained for their surface expression of CD8 (*x* axis) and their intracellular expression of IFN-γ (*y* axis). The numbers shown indicate the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cells. The data shown are representative of three independent experiments. At least three mice per group were used in each experiment. (B) The data are shown as the mean ± SD of all mice used in the experiments. One-way ANOVA was performed for comparison of data between vaccination groups.*, $p < 0.01$; **, $p < 0.001$.

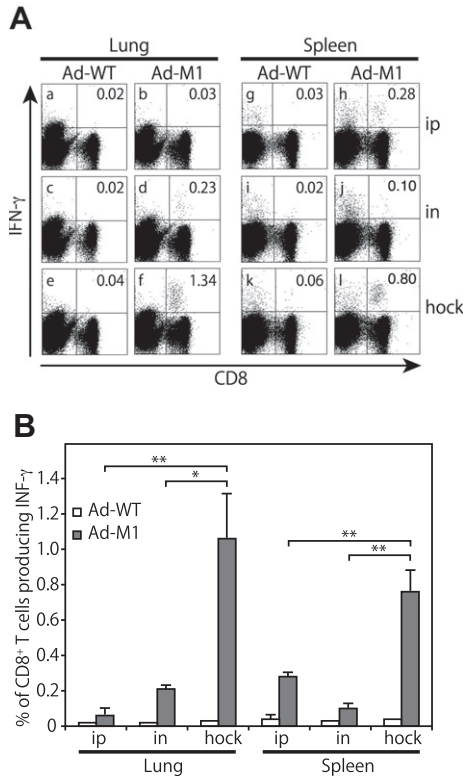

Fig. 3. Intracellular IFN-γ staining of $CD8^+$ T cells specific for the M1 128–135 peptide in the lungs and spleen of C57BL/6 mice infected with Ad-M1. (A) C57BL/6 mice were infected with 1×10^8 $CCID_{50}$ of either Ad-WT (a, c, e, g, i, and k) or Ad-M1 (b, d, f, h, j, and l) via various immunization routes including i.p. (a, b, g, and h), i.n. (c, d, i, and j), and s.c. hock (e, f, k, and l) administration. Two weeks after immunization, lymphocytes were prepared from lungs (a–f) or spleens (g–l), and stimulated with or without (data not shown) the M1 128–135 peptide for 5 h. Cells were then stained for their surface expression of CD8 (*x* axis) and their intracellular expression of IFN-γ (*y* axis). The numbers shown indicate the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cells. The data shown are representative of three independent experiments. At least three mice per group were used in each experiment. (B) The data are shown as the mean ± SD of all mice used in the experiments. One-way ANOVA was performed for comparison of data between vaccination groups. *, $p < 0.01$; **, $p < 0.001$.

for their surface expression of CD8 and antigen-induced intracellular expression of IFN-γ. In the spleen (Fig. 2A-k–t), the highest frequency of IFN-γ-producing $CD8^+$ T cells was observed in mice that had been immunized s.c. in the hind hock with Ad-M1 (9.43%) (Fig. 2A-t). Further, i.p. (2.43%) (Fig. 2A-l), i.v. (1.23%) (Fig. 2A-p), and i.m. (2.35%) (Fig. 2A-r) injections of Ad-M1 induced high percentages of IFN-γ-producing $CD8^+$ splenic T cells although i.n. immunization with Ad-M1 led to much less IFN-γ-secreting $CD8^+$ T cells in the spleen (0.19%) (Fig. 2A-n). In contrast, the induction pattern of CTLs in the lung (Fig. 2A-a–j) was completely distinct from that in the spleen. First of all, the frequency of IFN-γ-producing $CD8^+$ T cells in the lung was substantially lower than that in the spleen of HHD mice receiving i.p., i.v. or i.m. injection with Ad-M1 (Fig. 2A-b, -f, and -h). Surprisingly, however, s.c. hock immunization elicited a large number of IFN-γ-producing $CD8^+$ T cells in the lung (14.5%) (Fig. 2A-j). It should also be noted that the percentage of IFN-γ-secreting $CD8^+$ T cells in the lung (0.59%) (Fig. 2A-d) was higher than that in the spleen of i.n. immunized mice. Similar results were obtained in mice immunized with various doses ranging from 10^7 to 10^9 $CCID_{50}$ of Ad-M1 or Ad-WT via the various immunization routes indicated above.

A similar pattern was also observed in C57BL/6 mice. Two weeks after immunization with Ad-M1, lymphocytes in the spleen and the lung were stimulated with the M1 128–135 peptide, and then stained for the surface expression of CD8 and the intracellular expression of IFN-γ. As shown in Fig. 3A and B, s.c. hock immunization efficiently induced high frequencies of IFN-γ-producing $CD8^+$ T cells in the lung (Fig. 3A-f) as well as in the spleen (Fig. 3A-l). Further, IFN-γ^+ $CD8^+$ T cells were significantly detected in the lungs of i.n. immunized mice (Fig. 3A-d) and in the spleen of i.p. immunized mice (Fig. 3A-h), whereas these cells were almost not found either in the spleen of i.n. immunized mice (Fig. 3A-j) or in the lungs of i.p. immunized mice (Fig. 3A-b).

Taken together, these data demonstrate that the recruitment of antigen-driven IFN-γ-producing $CD8^+$ T cells in the lung is dependent on the route of adenoviral immunization. Notably, hock immunization induced a large number of antigen-driven IFN-γ-producing $CD8^+$ T cells in the lung, suggesting that this immunization may provide potent protection from viral respiratory infection such as influenza.

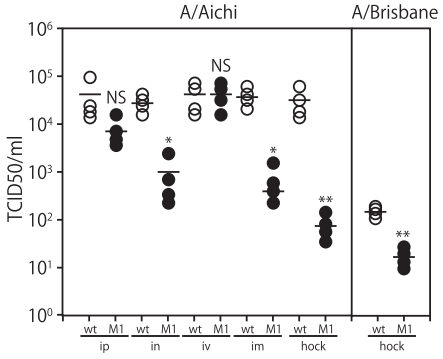

Fig. 4. Viral titers after influenza virus challenge in mice that immunized with Ad-M1 via various routes. HHD mice were immunized with 1×10^8 $CCID_{50}$ of either Ad-WT (wt) or Ad-M1 (M1) via various immunization routes including i.p., i.n., i.v., i.m., and s.c. hock administration. Two weeks later, mice were challenged with 1×10^4 $CCID_{50}$ of H3N2 (A/Aichi) or H1N1 (A/Brisbane) virus. Mice were then sacrificed on day 5 after the challenge, and the virus titers in their lungs were determined by calculating $CCID_{50}$ using MDCK cells. Four mice were used in each experimental group. The experiment was repeated twice with similar results. Horizontal bars represent the mean. *, $p < 0.05$; **, $p < 0.01$; NS, not significant; compared to mice immunized with Ad-WT via each immunization route.

3.3. Influence of the immunization route on the resistance to influenza virus

Since the level of pulmonary CTL induction varied depending on the immunization routes, we hypothesized that the route of immunization had an effect on the resistance to influenza A virus as well. To verify this hypothesis, HHD mice were immunized with Ad-M1 via various immunization routes, and then challenged i.n. with influenza virus A/Aichi/2/68 (H3N2). After 5 days following the challenge, virus titers in the lung were determined by calculating $CCID_{50}$ using MDCK cells. As expected, hock immunization with Ad-M1 was most efficient to suppress viral growth in the lung (Fig. 4). It was also demonstrated that viral titers of both i.n. and i.m. immunized mice were 1–2 log lower than those of the control mice (Fig. 4) presumably due to the substantial induction of virus-specific CTLs in the lungs of these mice (Figs. 2 and 3). On the other hand, i.v. injection of Ad-M1 failed to prove effective for the elimination of virus (Fig. 4). In addition, i.p. immunization could not offer statistically significant protection against i.p. virus infection ($p = 0.17$) (Fig. 4). Similar results were obtained in C57BL/6 mice (data not shown), or with a different subtype of influenza virus A/Brisbane/59/2007 (H1N1) (Fig. 4). Taken together, these data suggest that certain immunization routes which can sufficiently induce virus-specific CTLs in the lung are effective for the elimination of influenza A virus.

3.4. Killing activities of pulmonary CTLs

Epitope-specific IFN-γ^+ $CD8^+$ T cells in the lung were further characterized for their cytolytic activity in the CD107 mobilization assay. The transient expression of CD107 on the surface of $CD8^+$ T cells is correlated well with cytotoxic activity for antigen-specific CTLs (Betts et al., 2003). At 2 weeks after hock immunization with Ad-M1, lymphocytes in the lung were stimulated with either the M1 58–66 peptide for HHD mice or the M1 128–135 peptide for C57BL/6 mice, and stained for the expression of CD8, CD107 and intracellular IFN-γ. As shown in Fig. 5A, the majority of IFN-γ-producing $CD8^+$ T cells expressed CD107 on the cell surface, indicating that most IFN-γ^+ $CD8^+$ T cells in the lung possess M1-specific cytolytic ability. Furthermore, it was revealed that hock immunization was more effective to induce IFN-$\gamma^+$$CD107^+$$CD8^+$ T cells in the lung than i.p. and i.n. immunizations (Fig. 5B). To confirm these results,

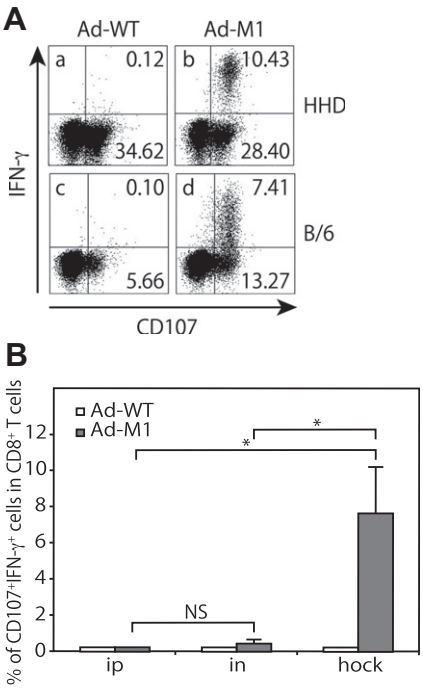

Fig. 5. Coordinate expression of CD107 on IFN-γ-secreting $CD8^+$ T cells in mice immunized with Ad-M1. (A) HHD mice (a and b) and C57BL/6 (B/6) mice (c and d) were immunized s.c. in the hind hock with 1×10^8 $CCID_{50}$ of either Ad-WT (a and c) or Ad-M1 (b and d). Two weeks later, lymphocytes in the lung were prepared, stimulated with either the M1 58–66 peptide for HHD mice or the M1 128–135 peptide for C57BL/6 mice, and stained for the expression of CD107 (*x* axis) and intracellular IFN-γ (*y* axis) in $CD8^+$ T lymphocytes. The numbers shown in the upper right and the lower right quadrants indicate the percentages of intracellular IFN-γ^+ $CD107^+$ cells and IFN-γ^- $CD107^+$ cells within $CD8^+$ T cell, respectively. Three mice were used in each experimental group. The experiment was repeated twice with similar results. (B) The data are shown as the mean ± SD of all HHD mice immunized with Ad-WT (white bars) or Ad-M1 (gray bras) via various immunization routes including i.p., i.n., and hock administrations. One-way ANOVA was performed for comparison of data between vaccination groups. *, $p < 0.01$; NS, not significant.

we next performed the ^{51}Cr-release assay using pulmonary lymphocytes in HHD mice immunized with Ad-M1. In agreement with the data shown in Fig. 5, M1-specific killing activities were extensively detected in lymphocytes prepared from the lung of hock-immunized mice (Fig. 6). In addition, it was also shown that hock immunization was most effective for the induction of M1-specific killing activities of various routes of immunization (Fig. 6).

3.5. Induction of long-lasting pulmonary CTLs

We next examined whether long-lasting M1-specific CTLs could be elicited in the lung with Ad-M1. HHD mice were immunized once with either Ad-WT or Ad-M1 via various routes. Lymphocytes in the lung were then prepared on day 84 after the immunization, and stimulated in vitro with the M1 58–66 peptide for 5 h at 37 °C. $CD8^+$ T cells were then analyzed for the peptide-specific expression of intracellular IFN-γ. It was shown that hock immunization was most efficient to induce IFN-γ-producing $CD8^+$ T cells in mice even on day 84 after immunization with Ad-M1 among various immunization routes (Fig. 7). These results demonstrate that s.c. hock immunization with Ad-M1 efficiently generates long-lasting memory CTLs in the lung.

4. Discussion

In the current study, we found that the route of adenoviral immunization dramatically influenced the recruitment of

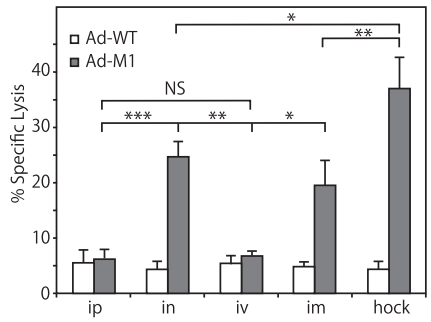

Fig. 6. Detection of M1-specific killing activity in lymphocytes prepared from the lung of mice immunized with Ad-M1 in the hind hock. HHD mice were immunized with 1×10^8 $CCID_{50}$ of either Ad-WT (white bars) or Ad-M1 (gray bars) via various immunization routes including i.p., i.n., i.v., i.m., and hock administration. After 2 weeks following the immunization, lymphocytes were prepared from the lung, and stimulated in vitro with irradiated syngeneic spleen cells pulsed with the M1 58–66 peptide for 7 days. After antigen stimulation, ^{51}Cr-release assays were performed at an E/T ratio of 150, using RMA-HHD cells pulsed with the M1 58–66 peptide as targets. Data are shown as the mean ± SD of triplicate wells. Similar results were obtained in two independent experiments. Five mice per group were used in each experiment and lymphocytes of mice per group were pooled. One-way ANOVA was performed for comparison of data between vaccination groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

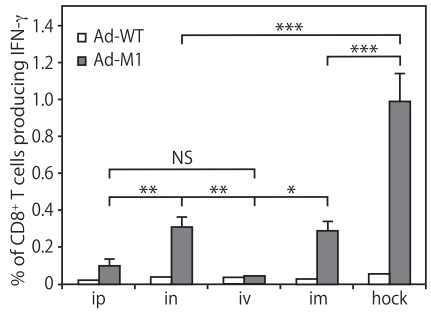

Fig. 7. Induction of long-lasting M1-specific CTLs in the lung of mice immunized with Ad-M1. HHD mice were immunized with 1×10^8 $CCID_{50}$ of either Ad-WT (white bars) or Ad-M1 (gray bars) via various immunization routes including i.p., i.n., i.v., i.m., and hock administration. At day 84 after the immunization, lymphocytes in the lung were prepared, and cultured in vitro with the M1 58–66 peptide. Cells were then stained for surface expression of CD8 and intracellular expression of IFN-γ. The experiment was repeated twice with similar results. Three mice per group were used in each experiment. The data indicating the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cell are shown as the mean ± SD of all mice used in the experiments. One-way ANOVA was performed for comparison of data between vaccination groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; NS, not significant.

M1-specific IFN-γ^+ $CD8^+$ T cells in the lung as well as in the spleen. It should be noted that hock immunization most effectively generated a large number of M1-specific IFN-γ^+ $CD8^+$ T cells corresponding to M1-specific CTLs that trafficked readily to local and systemic compartments. It was also shown that M1-specific CTLs in the lung, but not in the spleen, were likely to be correlated with protective immunity against challenge with influenza A virus.

Hock immunization (Kamala, 2007) has been reported to be a more humane alternative to footpad immunization that is a commonly used immunization method in mice. Footpad immunization is relatively easy to do and provides a robust immune response in mice. However, this immunization often results in the inflammation and swelling at the hind feet, and thereby, a mouse immunized in the footpad becomes progressively unable to bear weight on its injected foot, sometimes resulting in being lame. On the other hand, hock immunization does not cause the inflammation of the hind feet, while the immune responses induced by this protocol were quite comparable to those by footpad immunization (Kamala, 2007). Hock immunization as well as footpad immunization targets the three major lymph nodes including the popliteal, medial iliac, and inguinal nodes. These three nodes would provide a relatively large number of effector T lymphocytes that migrate to the lung as well as to the spleen. In addition, skin is well equipped with the dendritic cell network (Takashima and Morita, 1999). Dendritic cells are able to transfer antigen to the draining lymph nodes, where antigen-specific $CD8^+$ T cells are primed. These explanations might answer the question of why hock s.c. immunization can efficiently recruit influenza M1-specific CTLs both in the lungs and spleen. Additionally, it was shown that hock immunization induced long-lasting M1-specific CTLs in the lung (Fig. 7) and the spleen (data not shown). Hock immunization has been described in sheep (Kerlin and Watson, 1987), but to our knowledge, has not been widely used in mice. Therefore, it is unknown whether our findings regarding the efficiency of CTL induction might be limited to the use of adenovirus immunization strategies or might also apply to other immunization strategies such as DNA vaccines. However, protein-adjuvant mixtures are likely to be efficiently delivered into mice (Kamala, 2007) and rats (Gorton et al., 2010) for the induction of immune responses by hock immunization. Furthermore, we have revealed that the hock route of liposomal vaccines composed of peptides chemically coupled to the surface of liposomes was most efficient to induce peptide-specific CTLs among various immunization routes (data not shown).

Lin et al. (2010) evaluated the protective efficacy of vaccination in mice with novel adenovirus vectors expressing the influenza A nucleoprotein. They found that all vectors induced similar CTL responses in the spleen, yet differed in their ability to protect immunized mice against challenge with a lethal dose of influenza A virus. This impaired protection was due to the fewer antigen-specific IFN-γ-secreting CTLs in the lung (Lin et al., 2010). Similar results were observed in the current study. As shown in Figs. 2 and 3, i.n. immunization induced a significant number of pulmonary CTLs, and acted effectively in the suppression of viral growth (Fig. 4), whereas a minimum number of M1-specific IFN-γ^+ $CD8^+$ T cells were detected in the spleen of i.n. immunized mice (Figs. 2 and 3). In contrast, i.v. immunization with Ad-M1 failed to elicit M1-specific IFN-γ^+ $CD8^+$ T cells in the lungs (Fig. 2) and did not work well for the elimination of virus in the lungs (Fig. 4) although this immunization obviously induced M1-specific IFN-γ^+ $CD8^+$ T cells in the spleen (Fig. 2). Similar results were obtained in i.p. immunized mice (Figs. 2–4). Thus, circulating systemic T lymphocytes often exhibit a limited capacity to traffic to local mucosa layers (Mora and von Andrian, 2006). Taken together, these data strongly suggest that the recruitment of virus-specific CTLs in the lung is closely correlated with better protection against challenge with influenza A virus.

In general, immune protection against influenza A virus is mediated predominantly by neutralizing Abs directed to the hemagglutinin (HA), and the coevolution of HA and neuraminidase (NA) generates variant strains that become resistant to neutralization (Brown and Kelso, 2009; Wei et al., 2010). In the current study, we did not measure M1-specific Ab levels arising as a result of the various immunization routes. It is possible that the hock route of immunization also resulted in enhanced M1-specific Ab titers. However, it is unlikely that M1-specific Abs were responsible for the enhanced viral clearance because internal viral proteins such as M1 do not provide neutralizing Abs. It is noteworthy that non-neutralizing Abs against an internal nucleoprotein (NP) of influenza A virus provided protection against virus in mice through an unknown mechanism (Carragher et al., 2008). However, it is supposed that NP vaccination can convey protection primarily

via CD8 effector mechanisms and natural infection with influenza A virus does not generate a large amount of anti-NP Abs. Anyhow, it might be necessary to investigate the role of M1-specific Abs in the protection against influenza A virus.

Most current vaccines are administered parenterally through i.m. or s.c. injection. However, several reports advocate that mucosal i.n. vaccination is superior to parenteral vaccination for the induction of mucosal immunity (Bivas-Benita et al., 2010; Santosuosso et al., 2005; Wang et al., 2004). The nasal mucosa is important for the mucosal immune system because this is the first contact point for influenza A virus. In the current study, i.n. immunization was found to be considerably effective for the clearance of influenza A virus, and the level of viral clearance in i.n.-immunized mice was comparable to that in i.m.-immunized mice (Fig. 4). This respiratory mucosal immunization with Ad-M1 resulted in a preferential accumulation of M1-specific CTLs in the lung but not in the distant lymphoid organs (Figs. 2 and 3). In addition, the nose can be used for the easy immunization of large population groups without requiring needles (Davis, 2001). Thus, the nose is considered to be an attractive route for immunization against respiratory viruses. On the other hand, parenteral i.m. immunization with Ad-M1 leads to the accumulation of M1-specific CTLs predominantly in the spleen (Fig. 2), and at the same time allows M1-specific CTLs to migrate to mucosal surfaces to establish potent, durable mucosal cellular immunity (Fig. 4).

In conclusion, we have shown that the route of adenoviral immunization dramatically impacted the recruitment of M1-specific CTLs and the resistance to challenge with influenza A virus. Hock immunization was shown to be a promising method for the induction of both local and systemic immune responses in mice (Figs. 2 and 3). I.n. immunization without requiring needles was also found to be effective for protection from influenza A virus (Figs. 2–4). Various routes of adenoviral immunization generated antigen-specific CTL responses with strikingly different magnitudes and anatomic distributions (Figs. 2 and 3). As shown in Fig. 4, our findings suggest that systemic T cell activation may not be absolutely required for local immune protection against influenza A virus in the lung, and therefore, emphasize the importance of examining T cell responses locally in the lung after vaccination. These results may improve our ability to design vaccines that target virus-specific CTL responses to particular anatomic compartments.

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