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The innate immune system in host mice targets cells with allogenic mitochondrial DNA

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Mitochondrial DNA (mtDNA) has been proposed to be involved in respiratory function, and mtDNA mutations have been associated with aging, tumors, and various disorders, but the effects of mtDNA imported into transplants from different individuals or aged subjects have been unclear. We examined this issue by generating trans-mitochondrial tumor cells and embryonic stem cells that shared the syngenic C57BL/6 (B6) strain–derived nuclear DNA background but possessed mtDNA derived from allogenic mouse strains. We demonstrate that transplants with mtDNA from the NZB/B1NJ strain were rejected from the host B6 mice, not by the acquired immune system but by the innate immune system. This rejection was caused partly by NK cells and involved a MyD88-dependent pathway. These results introduce novel roles of mtDNA and innate immunity in tumor immunology and transplantation medicine.

Because nuclear-transplanted embryonic stem (ES) cells and induced pluripotent stem (iPS) cells share their nuclear DNA background with their nuclear donors, they are considered to be promising cellular systems for generating tissues that will not be rejected upon transplantation into a host (French et al., 2006; Byrne et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008). However, these cells are not complete clones of the hosts with respect to their mitochondrial DNA (mtDNA). The mtDNA in nuclear-transplanted ES cells is derived from the donors of the ES cells and thus differs from the mtDNA of their nuclear donors (Evans et al., 1999; Hall et al., 2006). Even in the case of iPS cells, the mtDNA accumulates specific somatic mutations during aging of the donors. Mutations in mtDNA have been proposed to be associated with mitochondrial diseases, aging, tumor formation, and various age-associated disorders such as diabetes mellitus and Parkinson’s disease (Larsson and Clayton, 1995; Wallace, 1999). Moreover, our recent studies (Ishikawa et al., 2008a,b) showed that mtDNA mutations that induce overproduction of reactive oxygen species (ROS) are responsible for the development of metastatic potential of some tumor cells. Some mtDNA polymorphisms can be recognized by the acquired immune system of the host (Loveland et al., 1990). Thus, it is possible that the mtDNA in specific somatic mutations during aging of the donors. Mutations in mtDNA have been proposed to be associated with mitochondrial diseases, aging, tumor formation, and various age-associated disorders such as diabetes mellitus and Parkinson’s disease (Larsson and Clayton, 1995; Wallace, 1999). Moreover, our recent studies (Ishikawa et al., 2008a,b) showed that mtDNA mutations that induce overproduction of reactive oxygen species (ROS) are responsible for the development of metastatic potential of some tumor cells. Some mtDNA polymorphisms can be recognized by the acquired immune system of the host (Loveland et al., 1990). Thus, it is possible that the mtDNA in...
nuclear-transplanted ES cells and in iP cells could influence mitochondrial respiratory function, tumor phenotypes, and the host–donor compatibility of the transplants differentiated from these cells.

RESULTS AND DISCUSSION

Isolation of the trans-mitochondrial tumor cells (tumor cybrids) with allogenic mtDNA

To examine the effects of mtDNA derived from allogenic strain mice on respiratory function, tumor phenotypes, and host–donor compatibility, we have to exclude the effects of nuclear DNA variations on these phenotypes. We resolved this problem by generating trans-mitochondrial tumor cells (tumor cybrids; see Materials and methods), which shared the C57BL/6 (B6) strain–derived nuclear DNA but possessed mtDNA polymorphisms (Bayona–Bafaluy et al., 2003) derived from different allogenic strain mice. As nuclear DNA donors and mtDNA recipients, we used mouse mtDNA-less P29 cells (ρ0P29 cells), which were isolated previously (Ishikawa et al., 2008a) from poorly metastatic Lewis lung carcinoma P29 cells derived from a B6 strain (Koshikawa and Takenaga, 2005).

The tumor cybrids were obtained by fusion of the ρ0P29 cells with enucleated cells carrying mtDNA from the syngenic strain B6 or the allogenic strain C3H/An (C3H) or NZB/B1NJ (NZB; Fig. S1 A). The resultant tumor cybrids were named P29mtB6, P29mtC3H, and P29mtNZB, respectively. Complete mtDNA replacement in the tumor cybrids was confirmed by restriction enzyme digestion of the PCR products (Fig. S2 A).

Effects of allogenic mtDNA on respiratory function, tumor phenotypes, and host–donor compatibility

First, we examined the effects of the allogenic mtDNA in the tumor cybrids on respiration and the production of ROS. We found no significant differences among the tumor cybrids in respiration complex activities, ATP contents, or O2 consumption rates (Fig. S2 B), although a slight increase in the amounts of ROS was observed in P29mtC3H and P29mtNZB (Fig. S2 C), which is consistent with a previous study (Moreno-Loshuertos et al., 2006).

Our previous study (Ishikawa et al., 2008a) showed that ROS production confers metastatic potential in tumor cells. Therefore, we examined the effect of the augmented ROS production on experimental and spontaneous metastases by inoculating P29mtB6, P29mtC3H, and P29mtNZB into the tail vein and under the back skin of recipient B6 mice (Fig. 1 A). As a positive control, we used P29mtA11, which was generated previously by ρ0P29 cells with highly metastatic Lewis lung carcinoma A11 cells (Ishikawa et al., 2008a). It expressed highly metastatic potentials caused by mtDNA with a G13997A mutation, which is derived from A11 cells, and induced sufficient ROS overproduction required for inducing metastatic potential (Ishikawa et al., 2008a). We found that only P29mtA11 formed lung nodules (Fig. 1 A), which suggested that the increase in ROS production by the allogenic mtDNA from the C3H and NZB strains (Fig. S2 C) was not sufficient to induce lung metastasis.

We next examined the effects of allogenic mtDNA on the host–donor compatibility by inoculating the tumor cybrids under the back skin of the B6 mice (Fig. 1 B). There was a marked difference in the latency for tumor formation between P29mtB6 cybrids and the cybrids with allogenic mtDNA. Although P29mtB6 formed tumors within 10 d of the inoculation, P29mtC3H took >25 d to form tumors, and P29mtNZB did not form them at all. Considering the 99.98% identity between the B6 and C3H mtDNA sequences, it was conceivable that the longer latent period of P29mtC3H was caused by the 0.02% sequence difference between the B6 and C3H mtDNAs. Meanwhile, it is plausible that the greater divergence between the NZB mtDNA and the B6 mtDNA (0.5% sequence difference between B6 and NZB) prevented tumor formation by the P29mtNZB cybrid. Moreover, the injection of a mixture of P29mtB6 and P29mtNZB resulted in primary tumors (Fig. 1 B, left) that consisted exclusively of P29mtB6 cells (Fig. 1 B, right). The slower tumor growth of the P29mtB6 and P29mtNZB mixture than of P29mtB6 alone may be attributable to the P29mtB6 and P29mtNZB mixture containing only half as many P29mtB6 cells as the P29mtB6-only inoculant. These results strongly suggest that the cybrids possessing allogenic mtDNA were selectively hindered from forming tumors in the host B6 mice.

To address the mechanism of this selective suppression of tumor formation, we performed the same experiment using B6mtNZB congenic mice as the hosts. They share the B6 nuclear DNA background but possess mtDNA that is exclusive to the NZB strain mice. When the B6mtNZB mice with NZB mtDNA were used as the hosts, P29mtNZB formed tumors, but P29mtB6 and P29mtC3H did not (Fig. 1 C), suggesting the self-tolerance of cells with syngenic mtDNA. Probably, mtDNA polymorphism might elicit host immune responses to selectively suppress the growth of the tumor cybrids possessing allogenic mtDNA.

Determination of the immune systems that suppress tumor formation

Cytotoxic T cells (CD8+ T cells) recognize an amino acid change caused by the A2766G and T2767C mutations in the gene encoding ND1 (NADH dehydrogenase subunit 1) of NZB mtDNA (I6A) and kill lymphocytes containing the NZB mtDNA in vitro (Loveland et al., 1990). Therefore, the selective suppression of tumor formation by P29mtNZB in B6 mice (Fig. 1 B) could have been caused by the acquired immune system, including CD8+ T cells. To test this possibility, we inoculated P29mtNZB into B6 β2m−/− mice, which lack CD8+ T cells because of a lack of MHC class I expression (Table S1). However, P29mtNZB did not form tumors in these mice (Fig. 2 A). The results were similar when we used the B6 Tap1−/− mice, another MHC class I–deficient model (Fig. 2 A). P29mtNZB was also rejected in the B6 Ahβ−/− mice, which are CD4+ T cell–deficient mice lacking MHC class II molecules (Fig. 2 B), and in the
Involvement of NK cells in the rejection of the tumor cybrids with allogenic mtDNA was confirmed by experiments in NKRed mice, which have a reduced number of NK cells (Fig. S3) as the result of i.p. injection of anti-NK1.1 antibody. Although tumor growth was very slow, P29mtNZB did form tumors in the B6 NKRed mice (Fig. 2 E). Similarly, P29mtB6 formed tumors in the B6mtNZB NK Red mice (Fig. 2 F). Furthermore, co-cultivation of B6-derived NK cells with the tumor cybrids in vitro showed that the NK cells were more cytotoxic to P29mtNZB than to P29mtB6 (Fig. 2 G). This cytotoxicity was significantly inhibited by the perforin inhibitor concanamycin A, but the anti-FasL antibody had no effect. These observations indicate that the cytotoxicity of B6-derived NK cells to P29mtNZB was partly dependent on perforin but independent of the Fas-FasL system (Fig. 2 G).

Although our data do not exclude possibilities other than direct killing of the tumor cybrids by NK cells, they support the idea that preferential killing of P29mtNZB by B6 NK

Figure 1. Effects of allogenic mtDNA on the expression of tumor phenotypes. (A) Experimental (left) and spontaneous (right) lung metastasis phenotypes of the tumor cybrids. Metastatic phenotypes were examined by inoculation of the tumor cybrids containing allogenic mtDNA derived from different strain mice into the tail veins or under the skin of B6 mice. (B) Tumor formation phenotype of the tumor cybrids. Total numbers of 5 × 10^6 tumor cybrids were inoculated under the skin of B6 mice (left). A mixture of P29mtB6 (2.5 × 10^6) and P29mtNZB (2.5 × 10^6) was also inoculated (left) to confirm the selective tumor formation of P29mtB6 (n = 6 for each group). Data are representative of two independent experiments (*, P < 0.005; **, P < 0.0005). Restriction enzyme (SphI) digestions of PCR products were performed to identify the mtDNA genotypes (right). After the digestion, NZB mtDNA produced 143- and 31-bp fragments, whereas B6 mtDNA remained 174-bp PCR products. Mixed cybrids represent a simple mixture of P29mtB6 and P29mtNZB. Tumors represent primary tumors formed by inoculation of the mixed cybrids. (A and B) Error bars represent SD. (C) Tumor formation phenotype of the tumor cybrids inoculated under the skin of the B6mtNZB mice. Data represent the mean ± SD (n = 6). Similar results were obtained in two independent experiments.

B6 recombination-activating gene–deficient (Rag2−/−) mice (T cell– and B cell–deficient mice; Fig. 2 C). Thus, the acquired immune system does not appear to be responsible for the rejection of the tumor cybrids with allogenic mtDNA (Fig. 1, B and C).

Surprisingly, the P29mtNZB cybrids could form tumors when used to inoculate B6 Rag2−/−/γc−/− mice, which are deficient in both RAG2 and the common cytokine receptor γc (Fig. 2 D). However, P29mtNZB recovered from the tumor masses formed in the B6 Rag2−/−/γc−/− mice did not make tumors in the B6 mice. This finding excluded the possibility that some revertants in the injected P29mtNZB population selectively grew in the B6 Rag2−/−/γc−/− mice. The B6 Rag2−/−/γc−/− mice are severe combined immunodeficiency models that have macrophages and granulocytes but no T cells, B cells, NK cells, or functional DCs (Table S1). Thus, the innate immune system, including NK cells and DCs, and not the acquired immune system, is responsible for the rejection of P29mtNZB in B6 mice.

Involvement of NK cells and DCs in the suppression of tumor formation

Involvement of NK cells in the rejection of the tumor cybrids with allogenic mtDNA was confirmed by experiments in NKRed mice, which have a reduced number of NK cells (Fig. S3) as the result of i.p. injection of anti-NK1.1 antibody. Although tumor growth was very slow, P29mtNZB did form tumors in the B6 NKRed mice (Fig. 2 E). Similarly, P29mtB6 formed tumors in the B6mtNZB NKRed mice (Fig. 2 F). Furthermore, co-cultivation of B6-derived NK cells with the tumor cybrids in vitro showed that the NK cells were more cytotoxic to P29mtNZB than to P29mtB6 (Fig. 2 G). This cytotoxicity was significantly inhibited by the perforin inhibitor concanamycin A, but the anti-FasL antibody had no effect. These observations indicate that the cytotoxicity of B6-derived NK cells to P29mtNZB was partly dependent on perforin but independent of the Fas-FasL system (Fig. 2 G). Although our data do not exclude possibilities other than direct killing of the tumor cybrids by NK cells, they support the idea that preferential killing of P29mtNZB by B6 NK
Figure 2. Determination of the immune system that suppresses tumor formation of the tumor cybrids containing allogenic mtDNA.

(A–F and H) The tumor cybrids were inoculated under the skin of B6 β2m−/− and B6 Tap1−/− mice (A), B6 Abβ−/− mice (B), B6 Rag2−/− mice (C), B6 Rag2−/−/yc−/− mice (D), B6 NKred mice (E), B6mtNZB NKred mice (F), and B6 Myd88−/− and B6 Myd88−/−/NKred mice (H) to study the involvement of the immune system and immune cells in the suppression of tumor formation (n = 6). Similar results were obtained in two independent experiments. Error bars represent SD. (G) Contribution of perforin but not FasL in preferential killing of P29mtNZB by NK cells derived from B6 Rag2−/− mice. Cytotoxic assay of B6 Rag2−/− splenocytes that had been stimulated with poly I:C in vivo for 20 h was tested against P29mtB6 or P29mtNZB at the indicated E/T ratios. An 8-h 51Cr releasing assay was performed in the presence or absence of 20 nM concanamycin A or 10 µg/ml anti-FasL antibody. In all experiments, spontaneous 51Cr release from the P29 cybrids was <15%. Data represent the mean ± SD of triplicate samples. Similar results were obtained in two independent experiments (*, P < 0.0005; **, P < 0.0001).
growth was very slow, P29mtNZB did form tumors in the B6 Myd88−/− mice (Fig. 2 H). Thus, MyD88-associated innate receptors such as TLRs played an important role in the rejection of P29mtNZB. We next used B6 Myd88−/− NKRed mice, which simultaneously lacked most NK cells and most functional TLRs, and found significant tumor growth of P29mtNZB (Fig. 2 H). These observations, together with the results shown in Fig. 2 D, suggested that NK cells and other innate immune cells that can mediate MyD88-dependent innate immune responses such as DCs are involved in suppressing the growth of the tumor cybrids containing allogenic mtDNA.

The involvement of the innate immune system in suppressing the growth of tumor cybrids containing allogenic mtDNA was supported by immunohistochemical analyses of the cell clusters that formed under the skin of the B6 mice 4–12 d after inoculation of P29mtB6 and P29mtNZB (Fig. 2 E and F). The slow growth of tumor cybrids with allogenic mtDNA in the NKRed mice (Fig. 2 E and F) could be caused by the involvement of innate immune cells other than NK cells in the rejection of the tumor cybrids. We examined this possibility by inoculating P29mtNZB into the B6 Myd88−/− mice, which lack a functional innate immune system because they do not have functional Toll-like receptors (TLRs) except for TLR3 (Adachi et al., 1998). Although the tumor growth was very slow, P29mtNZB did form tumors in the B6 Myd88−/− mice (Fig. 2 H). Thus, MyD88-associated innate receptors such as TLRs played an important role in the rejection of P29mtNZB. We next used B6 Myd88−/− NKRed mice, which simultaneously lacked most NK cells and most functional TLRs, and found significant tumor growth of P29mtNZB (Fig. 2 H). These observations, together with the results shown in Fig. 2 D, suggested that NK cells and other innate immune cells that can mediate MyD88-dependent innate immune responses such as DCs are involved in suppressing the growth of the tumor cybrids containing allogenic mtDNA.

The involvement of the innate immune system in suppressing the growth of tumor cybrids containing allogenic mtDNA was supported by immunohistochemical analyses of the cell clusters that formed under the skin of the B6 mice 4–12 d after inoculation of P29mtB6 and P29mtNZB (Fig. 3 A). An increased infiltration of mononuclear cells, including DCs, into the cell clusters of P29mtNZB was observed 4 d after inoculation. Quantitative flow cytometric analyses of the infiltrated cells (Fig. 3 B) indicated that the infiltration of DCs into the P29mtNZB clusters was followed by an accumulation of NK cells on day 12. On the contrary, the transcription of IL-12p40, which is produced by DCs and encodes a product important for NK cell activation (Yu et al., 2001; Miller et al., 2003), was detectable in both P29mtNZB and P29mtB6 cell clusters (Fig. 3 C), suggesting that the s.c. inoculation with the cybrids enhanced IL-12 production independent of the mtDNA allogenicity. The injection of the cybrids might cause the release of mitochondria-derived damage-associated molecular patterns, which induce damage-dependent inflammation (Rabiet, et al., 2005,
whereas ESmtNZB but not ESmtB6 formed tumors in the B6mtNZB mice (Fig. 4 C). Moreover, ESmtNZB did not form tumors in the B6 β2m−/− mice (Fig. 4 D) but did form tumors in the B6 NKRed mice (Fig. 4 E), which suggests the involvement of NK cells in the rejection of the ES cybrids. Therefore, even when tissues are reconstructed from stem cells, they could be rejected if they possess mtDNA with mutations that trigger the innate immune system.

Using our complete mtDNA replacement technology (Ishikawa et al., 2008a,b), this study uncovered novel roles of mtDNA and innate immunity in tumor suppression. We showed that the host innate immune system cannot recognize tumor and ES cells carrying a syngenic nuclear DNA and a syngenic mtDNA but can recognize tumor and ES cells carrying a syngenic nuclear DNA and an allogenic mtDNA without priming. Although the acquired immune system, including effector CD8+ T cells, can be elicited in the case of repeated immunization (Loveland et al., 1990), innate recognition of tumor cells and ES cells with allogenic mtDNA should be a focus of our attention in the context of interactions between the hosts and tumor cells and between the hosts and grafts differentiated from somatic cells. In fact, graft rejection was reported in the renal transplants with allogenic mtDNA of swine (Hanekamp et al., 2009). Even the mtDNA of iPS cells might accumulate certain somatic mutations during the aging of the donor, and it could be allogenic mtDNA that influences host–donor compatibility.

Many questions remain about the mechanism of the innate recognition of the cells with allogenic mtDNA. For example, it is not known which products of the allogenic mtDNA are responsible for its innate recognition or which molecules sense the allogenicity of the mtDNA. Our experiments of inoculation of the mixture of P29mtB6 and P29mtNZB demonstrated selective elimination of P29mtNZB from the same cell clusters in B6 mice (Fig. 1 B), strongly suggesting that the P29mtNZB cybrids were eliminated
and the P29mtB6 cybrids persisted in the same cytokine environment. Thus, the immunological discrimination of mtDNA allogenicity by effector cells is an important mechanism for eliminating foreign cells. Collectively, with the findings that B6-derived NK cells were more cytotoxic to P29mtNZB than to P29mtB6 (Fig. 2 G) and that NK cells were detectable in the cell clusters that formed after cybrid inoculation (Fig. 3 B), NK cells seem to be important for recognizing mtDNA allogenicity. Thus, how the NK cells recognize cells with allogenic mtDNA is another critical question. It is also important to elucidate whether allogenic mtDNA recognition also occurs in vivo under physiological conditions. Although the precise mechanism by which the innate immune system, including NK cells and DCs, recognizes cells containing allogenic mtDNA needs to be further investigated, this study provides potentially important information for the fields of tumor immunology and transplantation medicine.

MATERIALS AND METHODS

Cell lines and cell culture. Low metastatic P29 cells are originated from B6 mouse–derived Lewis lung carcinomas. B6G-2 ES cells are B6 mouse–derived ES cells obtained from the RIKEN BioResource Center (Shimizuoka, Japan). All of the cell except for ES cells and their cybrids were grown in normal medium (DME + 1 mM sodium pyruvate + 50 µg/ml uridine + 10% FBS). B6G-2 ES cells and their cybrids were grown in ES medium (high glucose DME + 1 mM sodium pyruvate + 50 µg/ml uridine + 15% FBS + 0.1 mM of nonessential amino acid + 0.1 mM 2-mercaptoethanol + 1,000 U/ml leukemia inhibitory factor [Invitrogen]).

Isolation of the cybrids. As nuclear donors and mtDNA recipients for isolation of the tumor cybrids, we used pP29 cells (Fig. S1 A). We also used ES cells pretreated with 1 µg/ml R6G (rhodamine 6G) for 48 h to eliminate their endogenous mitochondria and mtDNA as nuclear donors and mtDNA recipients for isolation of the ES cybrids (Fig. S1 B). As mtDNA donors, we used B82 cybrids (B82mtB6, B82mtC3H, and B82mtNZB; see Fig. S1) because they express HAT-sensitive phenotypes and can be excluded in the presence of HAT (Ishikawa et al., 2008a). Moreover, B82 cybrids share the C3H strain nuclear DNA background and thus can exclude the possibility of participation of other cytoplasmic materials than mtDNA and its products in the rejection of the cybrids. B82mtB6, B82mtC3H, and B82mtNZB cybrids were isolated by the fusion of the pB82 cells with the platelets from B6, B6mtC3H, and B6mtNZB mice, which possess the B6-strain nuclear DNA background but possess mtDNA from B6, C3H, and NZB strain mice, respectively. Encapsulation of the B82 cybrids was performed by their pretreatment with 10 µg/ml covalymycin B for 10 min and centrifugation at 22,500 g for 30 min. Resultant cytoplasts were fused with pP29 cells or with R6G-pretreated B6G-2 ES cells by polyethylene glycol. Unfused pP29 cells or R6G-pretreated ES cells failed to grow as a result of the absence of mtDNA or mitochondria, and unencapsulated B82 cybrids failed to grow by the HAT selection.

Genotyping of mtDNA. The mtDNA genotypes of the isolated cybrids were confirmed by KFEP analysis of the PCR products because there are strain-specific polymorphisms: the G9461T mutation for B6 mtDNA, the G9348A mutation for C3H mtDNA, and the A2766G/T2767C mutations for NZB mtDNA. To identify the B6 mtDNA, the nucleotide sequences from nucleotide position 9434 to 9460 (5' - TCTCTGAATACCC-CAGAAGAGTATGAT-3' [lowercase indicates the mismatch site]) and nucleotide position 9,584 to 9,588 (5' - GTGCAATCCGGATCATATG-GATTTGC-3') were used as primers, and the PCR products were digested by Thr1111. For recognition of the NZB mtDNA, the nucleotide sequences from nucleotide position 2736 to 2765 (5' - ATCTCCGCTCCA- ATAGTGGCTTTATAGATGAT-3' [lowercase indicate mismatch sites]) and nucleotide position 2909 to 2889 (5' - TATGGGCTGCTGAAATGTT- TTG-3') were used as primers, and the PCR products were digested by SphI.

Biochemical measurement of respiratory enzyme activities. Cells in log-phase growth were harvested, and the mitochondrial respiratory function was examined as described previously (Miyabayashi et al., 1989).

Mouse strains and crosses. Mice of the inbred strain B6 and NZB were obtained from CLEA Japan, Inc. and the National Institute of Genetics (Shizuoka, Japan), respectively. Using these strains, we obtained B6mtNZB mice by repeated backcrosses (female progenies [NZB female × B6 male] × B6 male) >20 times. NZB mtDNA provided normal respiratory function in the cybrids with B6 nuclear DNA (Fig. S2 B). Thus, they possess intact nuclear genome from the inbred B6 strain and intact mitochondrial genome from NZB strain. The immunodeficient B2m−/−, A bg−/−, and Rag2−/− mice were obtained from Taconic. The TgI−/+ mice were purchased from The Jackson Laboratory. The Rag2−/−/γ−/− mice were provided by S. Koyasu, and S. Nagai (Kojo University School of Medicine, Shimo-ku, Tokyo, Japan). The Myd88−/− mice were obtained from T. Kashi (RIKEN Research Center for Allergy and Immunology, Tsurumiku, Yokohama City, Japan). All of these immunodeficient mice share B6 nuclear DNA background. To obtain NKRed, B6mtNZB NKRed, and Myd88−/− NKRed mice, each mouse received repeated i.p. injection of anti-NK1.1 antibody (clone PK136; Acros Antibodies GmbH) from 1 wk after the birth (10 µg/wk for 1 wk old, 20 µg/wk for 2 wk old, 50 µg/wk for 3 and 4 wk old, and 100 µg/wk for >5 wk old). Experiments were performed according to the guidelines for animal use and experimentation of the University of Tsukuba and the International Medical Center of Japan.

Assays of metastatic potentials and tumor formation. For testing experimental metastasis, 5 × 10⁵ cells in 100 µl PBS were injected into the tail vein of 6-wk-old male B6 mice. The mice were sacrificed 18 d later, and their lungs were removed, and then parietal nodules were counted. In an experimental metastasis model, cells are intravenously inoculated. Thus, this model bypasses the initial steps of the metastatic cascade, including primary tumor growth, invasion, and intravasation, and has been termed experimental metastasis to differentiate it from spontaneous metastasis (Cricuolo et al., 2005). To examine spontaneous metastasis and tumor formation, 5 × 10⁶ cells in 100 µl PBS were injected s.c. into the back of 6-wk-old male mice. The recipient mice were sacrificed when the tumor volume reached 5,000 mm³, and their lungs were removed, and then parietal nodules were counted. In a spontaneous metastasis model, all of the steps of the metastatic cascade from primary tumor growth to secondary growth in distant organs were traversed.

Histochemical analyses. Removed cell clusters were either fixed in 10% formalin for hematoxylin and eosin (H&E) staining or immediately frozen in the Tissue–Tek OCT compound (Sakura Finetek) for immunohistochemical analyses. Frozen cell clusters were sliced into 10-µm-thick cryosections and fixed in cold acetone. Anti-CD11c antibodies (BD) were used as primary antibodies and then visualized by the HRP Detection kit (BD).

Cytotoxic assay. A ⁵¹Cr release assay was performed to examine cytotoxic activity of cells (Mazumder et al., 1983). For preparation of polyinosinic:polycytidylic acid (poly I:C)–activated NK cells, 100 µg of poly I:C (P-0913; Sigma-Aldrich) was i.p. injected into B6 Rag2−/− mice, and spleen cells were isolated 20 h after the injection. The target cells (P29mtB6 and P29mtNZB) were labeled with ⁵¹Cr (3,700 KBq/10⁶ cells) for 30 min at 37°C and used as target cells in the killer assay. After removing the excess ⁵¹Cr by washing cells with the complete medium, the target cells were plated in V-bottom 96-well plates at 10⁴ cells/well and mixed with effector cells in a final volume of 200 µl. After 8 h of incubation, cell-free supernatants were collected, and radio activities were measured in a gamma counter. In some
experiments, the effector cells were pretreated with 20 nM concanavalin A (Wako Pure Chemicals) for 2 h to inactivate perforin (Kayagaki et al., 1999), or anti-mFas, monoclonal antibody (MFL2) was added to a final concentration of 10 μg/ml at the start of the cytotoxic assay.

RT-PCR analyses. Total RNA was prepared from cell clusters, and RT-PCR analyses were performed using the following primers: 5′-CTGCCATCATGTCGTCGCTCACAAG-3′ (sense) and 5′-ACTCCTCATAGTGCCTTTGCCCGAGC-3′ (antisense) for IL-12p40, 5′-CATCACCTTCTGTGCCTTCTTCCG-3′ (antisense) for IL-15, 5′-GGCTGCTACATTTTGCGGCTTGGTCCAG-3′ (sense) and 5′-GAAGTTTCTCTGCCAGCGACCTTTGG-3′ (antisense) for IL-18, and 5′-TTCTTTGTCAAGCTTCCTTGGTGTTG-3′ (sense) and 5′-GATGGCTACGTCATTGAGGCTTGCTGG-3′ (antisense) for β-actin.

Statistical analysis. The data were analyzed with a Student’s t test. All values are the mean ± SD, and values with P < 0.05 were considered significant.

Online supplemental material. Fig. S1 is the scheme explaining how to generate the mtDNA-exchanged cybrids. Fig. S2 shows the mtDNA genotypes and respiratory phenotypes of the cybrids. Fig. S3 shows efficient depletion of NK cells by injecting anti-NK1.1 antibody PK136. Table S1 explains the genetic and immune characteristics of the recipient genotypes and respiratory phenotypes of the cybrids. Fig. S3 shows efficient depletion of NK cells by injecting anti-NK1.1 antibody PK136. Table S1 explains the genetic and immune characteristics of the recipient genotypes and respiratory phenotypes of the cybrids. Fig. S3 shows efficient depletion of NK cells by injecting anti-NK1.1 antibody PK136. Table S1 explains the genetic and immune characteristics of the recipient genotypes and respiratory phenotypes of the cybrids.
