

Induction of Natural Killer Cell-dependent Antitumor Immunity by the *Autographa californica* Multiple Nuclear Polyhedrosis Virus

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Wild-type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) infects a variety of mammalian cell types *in vitro*, but does not replicate in these cells. We investigated the effects of AcMNPV in the induction of the immune response and tumor metastasis in mice. After intravenous injection, AcMNPV was taken up by the liver and spleen, and preferentially infected dendritic cells (DCs) and B cells in the spleen; costimulatory molecules CD40, CD80, and CD86 were upregulated in the DCs. The hepatic mononuclear cells (MNCs) in these animals were highly cytotoxic to natural killer (NK)-sensitive YAC-1 and B16 melanoma cells, but not to NK-resistant EL4 cells. Intravenous injection of AcMNPV-induced NK cell proliferation in the liver and spleen, and enhanced antitumor immunity in mice with B16 liver metastases. Furthermore, such treatment increased the survival of C57BL/6, $J\alpha 281^{-/-}$, and interferon (IFN)- $\gamma^{-/-}$ mice that were previously injected with B16 tumor cells. AcMNPV injection did not enhance the survival of NK cell-depleted mice. Moreover, one AcMNPV treatment effectively prolonged survival in a B16 liver metastasis model, and was equivalent to five treatments with recombinant interleukin-12 (IL-12) protein. These findings suggest that AcMNPV efficiently stimulates NK cell-mediated antitumor immunity.

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INTRODUCTION

The ability of baculoviruses, including *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), to infect insect cells has led to their use in multiple protein expression systems^{1,2} and as plant insecticides.^{3,4} AcMNPV, the genome of which consists of a circular, double-stranded DNA that contains ~130 kilobase pair⁵ surrounded by a large envelope, infects a variety of mammalian cell types, with the exception of certain hematopoietic cell lines, although its genome does not replicate or integrate into

mammalian chromosomes.^{6,7} Recently, the potential use of these viruses as vectors was explored.^{8,9} Unfortunately, except in the case of the mouse brain and testes, these viruses are rapidly inactivated by the classical complement pathway following *in vivo* injection.¹⁰⁻¹² While considerable effort has been expended in an attempt to understand the nature of the host immune response to baculovirus, only a few definitive findings have been reported. The virus induces antiviral cytokine production after being injected *in vivo*, resulting in the protection of cells from infection by the vesicular stomatitis and influenza viruses.^{13,14} Furthermore, infected hepatocytes stimulate Kupffer cells to produce tumor necrosis factor- α , interleukin (IL)-1 α , and IL-1 β when these cells are cocultured *in vitro*.¹⁵

The purpose of this study was to examine the effects of AcMNPV on immune responsiveness in general, and on antitumor immunity in particular, in mice. Intravenously-injected AcMNPV-infected splenic dendritic cells (DCs) and B cells, increased the percentage of liver mononuclear cells (MNCs) that exhibited natural killer (NK) cell activity, and increased serum interferon (IFN)- γ levels. In liver metastasis models, AcMNPV-induced V α 14 NKT cell-independent and IFN- γ -independent antitumor effects. These results indicate that AcMNPV might be a useful adjunct in the design of antiviral and antitumor therapies.

RESULTS

In vivo infectivity of AcMNPV in B6 mice

Although complement-resistant baculovirus can express inserted foreign genes *in vivo*,^{10,16} wild-type (complement-sensitive) baculovirus does not express inserted foreign genes *in vivo*. The tissue tropism of AcMNPV was examined by analyzing total DNA isolated from tissues in B6 mice intravenously-injected with AcMNPV [1×10^8 plaque forming units (PFU)] by means of the polymerase chain reaction (PCR) analysis, using AcMNPV-gp64 specific primers. The AcMNPV titer was based on producing an innate immune response and virus protection in a mouse model by intranasal, intramuscular, intradermal, and intraperitoneal injection.¹³ The PCR products of the *gp64* gene were predominantly detected in the liver and spleen following AcMNPV injection; no *gp64* PCR

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products were detected following vehicle injection (Figure 1a). A thin band of gp64 was also detected in the lung, which was likely infected by the pulmonary circulation. This finding suggests that the AcMNPV genome is contained in spleen-accumulated lymphocytes; therefore, we investigated whether AcMNPV infects various lymphocytes in the spleen. The splenocytes were stained with lineage markers CD3 (T, NKT cells), NK1.1 (NK, NKT cells), CD11c (DCs), and CD45R (B cells), and sorted by a cell sorter. AcMNPV-infected cells were examined by PCR using total DNA isolated from sorted cells with AcMNPV-specific primers. AcMNPV-specific bands were detected in total splenocytes and in the CD3⁺ NK1.1⁻ population. Total DNA from the T, NK, and NKT cells did not contain the AcMNPV genome (Figure 1b). These results indicate that AcMNPV did not infect T, NK, and NKT cells. AcMNPV-specific bands were present in DCs and B cells. The PCR analysis of total DNA from a CD11c⁻ CD45R⁻ population failed to amplify the AcMNPV genome (Figure 1c). These results suggested that in AcMNPV-injected mice, DCs and B cells as antigen-presenting cells are predominantly infected in the spleen *in vivo*.

We evaluated surface markers for activation on infected CD11c⁺ DCs by flow-cytometric analysis. Mice were injected intravenously with AcMNPV (1×10^8 PFU) or vehicle, and

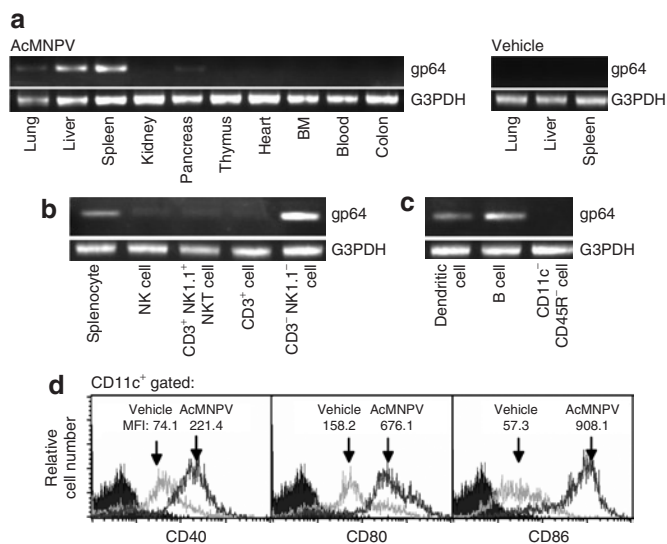


Figure 1 *In vivo* *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) infectivity. (a) Mice were injected with AcMNPV [10^8 plaque forming units (PFU)] or vehicle. After 24 hours, the tissues were harvested and total DNA was isolated. The presence of the AcMNPV genome was determined by polymerase chain reaction (PCR) using AcMNPV-gp64 specific primers. The data shown are from duplicate experiments that gave similar results. (b, c) Mice were injected with AcMNPV (10^8 PFU), and their splenocytes were harvested 6 hours later and sorted by fluorescence-activated cell sorting. AcMNPV genomic DNA was detected in the lymphocyte total DNA by PCR using specific primers. (d) Mice were injected intravenously with AcMNPV (10^8 PFU) or vehicle (phosphate-buffered saline). Splenocytes were isolated and examined by flow cytometry for gated CD11c (fluorescein isothiocyanate), and the activation markers CD40 [phycoerythrin (PE)], CD80 (PE), and CD86 (PE) 24 hours after AcMNPV injection. The mean fluorescence intensity (MFI) is depicted in each panel. The data shown are from duplicate experiments that gave similar results. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; NK cell, natural killer cell.

splenocytes were isolated 24 hours later. We then compared the DC phenotype from AcMNPV-infected and control mice. The mean fluorescence intensity of costimulatory molecules on infected CD11c⁺ DCs significantly increased with CD40 (threefold), CD80 (fourfold), and CD86 (16-fold) compared with control CD11c⁺ DCs (Figure 1d), and returned to baseline after 72 hours (data not shown). Costimulatory molecules CD40, CD80, and CD86 were upregulated in CD11c⁺ DC phenotypes in AcMNPV-treated mice. This result demonstrates that AcMNPV injection activates CD11c⁺ DCs in mice, thus suggesting that AcMNPV induces an immune response (*i.e.*, DC activation) by infecting DCs and B cells in the spleen and presumably the liver.

Effects of AcMNPV on IFN- γ production by lymphocytes

The Th1 immune response has both antiviral and antitumor effects, and is considered to be powerfully induced by the cytokine IFN- γ . The effect of AcMNPV stimulation on IFN- γ levels in mouse serum was investigated. Mice were injected intravenously with AcMNPV (1×10^8 PFU). The serum IFN- γ concentration increased after approximately 3 hours and peaked at 6 hours, returning to baseline levels after 24 hours (Table 1). The serum IFN- γ concentration was a function of the AcMNPV titer used (data not shown). The vehicle (phosphate-buffered saline or PBS) injection failed to induce detectable levels of IFN- γ under all the conditions tested. By contrast, IL-12p70 levels in the AcMNPV-treated animals were not significantly different from those in the PBS-injected control animals (data not shown).

To investigate the cytokine expression in AcMNPV-infected tissues, the mRNA levels of inflammatory cytokines (IFN- γ and IL-12p40) were examined in the spleen and liver 2 hours after AcMNPV or vehicle injection using reverse transcription (RT)-PCR methods (Figure 2a). We detected IFN- γ and IL-12p40 in the mRNA of the spleen and liver, but not the serum, from AcMNPV-injected mice. The antiviral cytokines IFN- α and IFN- β were also detected using the RT-PCR method (data not shown). The effect of AcMNPV stimulation on *in vivo* tissue lymphocyte IFN- γ production was assessed. AcMNPV stimulation significantly increased the amount of IFN- γ released by both splenocytes and liver MNCs (Figure 2b). The increase in IFN- γ released by liver MNCs was approximately sevenfold greater than that released by splenocytes.

Table 1 Effect of AcMNPV on serum concentration of IFN- γ in B6 mice^a

Treatment	Time (h)	IFN- γ (ng/ml)
Vehicle	6	<0.1
AcMNPV	1	<0.1
	3	1.48 \pm 0.2
	6	7.37 \pm 2.84
	12	6.24 \pm 1.77
	24	<0.1

^aMice were injected intravenously with *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) (10^8 plaque forming units) or vehicle (phosphate-buffered saline). The concentrations of interferon- γ (IFN- γ) in the sera of mice sacrificed at the times indicated following the injection of AcMNPV were determined by an enzyme-linked immunosorbent assay. Data are the mean \pm SD of four mice.

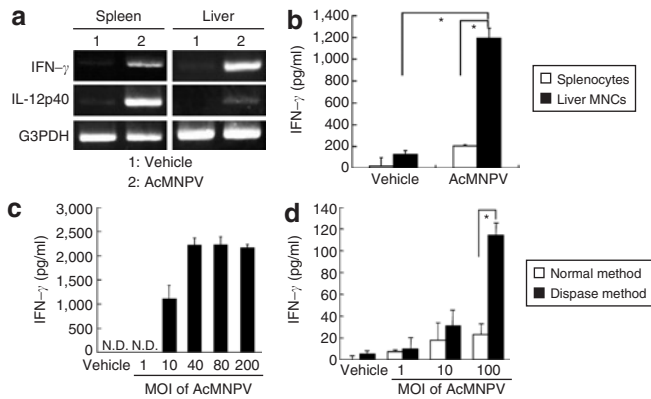


Figure 2 Effect of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) on interferon- γ (IFN- γ) production by splenocytes and liver mononuclear cells (MNCs) *in vivo* and *in vitro*. **(a)** Expression of various cytokine messenger RNAs (mRNAs) in the liver and spleen was detected 2 hours after mice were injected with AcMNPV (10^8 plaque forming units). mRNA expression was determined by reverse transcription polymerase chain reaction using the primers described in the Materials and Methods. The data shown are from duplicate experiments that gave similar results. **(b)** Concentrations of IFN- γ , as determined by enzyme-linked immunosorbent assay (ELISA), in the supernatants of 2-day-old cultures containing splenocytes and liver MNCs that were isolated from mice 6 hours after they were treated with AcMNPV. Data are the mean values \pm SEM; $P < 0.01$. **(c)** Production of IFN- γ by splenocytes stimulated *in vitro* with AcMNPV at the indicated multiplicities of infection (MOIs) for 72 hours, as determined by ELISA. The data represent the means \pm SEM. Similar results were obtained in two independent experiments. **(d)** Production of IFN- γ by liver MNCs, isolated with or without dispase, that were treated *in vitro* with AcMNPV at the indicated MOIs for 72 hours, as determined by ELISA. The data are the means \pm SEM; $*P < 0.01$. Similar results were obtained in two separate experiments. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; N.D., not detectable.

To examine IFN- γ production *in vitro*, the levels in the supernatants of cultured AcMNPV-stimulated splenocytes were measured by an enzyme-linked immunosorbent assay. IFN- γ production increased as a function of the AcMNPV titer (Figure 2c), reaching a plateau at a multiplicity of infection of 40, and remaining constant up to a multiplicity of infection of 200. Microscopic examination of stimulated splenocytes revealed cell aggregation (data not shown). Although *in vitro* AcMNPV stimulation increased IFN- γ levels in the supernatants of stimulated splenocytes, there was no detectable response in the supernatants of stimulated liver MNCs. We therefore compared the responses of liver MNCs, isolated with or without dispase to obtain the Kupffer cells, and found that substantially more IFN- γ was released by the cells isolated with dispase than from those isolated without dispase (Figure 2d). This result suggests that liver IFN- γ production in response to AcMNPV infection requires the release of inflammatory cytokines from virus-responsive lymphocytes (*i.e.*, Kupffer cells and/or hepatocytes), and that AcMNPV injection in mice induces IFN- γ production, which is known to have antiviral and antitumor effects.

Induction of NK cell activity by AcMNPV injection

We next examined the cytotoxicity of liver MNCs from AcMNPV-injected B6 mice on YAC-1 (NK-sensitive), EL4 (NK-resistant), and B16 (melanoma) cell lines. B6 mice were sacrificed 48 hours after AcMNPV injection and the cytotoxicity of their liver MNCs

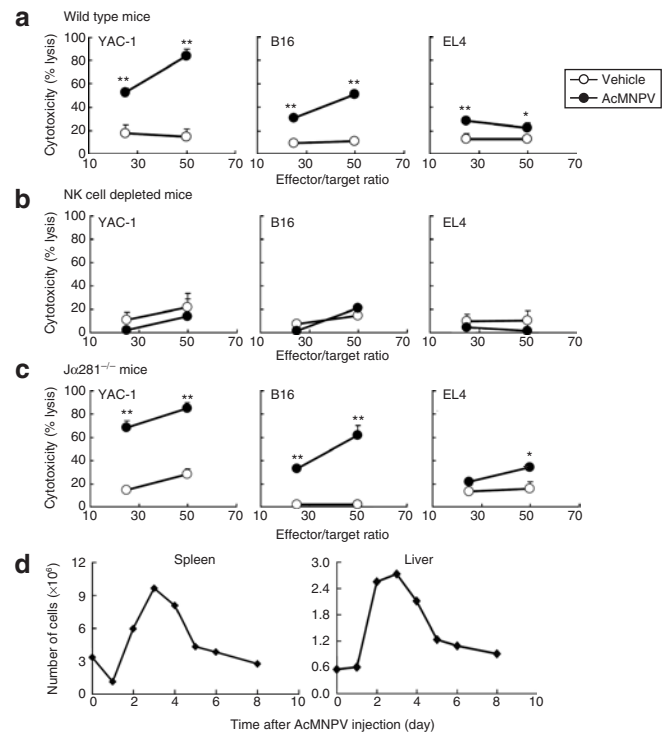


Figure 3 Effect of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) injection on cytotoxic activity and number of natural killer (NK) cells. **(a)** Cytotoxic activity of liver mononuclear cells (MNCs) from AcMNPV-treated or vehicle-treated mice against YAC-1, EL4, and B16 melanoma cells. **(b)** Cytotoxic activity of liver MNCs derived from mice that were pretreated with anti-asialoGM1 antibody before AcMNPV injection. **(c)** Cytotoxic activity of liver MNCs from *Ja281^{-/-}* mice treated with AcMNPV or vehicle. The data are the means \pm SD; $*P < 0.05$; $**P < 0.01$. Similar results were obtained in two separate experiments. **(d)** Numbers of NK cells (FITC-CD3⁻, PE-NK1.1⁺) in the spleen and liver of B6 mice on successive days following intravenous injection with AcMNPV, as calculated from flow cytometric data. Similar results were obtained in two independent experiments. FITC, fluorescein isothiocyanate; PE, phycoerythrin.

was tested. Cytotoxicity against YAC-1 and B16 cells increased (Figure 3a), whereas there was only minimal cytotoxicity against EL4 cells. AcMNPV injection of B6 mice, but not NK-depleted mice, produced by asialoGM1 injection (Figure 3b), increased the cytotoxicity against YAC-1 and B16 cells. Both IL-12 and α -galactosylceramide exert strong antimetastatic effects by inducing $V\alpha 14$ NKT cell-mediated IFN- γ production and NK cell activation.¹⁷⁻¹⁹ Therefore, we examined the cytotoxicity against YAC-1, EL4, and B16 cells using *Ja281^{-/-}* mice, which were devoid of $V\alpha 14$ NKT cells while having other lymphoid cell lineages intact, to determine whether AcMNPV induces cytotoxicity in $V\alpha 14$ *Ja281* NKT cells. In *Ja281^{-/-}* mice, the cytotoxicity against YAC-1, EL4, and B16 cells was similar to that in B6 mice (Figure 3c). Thus, the cytotoxicity of liver MNCs in response to AcMNPV depended on NK cells, but not NKT cells or other immune cells.

Moreover, to examine NK cell activation, we analyzed the time course of the number of CD3⁻NK1.1⁺ NK cells in the spleens and livers of B6 mice injected with AcMNPV (1×10^8 PFU) by flow cytometry and cell counting. There was a threefold to fourfold increase in the number of NK cells in the two organs that peaked at 3 days and returned to normal levels after 8 days (Figure 3d).

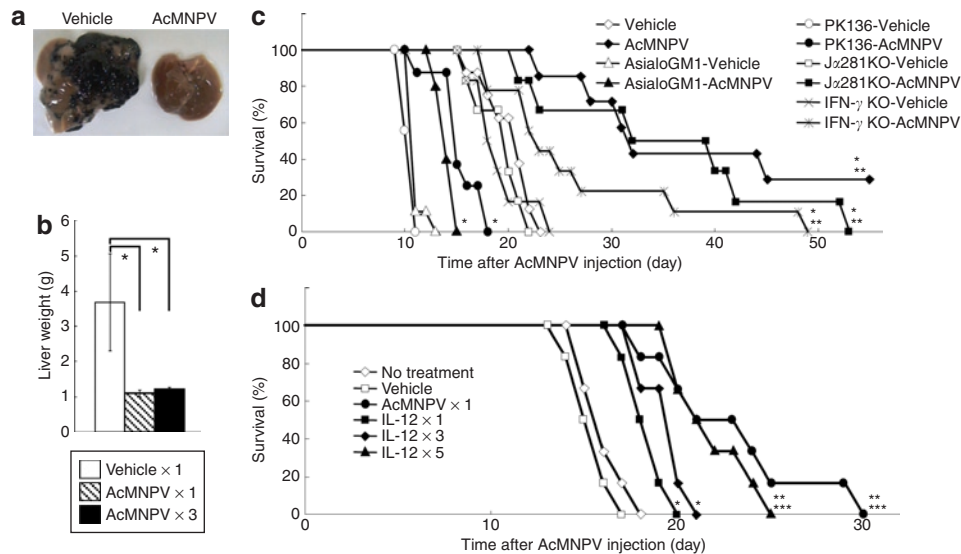


Figure 4 Effect of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) injection on B16 melanoma liver metastases in mice. Mice were injected intrasplenically on day 1 with 1×10^6 B16 cells in 0.1 ml phosphate-buffered saline (PBS). **(a)** Representative photographs of the livers of B6 mice on day 14 after they were treated with either vehicle or AcMNPV, showing typical metastases. **(b)** Liver weights of B16 melanoma cell-injected mice (eight per group) that were treated once with vehicle or AcMNPV (day 1), or three times with AcMNPV (days 1, 3, and 7). The data are the means \pm SD; * $P < 0.05$. **(c)** Survival rates of B6 mice that were injected with B16 melanoma cells, some of which were pretreated with anti-asialoGM1 antibody (Ab) (AsialoGM1) or anti-NK1.1 monoclonal Ab (PK136) to deplete them of natural killer (NK) cells or NK and NKT cells, respectively. Survival rates for $J\alpha 281^{-/-}$ and $IFN-\gamma^{-/-}$ mice that were injected with B16 melanoma cells. * $P < 0.05$ versus each vehicle-treated group. **Not significant according to the log-rank test. All groups contained between 6 and 11 mice. **(d)** Comparison of antitumor immunity induced by AcMNPV or interleukin-12 (IL-12) in B6 mice. Mice were injected intrasplenically on day 0 with 3×10^6 B16 cells in 0.1 ml PBS. Survival rates of B16 melanoma cell-injected mice with no treatment, or those treated with AcMNPV (day 1), vehicle (day 1), or IL-12 (day 1; days 1, 3, and 5; or days 1, 3, 5, 7, and 9). * $P < 0.05$; ** $P < 0.01$. ***Not significant according to the log-rank test. All groups contained six mice.

Moreover, NK cell numbers were similarly increased in the bone marrow and thymus of AcMNPV-treated mice (data not shown). This result indicates that liver and spleen NK cells are activated by AcMNPV injection in mice.

Treating liver metastatic mice with AcMNPV induces NK cell-dependent antitumor immunity

The antiliver metastatic effects of AcMNPV were assessed as follows. Mice were injected intrasplenically on day 0 with 1×10^6 B16 cells in 0.1 ml PBS. This was followed by intravenous injection of either AcMNPV (1×10^8 PFU) or vehicle on day 1. The mice were killed after day 14. **Figure 4a** shows the typical appearance of the livers at this time. Multiple tumor nodules were clearly visible on the livers of mice that received only vehicle (PBS), in marked contrast to those that received AcMNPV on day 1, which appeared normal. The degree of the antimetastatic effect was measured by weighing the livers. Livers of the AcMNPV-injected mice weighed significantly less than vehicle-injected mice (**Figure 4b**), and were comparable in weight to those of normal mice (~ 1 g). Tumor development was also inhibited in the livers of the mice that received three AcMNPV injections on days 1, 3, and 7, which did not induce toxicity or tumor growth (**Figure 4b**).

We then evaluated mouse survival rates after intrasplenic injection of B16 cells. This was followed by intravenous injection of either AcMNPV (1×10^8 PFU) or vehicle on day 1. Vehicle and AcMNPV injection had comparable effects on survival in wild-type, NK cell-depleted (anti-asialoGM1-treated), and NK/NKT cell-depleted (anti-NK1.1 monoclonal antibody-treated) mice. We confirmed that the antibody-induced depletion of NK

cells or NK/NKT cells was maintained for at least 3 days (data not shown). Animals in negative control groups that were pretreated with vehicle, and NK cell-depleted and NKT cell-depleted groups developed rapidly growing tumors that led to the death of the animals within 22 days (**Figure 4c**). By contrast, AcMNPV-treated wild-type mice had a significantly increased survival rate. The survival rates of $J\alpha 281^{-/-}$ or $IFN-\gamma^{-/-}$ mice were evaluated after intrasplenic injection of B16 cells to determine whether the AcMNPV-induced antimetastatic effects are dependent on $V\alpha 14$ NKT cells or $IFN-\gamma$. AcMNPV, but not vehicle, injection induced antimetastatic effects in $J\alpha 281^{-/-}$ and $IFN-\gamma^{-/-}$ mice, and the survival rates were similar to those of wild-type mice. These findings suggest that AcMNPV injection induces $V\alpha 14$ NKT cell-independent and $IFN-\gamma$ -independent NK cell cytotoxicity against tumor cells in mice.

Comparison of AcMNPV or IL-12-induced antitumor immunity

Cui and colleagues, and Nakagawa and co-workers, suggested that IL-12 and α -galactosylceramide induce antimetastatic effects by activating $V\alpha 14J\alpha 281$ NKT cells.^{18,20} To assess the effects of AcMNPV-induced antitumor immunity, we compared the antimetastatic effects by evaluating differences in survival rate following AcMNPV and recombinant IL-12 protein injections. The appropriate AcMNPV titer was determined for these experimental conditions (**Supplementary Figure S1**). This was accomplished by intravenous injection of either AcMNPV (1×10^8 PFU) or vehicle on day 1, and by intraperitoneal injection of recombinant IL-12 protein (1 μ g) on days 1; 1, 3, and 5; or 1, 3, 5, 7, and 9.²⁰

Here, a more malignant liver metastasis model was produced by increasing the number of injected B16 melanoma cells threefold to investigate the precise difference in the antimetastatic effects. One AcMNPV treatment induced similar survival compared to five recombinant IL-12 treatments (Figure 4d). These results suggest that the antimetastatic effects of one AcMNPV and five recombinant IL-12 protein treatments are equally efficient.

Analysis of AcMNPV-induced IFN- γ -independent antitumor immunity

IFN- γ is very important for the induction of cytotoxicity in liver MNCs in α -galactosylceramide-treated mice.¹⁹ We have established mouse survival rates after intrasplenic injection of B16 cells in IFN- $\gamma^{-/-}$ mice (Figure 4c). The survival rate in IFN- $\gamma^{-/-}$ mice was clearly increased due to the AcMNPV injection, but not due to the vehicle injection. We then evaluated the antimetastatic effects mediated by AcMNPV by examining the liver weight of IFN- $\gamma^{-/-}$ mice that were intrasplenically injected with B16 cells as described above. The antimetastatic effects of AcMNPV were assessed in mice that were injected intrasplenically on day 0 with 1×10^6 B16 cells, and with intravenous AcMNPV (1×10^8 PFU/mouse) or vehicle alone on day 1; Figure 5a shows the typical appearance of the liver on day 14 in these animals. The antimetastatic effect of AcMNPV was quantified by measuring liver weight, and the results indicated that the mean weight of the livers from AcMNPV-treated mice was significantly lower than that of mice with metastases (Figure 5b), and comparable to that in normal mice (~1 g), although a few tumor nodules were present in untreated mice, and this result was similar to the number of liver nodules and liver weight (Figure 4c).

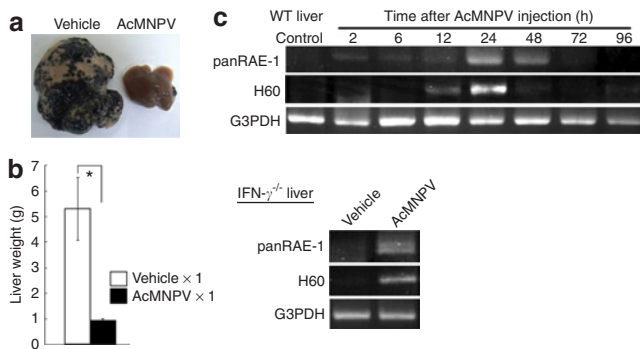


Figure 5 Analysis of antitumor mechanism of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) injection in mice. Mice were injected intrasplenically on day 0 with 1×10^6 B16 cells in 0.1 ml phosphate-buffered saline. (a) Representative photographs of the livers from interferon (IFN)- $\gamma^{-/-}$ mice on day 14 after they were treated with either vehicle or AcMNPV, showing typical metastases. (b) Liver weights of B16 melanoma cell-injected IFN- $\gamma^{-/-}$ mice (six mice per group) that were treated once with vehicle or AcMNPV (day 1). The data are the means \pm SD; * $P < 0.05$. (c) Kinetics of panRAE-1 and H60 messenger RNA (mRNA) expression in the liver induced by AcMNPV injection. mRNA expression was determined by reverse transcription polymerase chain reaction (RT-PCR) using the appropriate specific primers. The data shown are from duplicate experiments that gave similar results. IFN- $\gamma^{-/-}$ mice were injected with AcMNPV or vehicle and their livers were harvested 24 hours later. panRAE-1 and H60 mRNA were detected in the total RNA of their tissues by RT-PCR. The data shown are from duplicate experiments that gave similar results. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; WT liver, wild-type liver.

We demonstrated that AcMNPV, but not vehicle, injection induced antimetastatic effects in IFN- $\gamma^{-/-}$ mice. These findings suggest that AcMNPV-induced IFN- γ -independent NK cell cytotoxicity against tumor cells in mice.

Diefenbach and co-workers reported that the NKG2D ligand, but not cytokine-activated NK cells, inhibits tumor growth.²¹ It was possible that IFN- γ independent activation of NK cells by AcMNPV involves the NKG2D ligand, and we therefore examined the expression of NKG2D ligands in the liver of AcMNPV-treated mice by means of RT-PCR. Expression of the NKG2D ligands panRAE-1 and H60 mRNA was detected in total liver RNA of AcMNPV-treated mice, but not in vehicle-treated mice; expression peaked at 24 hours and returned to normal levels after 72 hours (Figure 5c). Expression of NKG2D ligands panRAE-1 and H60 mRNA was detected in total liver RNA of AcMNPV-injected IFN- $\gamma^{-/-}$ mice (Figure 5c). The data suggest that liver NK cells are IFN- γ -independently activated by the NKG2D ligands Rae-1 and H60 by injection of AcMNPV.

DISCUSSION

Insects are natural hosts for the baculovirus AcMNPV, which is widely used as a pesticide and is considered harmless to humans. Because insects coevolved with humans, it is likely that humans can also produce an efficient immune response against AcMNPV. Mice are unlikely to be naturally infected, but artificial infection with high titers (as used in our experiments) is expected to induce an antiviral (or antitumor) response.

It was previously reported that a recombinant AcMNPV containing a mammalian expression cassette failed to be expressed in immunocompetent recipient animals, and it was suggested that this might be due to viral inactivation by complement protein, an effect that was demonstrated both *in vivo* and *in vitro*.^{10,16} These data notwithstanding, in the present study, the virus infected cells in the liver and spleen of immunocompetent C57BL/6 mice (Figure 1a). Our results suggest that injection of a high titer of AcMNPV might exceed the ability of the complement to neutralize the virus or, alternatively, that the virus has a high infectivity rate in the liver and spleen. Cheng and colleagues reported that AcMNPV efficiently transduced a variety of adherent, but not suspension, cell lines *in vitro*.^{22,23} Wild-type AcMNPV-infected primary suspensions of mouse DCs and B cells *in vivo* (Figure 1b and c). Such infectivity is thought to result from endocytosis mediated by either DC complement receptors or pathogen-specific Toll-like receptor-recognition of the pathogen components, and by subsequently upregulating costimulatory molecules on DCs (Figure 1d). These data suggest that AcMNPV-induced immune activation and infected DCs and B cells in spleen and liver and, together with previous findings, indicate that it is safe to use as a vector in mammals.²⁴ Our results also suggest, however, that AcMNPV, when present in high titers, is recognized as a pathogen by DCs, and induces a DC-activated immune response in mice.

Baculovirus is rapidly inactivated by the classical complement pathway following *in vivo* injection.^{10,11} We demonstrate that intravenous injection of AcMNPV induces IFN- γ production in serum (Table 1). The increased production of IFN- γ by liver MNCs compared to splenocytes that was stimulated *in vivo* with AcMNPV (Figure 2a and b) might have been due to the fact that

intravenously injected substances tend to accumulate in the liver (Figure 1a). *In vitro* AcMNPV stimulation, however, induced lower IFN- γ levels in the liver than in splenocytes (Figure 2d). The results described above suggest that intravenously-injected AcMNPV is inactivated by the classical complement cascade in serum, after which it is clearly excluded by IFN- γ -producing hepatic MNCs that are activated by inflammatory cytokines produced by activated Kupffer cells, and/or hepatocytes activated by AcMNPV stimulation.¹⁵ Moreover, this result suggests that IFN- γ production in liver MNCs following AcMNPV stimulation requires other factors in the blood or liver. We assumed that AcMNPV might also induce strong antitumor and antiviral Th1 responses that are mediated, at least in part, by IFN- γ .

Nakagawa and co-workers, and Smyth and colleagues, suggested that IL-12 and α -galactosylceramide induce IFN- γ production by V α 14J α 281 NKT cells, and that NK cells have antimetastatic effects in the liver.^{20,25} Initially, we provided evidence for strong AcMNPV-induced cytotoxic activity of liver MNCs against NK cell-sensitive YAC-1 and B16 melanoma cell lines, but not against the NK-resistant EL4 cell line, using B6 (Figure 3a). AcMNPV-induced cytotoxicity disappeared in NK cell-depleted mice (Figure 3b), but not in J α 281^{-/-} mice (Figure 3c). In fact, AcMNPV injection increased the NK cell number in the liver and spleen (Figure 3d). Our results demonstrate that AcMNPV injection induces NK cell activation, and augments NK cell-dependent, V α 14 NKT cell-independent, cytotoxicity. NK cells in AcMNPV-treated mice were not infected (Figure 1b); therefore, these data suggest that NK cell activation mediates antigen-presenting cells because AcMNPV infects DCs.

Wild-type mice treated with AcMNPV had a significantly increased survival rate in a liver metastasis model (Figure 4c). This result is consistent with the finding that liver tumor nodules and liver weight in AcMNPV-treated mice are normal (Figure 4a and b). A similar antimetastatic effect (tumor nodules) was observed with AcMNPV injection 3 days after B16 injection (data not shown). Furthermore, liver metastasis after repeated AcMNPV injections (days 1, 3, and 7) induced similar antitumor effects without toxicity or tumor growth compared with one injection of AcMNPV (Figure 4b). These results demonstrate that repeated AcMNPV injection induces antiliver metastatic effects without toxicity. Vehicle or AcMNPV-treated NK cell or NK/NKT cell-depleted mice died more rapidly than vehicle-treated non-depleted wild-type mice. Treatment with AcMNPV in both NK cell and NK/NKT cell-depleted mice, however, prolonged survival by ~5 days, compared with vehicle-treated NK cell and NK/NKT cell-depleted mice (Figure 4c). This increased survival rate indicates that the AcMNPV-induced antimetastasis effects might involve unknown NK cell-independent mechanisms. The survival rate of J α 281^{-/-} and IFN- γ ^{-/-} mice was similar to that of wild-type mice in AcMNPV-treated and vehicle-treated mice. These results indicate that the antimetastatic effects of AcMNPV-induced NK cell activation differ from the antimetastatic effects of α -galactosylceramide-induced V α 14 NKT cell-dependent NK cell activation. Thus, the AcMNPV-induced antimetastasis effects in mice are NK cell-dependent, and V α 14 NKT cell and IFN- γ -independent.

To assess the strength of AcMNPV-induced antitumor immunity, we compared the antimetastatic effects by measuring

differences in survival between a single AcMNPV treatment and five treatments of recombinant IL-12 protein. The antimetastatic effect of AcMNPV was similar to that of recombinant IL-12 protein (Figure 4d). Thus, AcMNPV and IL-12 are equally effective in a liver metastasis model; however, the antimetastasis mechanisms for the NK cell-dependent, V α 14 NKT cell-independent, antitumor activity of AcMNPV differ from the V α 14 NKT cell-dependent antitumor activity of IL-12, and might be useful in the development of tumor immunity.

In this study, mRNA expression of the NKG2D ligand H60 and panRAE-1 was detected in the total RNA of livers from both B6 and IFN- γ ^{-/-} mice injected with AcMNPV (Figure 5c). Recently, Diefenbach and colleagues reported that NKG2D ligand, but not cytokine-activated NK cells, significantly inhibit tumor growth.²¹ By contrast, Hamerman and co-workers reported that lipopolysaccharides and poly(I:C) activate Toll-like receptor signaling and induce the expression of an NKG2D receptor ligand on macrophages.²⁶ More recently, T.A. and co-workers reported that AcMNPV was recognized by Toll-like receptor 9 expressed on DCs and macrophages, but heat-inactivated AcMNPV was not recognized.²⁷ Moreover, adenovirus containing genomic DNA and AcMNPV induces the expression of the NKG2D ligand H60 in mice, and activates NK cells,²⁸ suggesting that AcMNPV induced IFN- γ -independent cytotoxicity of NK cells by inducing the expression of an NKG2D ligand, such as H60 and Rae-1, on the virus-infected antigen-presenting cells in the liver. A more detailed investigation is needed to clarify this issue.

Insects are the natural hosts of AcMNPV, and the virus is thought to be safe in these organisms. The safety of the virus for applications in human is, however, not known, and toxicity in animals must be examined prior to its use in clinical trials. No activation of hepatic enzymes, such as plasma alanine aminotransferase and aspartate aminotransferase, was observed in serum 3 days after injection into B6 mice with AcMNPV (data not shown). The virus does not replicate in mammals and is not toxic to mammalian cells.⁷ Consequently, AcMNPV is clinically used in multiple protein expression systems^{5,22} and in plant insecticides, as the baculoviruses have been registered as insecticides with the US Environmental Protection Agency.^{3,4} Humans are the hosts of most other viruses used for therapy, which causes various problems, such as replication and toxicity, but humans are not hosts of baculovirus.

In conclusion, AcMNPV predominantly infected antigen-presenting cells, and activated DCs and NK cells in mice. Furthermore, AcMNPV efficiently stimulated NK cell-dependent antitumor immunity, but is V α 14 NKT cell and IFN- γ -independent. Therefore, AcMNPV might be potentially useful in clinical trials as an efficient antimetastatic agent, and is expected to be useful in the development of antitumor therapies.

MATERIALS AND METHODS

Animals and cell lines. Female C57BL/6 (B6) mice were purchased from Nippon SLC (Hamamatsu, Japan). B6.J α 281^{-/-} mice lacking the J α 281 T-cell receptor gene segment, which were devoid of V α 14 NKT cells while having other lymphoid cell lineages intact (hereafter referred to as J α 281^{-/-} mice), were generated as previously described.¹⁷ B6.IFN- γ ^{-/-} mice were obtained from Yoichiro Iwakura (Tokyo University, Tokyo, Japan). All mice used were between 5 and 10 weeks of age. The

B16 melanoma, YAC-1, and EL4 cell lines were obtained from the Riken Cell Bank (Wako, Japan). B16 melanoma and EL4 cells were maintained in Dulbecco's modified Eagle's medium (Sigma Chemical, St. Louis, MO), and YAC-1 cells were maintained in Rosewell Park Memorial Institute (RPMI) 1640 (Sigma Chemical), both of which were supplemented with 10% fetal bovine serum, 100 U/ml penicillin (Invitrogen, Carlsbad, CA) and 100 µg/ml streptomycin (Invitrogen).

Purified wild-type AcMNPV. Wild-type baculovirus AcMNPV was purchased from BD Bioscience (San Diego, CA). Sf-9 insect cells were cultured at 27°C in Sf-900IISFM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and 100 µg/ml kanamycin sulfate. Purification of the baculovirus by filtration and the sucrose gradient method was performed as previously described.²⁹ The virus stocks were endotoxin-free (<0.01 U/ml), as determined using a Pyrodict endotoxin measurement kit (Seikagaku, Tokyo, Japan).

Isolation of liver MNCs and splenocytes. Splenocytes were passed through a nylon mesh, centrifuged, and resuspended in red blood cell lysis solution [1.54 mol/l NH₄Cl, 14 mmol/l NaHCO₃, and 0.1 mmol/l EDTA₂Na (pH 7.3)], after which they were washed in PBS and resuspended in RPMI 1640 culture medium. Liver MNCs were prepared as described previously.³⁰ To obtain liver MNCs with Kupffer cells, the cells were harvested by agitating the liver in RPMI 1640 containing 10% fetal bovine serum and 2 U/ml dispase (Godo Shusei, Tokyo, Japan), which comprises a cocktail of proteolytic enzymes, principally collagenase, and is used for dispersing tissues and cells, at 37°C, after which they were passed through a nylon mesh. After three washes with RPMI 1640, the cells were resuspended in 33% Percoll solution (Amersham Biosciences, Piscataway, NJ), containing 100 U/ml heparin and centrifuged at 2,000 rpm for 20 minutes at room temperature. Pellets were resuspended in red blood cell lysis solution, washed three times in PBS, and resuspended in culture medium.

PCR and analysis of amplified products. Total DNA was purified from various tissues, and the presence of AcMNPV was detected by performing PCR for the viral gene AcMNPV-gp64. The sequences of the specific primers were as follows: AcMNPV-gp64, 5'-CTACTAGTAAATCAGTCACACC-3' (sense) and 5'-CCAAGTTTTTAATCTTGTACGG-3' (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5'-TCCACCACCCTGTTGCTGTA-3' (sense) and 5'-ACCACAGTCCATGCCATCAC-3' (antisense).

RT-PCR and analysis of amplified products. Total RNAs were extracted from cells using a GenElute Mammalian Total RNA Miniprep Kit (Sigma Chemical), according to the manufacturer's instructions. One-step RT-PCR was performed on RNA samples by RT-PCR high-Plus- (Toyobo, Osaka, Japan). The sequences of specific primers were as follows: IL-12p40, 5'-TCTGCAGAGAAGGTCACACTGGACCAAAG-3' (sense) and 5'-ATGCCACTTGCTGCATGAGGAATT-3' (antisense); IFN-γ, 5'-ACACA CTGCATCTGGCTTTGAGCT-3' (sense) and 5'-GGACCTGTGGGT TGTGACCTCAAACCTT-3' (antisense); IFN-α, 5'-TGAAGGACAGGA AGGACTTTGGATTCCC-3' (sense) and 5'-TCTCTCAGTCTTCCCAG CACATTGGCA-3' (antisense); IFN-β, 5'-TACAGGGCGGACTTCAAG ATCCCTATG-3' (sense) and 5'-CATCCAGGCGTAGCTGTTGTA CTG-3' (antisense); panRAE-1, 5'-GAAGTGGGGGAATGTTTGACA CAACC-3' (sense) and 5'-GGACCTTGAGGTTGATCTTGGCTTTTC-3' (antisense); and H60, 5'-TCTGGGCCATCAACACTGATGAACAG-3' (sense) and 5'-CACCAAGCGAATACCATGAATGCCA-3' (antisense).

Flow cytometry. Surface antigens were labeled with fluorescent monoclonal antibodies according to the protocols provided by BD Bioscience, which supplied all of our reagents. The monoclonal antibodies included fluorescein isothiocyanate-conjugated anti-CD3 (17A2), phycoerythrin (PE)-conjugated anti-NK1.1 (PK136), PE-conjugated anti-CD4 (H129.19), PE-conjugated anti-CD8α (53-6.7), PE-conjugated anti-CD45R (RA3-6B2), PE-conjugated anti-CD11b (M1/70), fluorescein isothiocyanate-conjugated

anti-CD11c (HL3), PE-conjugated anti-CD40 (GL1), PE-conjugated anti-CD80 (16-10A1), and PE-conjugated anti-CD86 (GL1). Cell subsets were analyzed using a fluorescence-activated cell sorting (FACS) Vantage cell sorter (Becton Dickinson, Mountain View, CA); all of the sorted populations were at least 90% pure.

Cytotoxicity assay. The effector cells in this assay were liver MNCs obtained from mice 48 hours after they had been intravenously-injected with AcMNPV (1×10^8 PFU), and the target cells were either B16 melanoma, YAC-1, or EL4 cells ($0.5\text{--}1 \times 10^6$ /well). The effector and target cells were coincubated in RPMI 1640 containing 10% fetal bovine serum (total volume of 100 µl) in a 96-well round-bottomed microtiter plate. The cytotoxicity was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI). The spontaneous release from target cells was determined to be less than 10% of the maximum release.

In vivo lymphocyte stimulation with baculovirus AcMNPV. Six hours after the mice were treated with AcMNPV (1×10^8 PFU/mouse) or vehicle, their lymphocytes were harvested and 5×10^5 cells were cultured in a total volume of 200 µl medium in a 96-well flat-bottomed microtiter plate, at 37°C in a humidified 5% CO₂ incubator. Forty-eight hours later, the supernatants were harvested and centrifuged, and the IFN-γ concentration was determined using an enzyme-linked immunosorbent assay kit (BD Bioscience).

In vitro lymphocyte stimulation with baculovirus AcMNPV. Isolated lymphocytes (5×10^5) were cultured in a total volume of 200 µl medium in a 96-well flat-bottomed microtiter plate, at 37°C in a humidified 5% CO₂ incubator, to which an aliquot of AcMNPV, vehicle (PBS), or control medium (culture medium) was added. Seventy-two hours later, the supernatants were harvested and centrifuged, and the IFN-γ concentration was determined using an enzyme-linked immunosorbent assay kit (BD Bioscience).

In vivo depletion of specific immune cells. Two or three days before the mice were treated with vehicle or AcMNPV, they were injected intraperitoneally with 50 µl anti-asialoGM1 antiserum (Wako Chemical, Osaka, Japan) or 100 µl ascites fluid from the PK136 hybridoma (anti-NK1.1; American Type Culture Collection). The depletion of NK cells and NK/NKT cells, as assessed by flow cytometry, was >90%.

B16 melanoma model of liver metastases. To induce liver metastases, mice were injected intrasplenically with $\sim 1\text{--}3 \times 10^6$ B16 cells in 0.1 ml PBS. The apertures of the injected spleens were surgically sutured after tying the vessels to arrest the blood flow. On day 1, the mice were intravenously injected with either AcMNPV (1×10^8 PFU/mouse) or vehicle. On day 1, days 1, 3, and 5, or days 1, 3, 5, 7, and 9 after injection of B16 cells, the animals were intravenously-injected with recombinant IL-12 protein (1 µg/mouse; R&D Systems, Minneapolis, MN) or vehicle.¹⁹ Fourteen days later, the mice were sacrificed, and their livers were weighed and examined for visible metastases.

Serum alanine aminotransferase and aspartate aminotransferase. The mice were intravenously-injected with either AcMNPV (1×10^8 PFU/mouse) or vehicle, and serum samples were collected 3 days later. The serum alanine aminotransferase activity and aspartate aminotransferase activity were determined by commercial kits (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. Data were analyzed using a two-tailed Student's *t*-test. The mouse survival rates were analyzed by the log-rank test. A *P* value <0.05 was considered to be statistically significant.

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SUPPLEMENTARY MATERIAL

Figure S1. Survival analysis of antitumor effect in titrated AcMNPV injection in mice.

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