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https://hdl.handle.net/2324/26620

出版情報：Journal of Virology. 84 (10), pp.5360-5367, 2010-05-01. American Society for Microbiology
バージョン：
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Measles Virus-Induced Immunosuppression in SLAM Knock-In Mice

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Running title: MV-INDUCED IMMUNOSUPPRESSION IN SLAM KNOCK-IN MICE

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Word count for abstract: 229 words

Word count for body: 3737 words

ABSTRACT

Measles virus (MV) causes transient severe immunosuppression in patients, which may lead to secondary viral and bacterial infections largely accounting for measles-related morbidity and mortality. MV is known to infect immune cells by using the human signaling lymphocyte activation molecule (SLAM, also called CD150) as a cellular receptor, but the mechanism by which MV causes immunosuppression is not well understood. We show that MV infection of SLAM knock-in mice, in which the V domain of mouse SLAM was replaced by the V domain of human SLAM, crossed with α/β-interferon receptor knock-out mice reproduced many immunological alterations observed in human patients. These included lymphopenia, inhibition of T cell proliferation and antibody production, increased production of the Th2 cytokine interleukin (IL)-4 and the immunosuppressive cytokine IL-10, and suppression of contact hypersensitivity. Gross redistribution of lymphocytes among lymphoid tissues was not apparent in infected mice, nor was an increase of regulatory T cells. The numbers of lymphocytes in lymph nodes remained almost unchanged after MV infection despite enhanced apoptosis, suggesting that lymph nodes were replenished with lymphocytes from the peripheral blood, which may have contributed to the observed lymphopenia in the spleen. Blocking of IL-10 using an anti-IL10 receptor antibody ameliorated suppression of contact hypersensitivity in infected mice. These results indicate that SLAM knock-in mice lacking the expression of α/β-interferon receptor serve as a useful small animal model to elucidate MV-induced immunosuppression.

INTRODUCTION

Measles virus (MV), a member of the *Morbillivirus* genus in the *Paramyxoviridae* family, causes an acute febrile disease with a generalized skin rash, accompanied with transient immunosuppression, which may lead to secondary viral and bacterial infections largely accounting for measles-related morbidity and mortality (8). von Pirquet first made a scientific observation of virus-induced immunosuppression when he showed that children who had been positive for the tuberculin skin test failed to mount a response to tuberculin during the course of measles (35). Additionally, children with measles were found to show a significantly reduced antibody response to immunization with the H and O antigens of *Salmonella typhi* (37). MV infection is also characterized by lymphopenia, suppression of mitogen-induced and antigen-specific lymphocyte proliferation *ex vivo*, and cytokine imbalance skewed towards a Th2 response (8, 13, 26). The exact mechanism underlying these immunological alterations and the extent to which they are related to immunosuppression *in vivo* are not well understood.

MV predominantly infects immune cells such as lymphocytes, dendritic cells (DCs) and macrophages by using the human signaling lymphocyte activation molecule (SLAM, also called CD150) as a receptor (4, 33, 38). Several groups have produced SLAM transgenic mice, using various promoters, as small animal models to study MV pathogenesis (11, 12, 27, 31, 36). To facilitate MV replication, these transgenic mice are made defective in α/β-interferon (IFN) signaling (12, 31, 36) or used when they are newborn (11, 27).

Recently, we reported that SLAM knock-in mice in which the V domain of mouse SLAM was replaced by the V domain of human SLAM efficiently support MV replication in lymphoid tissues when crossed with mice lacking the α/β-IFN receptor (IFNAR) (22). Splenocytes from these mice infected with MV demonstrated suppression of proliferative responses to concanavalin A (22).

In this study, we further examined MV-induced immunological alterations in IFNAR$^{-/-}$ SLAM knock-in mice (hereafter referred to as KI mice). We found that enhanced apoptosis of lymphocytes and increased production of the immunosuppressive cytokine interleukin (IL)-10 may be responsible for some of the alterations.

MATERIALS AND METHODS

Mice. Generation of KI mice (SLAM knock-in mice crossed with IFNAR$^{-/-}$ mice) has been described previously (22). All mice used were 8–10 weeks of age, and animal experiments were reviewed by the Institutional Committee of Ethics on Animal Experiments, and carried out according to the Guideline for Animal Experiments of the Faculty of Medicine, Kyushu University, Japan.

Viruses and cells. MV used throughout in this study was a recombinant virus (IC323) based on the wild-type IC-B strain (32), which has been shown to be virulent in monkeys (4, 32). The virus was grown on Vero/hSLAM cells (23) and virus titers were determined by plaque assay on Vero/hSLAM cells. For some experiments, IC-323 expressing enhanced

green fluorescence protein (EGFP) was used to monitor infection levels. Splenocytes and lymph node cells isolated from mice were cultured in RPMI 1640 medium (MP Biomedicals LLC) containing 10% fetal bovine serum, 100 μM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. DCs were derived from bone marrow (BM) cells. Briefly, BM cells were prepared from mice, and red blood cells were removed by treatment with 1.66% NH_4Cl. The BM cells were then suspended with RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 μM 2-mercaptoethanol, 10 ng/ml recombinant mouse granulocyte-macrophage colony-stimulating factor (Peprotech), 100 U/ml penicillin and 100 μg/ml streptomycin. Approximately 1 x 10^6 BM cells were seeded into each well of a 24-well plate. Non-adherent cells were discarded, and the remaining cells were fed with fresh medium. Following 6 days of incubation, non-adherent and loosely adherent cells were harvested and used as immature DCs. Mononuclear cells were prepared from the liver by Percoll density gradient (GE Healthcare), and those from the lung by collagenase treatment of the tissue.

MV Infection. For *in vivo* infection, 500 μl of the solution containing 1 x 10^6 pfu of MV was injected into the peritoneal cavity of each mouse. For *in vitro* experiments, DCs (stimulated with 100 ng/ml lipopolysaccharide for 16 h) were infected with MV at a multiplicity of infection (MOI) of 1. After 1 h of incubation, the cells were washed twice with phosphate-buffered saline (PBS) and cultured in medium for the indicated time periods.

Flow cytometry. Single-cell suspensions of splenocytes and lymph node cells were

prepared from mice, and incubated with anti-mouse CD16/32 monoclonal antibody (MAb, 2.4G2; Pharmingen) to block nonspecific immunoglobulin (Ig) G binding to Fc receptor. The following MAbs were used to detect the respective cell surface molecules: fluorescein isothiocyanate (FITC)-conjugated anti-Thy1.2 (53.2.1; eBioscience), FITC-conjugated anti-CD4 (GK1.5; eBioscience), FITC-conjugated anti-B220 (RA3-6B2; eBioscience), and anti-FoxP3 (3G3; Abcam). Phycoerythrin-conjugated secondary antibody was used following staining with anti-FoxP3 antibody. For the detection of apoptosis, lymph node cells were stained with FITC-conjugated Annexin V and propidium iodide (AbD serotec). Flow cytometric analysis was performed on a FACSCalibur instrument (BD Biosciences).

Cell proliferation assay. Splenocytes and lymph node cells were isolated from infected mice. $CD4^+$ T cells were prepared using a negative-selection column (Miltenyi Biotec), stimulated in plates coated with 3 μg/ml of anti-CD3 MAb (145.2C11; BD Pharmingen) for 48 h, and pulsed with 1 μCi of ^{3}H-thymidine for the last 6 h of incubation. The cells were then harvested on glass fiber filters and the incorporated ^{3}H-thymidine was measured using a liquid scintillation counter.

Measurement of antibody production. Mice were infected with MV or left uninfected, and at 5 days post infection (p.i.) individual mice were immunized intraperitoneally (i.p.) with 100 μg ovalbumin (OVA; Sigma) emulsified in Freund's complete adjuvant (Sigma). To determine the levels of anti-OVA antibodies in sera collected 2 weeks after immunization, the 96-well plates were coated with 10 μg/ml OVA (200 μl/well) and incubated overnight at 4°C. After being washed with PBS, the plates were blocked with 5%

non-fat milk at room temperature for 2 h. Serially diluted sera were added to the wells of plates (100 μl/well) and incubated at room temperature for 1 h. After washing, horseradish peroxidase-labeled anti-mouse IgG (Bio-Rad) was added and incubated at room temperature for 1 h, and peroxidase substrate (2,2'-Azino-bis 3-Ethylbenthiazoline-6-sulfonic acid tablets; Sigma) were added to each well. After a 30-min incubation, absorbance at a wavelength of 405 nm was determined with a microplate reader (Bio-Rad).

Measurement of cytokine production. CD4+ T cells were isolated from the spleen of infected mice using a negative-selection column, and stimulated in plates coated with 3 μg/ml of anti-CD3 MAb for 48 h. The culture supernatants were collected and analyzed for the presence of IL-4, IFN-γ, IL-10 and transforming growth factor (TGF)-β using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) according to the manufacturer's instructions. The culture supernatants of infected and uninfected DCs were collected at 24 and 48 h p.i. and analyzed for IL-10 and IL-12 using ELISA kits (eBioscience) according to the manufacturer's instructions.

Quantitative real-time reverse transcription (RT)-PCR. Total RNA was isolated from cells with Trizol (Invitrogen), and 2 μg of the RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) and random primers (Takara) after treatment with DNase I (Promega). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Takara) and a Light Cycler 1.5 (Roche). The following primers were used: 5'-ATGCTCCTAGAGCTGCGGACT-3' (forward) and

5’-CCTGCATTAAGGAGTCGGTTAG-3’ (reverse) for IL-10; 5’-CAGAAGCTAA CCATCTCCTGGTTTG-3’ (forward) and 5’-CCGGAGTAATTTGGTGCTCCACAC-3’ (reverse) for IL-12p40. All data were normalized with the expression level of β-actin.

Generation of anti-mouse IL-10 receptor (IL-10R) MAb. To generate anti-mouse IL-10R MAb, rats were immunized with 293 T cells transfected with cDNA encoding mouse IL-10R. Hybridoma cells producing anti-mouse IL-10R antibodies were screened for their ability to bind to 293T cells expressing mouse IL-10R. Antibodies were further selected for their ability to block IL-10-dependent proliferation of mouse MC/9 cells (34). Anti-IL-10R MAb was purified by ammonium sulfate fractionation and protein G affinity chromatography.

Assay for contact hypersensitivity. Contact hypersensitivity to 2,4-dinitro-1-fluorobenzene (DNFB; Sigma) was determined as described previously (20, 28) with modifications. Briefly, 50 μl of 0.5% DNFB in ethanol was applied to the shaved ventral skin of mice (day 0). At day 1, sensitized mice were infected with MV or left uninfected. At day 6, 10 μl of 1% DNFB in olive oil was applied to both sides of the right ear and olive oil alone to the left ear. For some experiments, 500 μg of rat anti-mouse IL-10R MAb or control rat IgG was injected i.p. into individual mice at day 4. Either the clone YL03.1B1.3 (kindly provided by Schering-Plough Biopharma) or our own preparation (described above) was used as an anti-mouse IL-10R MAb. The ear thickness of mice was measured before and after challenge by an examiner who was blinded. Ear swelling was calculated as ([E-E0] right ear) – ([E-E0] left ear), where E and E0 represent

the ear thickness after and before challenge, respectively. Ears treated with DFNB were also examined histopathologically.

RESULTS

MV-induced immunosuppression in KI mice. We first examined whether MV infection affects lymphocyte counts in KI mice as is the case in humans (8). Spleens were obtained from KI mice at various time points after MV infection, and the total numbers of splenocytes (lymphocytes and monocytes/macrophages) were determined. We found that the numbers of splenocytes did not change significantly until 14 days p.i. However, the proportions of different cell types as examined by flow cytometry analysis were varied. At 5 days p.i., the proportion of T cells ($Thy1.2^+$ cells) in KI mice decreased to less than one third of that in uninfected KI mice, whereas it was hardly altered in unsusceptible $IFNAR^{-/-}$ mice (hereafter referred to as wild-type (WT) mice) (Fig. 1A). In KI mice, the absolute numbers of T cells did not change significantly at 2 days p.i., but decreased at 5 days p.i. and returned to its original level at 14 days p.i. (Fig. 1B). The results indicate that there was a transient decrease in the proportion as well as the absolute number of T cells in the spleens of MV-infected KI mice. On the other hand, the absolute numbers of B cells ($B220^+$ cells) remained more or less constant in those mice (Fig. 1B).

Next, we examined whether MV infection affects T cell proliferation in these mice. KI and WT mice were infected with MV or left uninfected. $CD4^+$ T cells were isolated from the

spleens of those mice at 5 days p.i. and stimulated with anti-CD3 antibody. Proliferation of $CD4^+$ T cells was significantly suppressed in infected KI mice, but not in infected WT mice (Fig. 2A), consistent with our previous observations at 4 days p.i. obtained using a different assay (22). Similar to the decrease in splenic T cells, suppression of $CD4^+$ T cell proliferation was observed only at 5 days p.i. (Fig. 2B). Although splenic B cell counts were not significantly altered (Fig. 1B), production of anti-OVA antibody was suppressed in MV-infected KI mice (Fig. 2C).

We also examined the effect of MV infection on the contact hypersensitivity response in mice. KI mice were sensitized with DNFB, infected with MV on the next day or left uninfected, and then challenged on their ears with DNFB at 5 days p.i. Ear thickness of MV-infected KI mice at 48 h after the challenge was significantly suppressed compared with that of uninfected mice (Fig. 3A). Although infiltration of mononuclear cells varied even among the same group of mice, it tended to be diminished in the ears of infected mice (Fig. 3B).

Profiles of cytokines produced by $CD4^+$ T cells and DCs from MV-infected mice. It has been reported that cytokine production is altered in measles patients (9, 19, 39). $CD4^+$ T cells were isolated from the spleens of KI and WT mice at 5, 8 and 15 days p.i., and stimulated with anti-CD3 antibody for 48 h. The supernatants of the stimulated $CD4^+$ T cells were examined by ELISA for a Th1-type cytokine IFN-γ, a Th2-type cytokine IL-4 and immunosuppressive cytokines IL-10 and TGF-β. $CD4^+$ T cells from KI mice at 5 and 8 days p.i. produced similar levels of IFN-γ to those from similarly infected WT mice, but

produced much higher levels of IL-4 (Fig. 4A), indicating that MV infection causes a Th2 shift of the T cell response in susceptible mice. Furthermore, production of IL-10, but not TGF-β, by $CD4^+$ T cells was greatly increased in KI mice at 5 and 8 days p.i. compared with similarly infected WT mice (Fig. 4A). At 15 days p.i., differences were no longer found in cytokine productions between splenic $CD4^+$ T cells from WT and KI mice (Fig. 4A). Expression of IL-10 mRNA was also increased in lymph nodes obtained from infected KI mice at 5 and 8 days p.i. compared with lymph nodes from infected WT mice (Fig. 4B), indicating that production of IL-10 was also increased *in vivo* after MV infection. However, there was no difference in IL-10 mRNA levels between lymph nodes from two groups of mice at 14 days p.i. (Fig. 4B). BM-derived DCs were prepared from WT and KI mice, and infected with MV *in vitro*. Only ~1% of DCs prepared from KI mice were infected as determined by using the MV expressing EGFP, while no DCs from WT mice were infected (data not shown). There were no apparent differences in IL-10 and IL-12 productions at the protein level between DCs from WT and KI mice at 24 h p.i. (Fig. 4C) and 48 h p.i. (data not shown). However, DCs from KI mice produced increased levels of IL-10 mRNA, but decreased levels of IL-12 mRNA, compared with those from WT mice (Fig. 4C). All these results indicate that MV infection of KI mice reproduce many immunological abnormalities observed in human patients.

Enhanced apoptosis but no increase in regulatory T cells in MV-infected mice. Under the experimental conditions used, less than 1% of cells in the spleen and lymph nodes from MV-infected KI mice were infected with MV at any time point, as determined

using a fluorescent MV or infectious center assay (22). Therefore, direct infection *per se* cannot account for the observed immunological abnormalities. To gain an insight into the mechanism of the decrease in splenic T cells, the spleens, lungs, livers and inguinal lymph nodes were recovered from KI and WT mice at 5 days p.i., and the total numbers of cells in those organs were counted. In the case of the liver and lungs, mononuclear cells were further isolated and counted. The proportions of T and B cells were determined by flow cytometry to calculate the absolute numbers of the respective cell types. The total numbers of mononuclear cells were not significantly different in any organs between KI and WT mice (Fig. 5A). The absolute numbers of T cells were decreased in the spleen and lungs, but not in the liver and lymph nodes of KI mice, compared with those in the corresponding organs of WT mice (Fig. 5B). The results indicate that the gross redistribution of T cells within the body (from the spleen to other organs) alone cannot account for the decrease in splenic T cells in MV-infected KI mice. There was no significant difference in the absolute numbers of B cells in any organs between KI and WT mice (Fig. 5C).

It has been reported that MV infection causes apoptosis of infected as well as uninfected bystander cells (1, 6, 29). The proportion of cells undergoing apoptosis among the total cells was significantly increased at 5 and 8 days p.i. in lymph nodes from KI mice, but not in those from WT mice (Fig. 5D).

Regulatory T cells have been implicated in immunosuppression during MV infection (28, 39). We examined the proportion of $CD4^+FoxP3^+$ regulatory T cells among splenocytes and inguinal lymph node cells, but no increase in $CD4^+FoxP3^+$ cells was observed in KI mice

after MV infection (Fig. 5E).

Amelioration of MV-induced inhibition of the contact hypersensitivity response with anti-IL 10R antibody. As described above, cells from MV-infected KI mice produced higher amounts of the immunosuppressive cytokine IL-10, compared with control mice. To determine whether IL-10 is involved in MV-induced immunosuppression, the effect of anti-IL-10R antibody on the contact hypersensitivity response was examined. The administration of anti-IL-10R antibody at 3 days p.i. (2 days before the challenge with DNFB) was included in the experimental protocol for the measurement of the contact hypersensitivity response. Due to treatment with anti-IL-10R antibody, ear swelling in MV-infected KI mice was restored to the same levels as that in uninfected mice (Fig. 6A). In contrast, the administration of anti-IL-10R antibody did not affect the number and proliferation of T cells in MV-infected KI mice (Fig. 6B and C).

DISCUSSION

In this study, we have shown that MV-infected KI mice reproduce many immunological alterations observed in human patients, including T lymphopenia, inhibition of T cell proliferation and antibody production, increased production of the Th2 cytokine IL-4 and the immunosuppressive cytokine IL-10, and suppression of contact hypersensitivity (8, 13, 26). Thus, KI mice serve as a useful small animal model to study MV-induced immunosuppression.

Although both T and B cells have been shown to express the human-type SLAM and be susceptible to MV in KI mice (22), only the decrease in splenic T cells, but not in B cells, was apparent after MV infection. A recent study also showed that in mink infected with canine distemper virus, another morbillivirus causing immunosuppression, T cells were depleted to a higher extent than B cells (21). The reason for the discrepancy between T and B cells in our KI mice is unknown at present, as we do not understand how T cells were reduced in the spleen. Infection-induced cell death cannot explain the decrease, as only a small percentage of cells were infected under the experimental conditions used (22). Redistribution of lymphocytes from the peripheral blood to lymphoid tissues was suspected to account for the observation, but gross change in lymphocyte distribution was not observed. However, it was notable that lymph node cells in KI mice exhibited enhanced apoptosis at 5 and 8 days p.i., even though the numbers of lymph node cells in KI mice remained almost the same as those in WT mice. The results suggest that lymph nodes in KI mice were replenished with lymphocytes from the peripheral blood to compensate for the loss of cells due to enhanced apoptosis. This may contribute at least partly to the observed lymphopenia in the spleen.

MV-infected KI mice exhibited inhibition of $CD4^+$ T cell proliferation in response to anti-CD3 antibody as well as antibody production to an antigen OVA after immunization. Since direct infection of lymphocytes or a decrease in cell numbers cannot explain these findings, there must be indirect mechanisms that cause functional impairment of lymphocytes presumably through surface contact or soluble factor-mediated means. In

humans, lymphopenia and suppression of T cell proliferation and antibody production last several weeks after infection (8), whereas these manifestations were apparent only around 5 days p.i. in MV-infected KI mice. It should be noted that in our mouse model, MV is first detected in lymph nodes at 2–5 days p.i., attains a peak in titer at 7 days p.i., and completely disappears at 11 days p.i. (22). The difference in the course of MV replication and/or host immune responses may be responsible for the different time course of immunological alterations between humans and KI mice.

During the past several years, $CD4^+FoxP3^+$ regulatory T cells have been shown to play an important role in the control of autoimmunity as well as antimicrobial immune responses (18, 25). Regulatory T cells were reported to be increased not only in measles patients (39) but also in MV-infected SLAM transgenic mice (28), suggesting that regulatory T cells are responsible for MV-induced immunosuppression. In the present study, we did not observe an increase in $CD4^+FoxP3^+$ T cells in the spleen and lymph nodes for 8 days after MV infection, during which immunological alterations were observed in mice. Thus, it is unlikely that immunological alterations in our model were caused by the increased activity of regulatory T cells. In the above study with SLAM transgenic mice, the percentages of $FoxP3^+$ regulatory T cells were examined at 11 or 13 days p.i. (28).

It is notable that $CD4^+$ T cells from MV-infected KI mice exhibited the Th2 shift of the T cell response and increased production of IL-10. Similar observations have been seen in humans (9, 19, 39), and can at least partly explain MV-induced immunosuppression. We examined the levels of IL-10 in the blood from MV-infected KI mice, but a significant

difference was not found when compared with uninfected mice (data not shown). Therefore, if IL-10 is involved in immunological alterations of KI mice, it may be acting locally, not systemically. The increase of IL-10 production was indeed detected in lymph node cells taken from MV-infected KI mice *ex vivo* and in KI mice-derived DCs after MV infection *in vitro*. Thus, both T cells, although not necessarily regulatory T cells, and DCs could be the source of IL-10. Recently, MV was shown to activate the serine and threonine kinase Raf-1 via the C-type lectin DC-SIGN, which subsequently leads to acetylation of the NF-κB subunit p65and increased production of IL10 mRNA (10). Furthermore, DCs from KI mice produced lower levels of IL-12 mRNA after MV infection than those from WT mice, consistent with previous studies (17, 30). The reduction in IL-12 may be a mechanism for the Th2 shift in MV-infected KI mice, although the exact mechanism by which MV infection leads to suppression of IL-12 production remains to be determined.

One of the most interesting observations in this study was that blocking of IL-10 using anti-IL10R antibody ameliorated suppression of contact hypersensitivity in infected KI mice. IL-10 can suppress cellular immune responses by modulating the function of T cells and DCs (16). IL-10 has been implicated in immunosuppression during persistent infection with lymphocytic choriomeningitis virus (LCMV) (3, 5). Unlike C57/BL6 mice, IL-10-deficient mice maintained robust effector T cell responses and rapidly eliminated LCMV. Administration of anti-IL10R antibody into C57/BL6 mice persistently infected with LCMV restored T cell function and eliminated viral infection. In our model, anti-IL-10R antibody only restored contact hypersensitivity, but not T lymphopenia and

inhibition of T cell proliferation. Thus, mechanisms other than IL-10-mediated signaling are also responsible for the immunosuppression observed in MV-infected KI mice.

Other mechanisms proposed include interactions of different MV proteins with cell surface proteins (13, 26). MV envelope proteins, hemagglutinin and fusion protein, have been shown to induce a surface-contact-mediated signaling, resulting in the disruption of Akt kinase activation and inhibition of T cell proliferation (2). The interaction of the nucleoprotein with Fcγ receptor II on antigen-presenting cells or with an unidentified nucleoprotein receptor on various types of cells has been shown to affect cell functions (14, 15, 24). MV-induced ceramide accumulation is also implicated in MV-induced immunosuppression by preventing actin cytoskeletal dynamics (7, 26). Our KI mice, in conjunction with recombinant MVs containing a variety of mutant MV proteins, have the potential to be a powerful tool in unraveling MV-induced immunosuppression mechanisms.

ACKNOWLEDGMENTS

We thank T. Iwaki and H. Kishimoto for invaluable comments and Schering-Plough Biopharma for providing rat anti-mouse IL-10R MAb YL03.1B1.3. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

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FIGURE LEGENDS

Figure 1. Lymphocyte counts in the spleens of MV-infected mice. (A) Single cell suspensions of splenocytes were prepared from uninfected WT and KI mice or from WT and KI mice 5 days after MV infection, stained with FITC-conjugated anti-Thy1.2 antibody and analyzed by flow cytometry. The data shown are representative of > 10 mice for each group. (B) Splenocytes were prepared from KI mice at indicated time points after MV infection and counted. Cells were stained with FITC-conjugated anti-Thy1.2 or anti-B220 antibody. The absolute numbers of T (left) and B cells (right) were calculated from total cell numbers and proportions of $Thy1.2^+$ and $B220^+$ cells, respectively. The value at each time point represents the mean ± the standard deviation of four mice, and the data shown are representative of three different experiments. ** $P < 0.01$, * $P < 0.05$ (significant differences based on Student's *t* test).

Figure 2. T cell proliferation and antibody production in MV-infected mice. (A) $CD4^+$ T cells were isolated from the spleens of uninfected WT and KI mice or from those of WT and KI mice 5 days after i.p. infection with MV. The cells (2×10^5 cells/well) were stimulated in plates coated with anti-CD3 antibody for 48 h and pulsed with ^{3}H-thymidine for the last 6 h of incubation. Thymidine uptake was measured in triplicate for each sample.

The value for each group represents the mean ± the standard deviation of four mice, and the data shown are representative of three different experiments. (B) $CD4^+$ T cells were isolated from the spleens of KI mice at indicated time points after MV infection. The cells were stimulated in plates coated with anti-CD3 antibody for 48 h and pulsed with ^{3}H-thymidine for the last 6 h. Thymidine uptake was measured in triplicate for each sample. The value at each time point represents the mean ± the standard deviation of four mice, and the data shown are representative of three different experiments. (C) KI mice were infected with MV or left uninfected, and immunized with OVA 5 days p.i. The levels of anti-OVA antibodies were determined 2 weeks after immunization. Symbols indicate individual mice. The data shown are representative of two different experiments. ** $P < 0.01$ (significant difference based on Student's *t* test).

Figure 3. Contact hypersensitivity in MV-infected KI mice. (A) KI mice were sensitized with DNFB and infected with MV or left uninfected the next day. At 5 days p.i., DNFB was applied to the ears of mice. Ear thickness was measured at 0 and 48 h after challenge. The value for each group represents the mean ± the standard deviation of five mice, and the data shown are representative of three different experiments. ** $P < 0.01$ (significant difference based on Student's *t* test). (B) Tissue sections of the ears (challenged right and unchallenged left ears) were prepared from uninfected and infected KI mice at 48 h after DNFB challenge and stained with hematoxylin and eosin. Bar, 100 μm.

Figure 4. Cytokine production in MV-infected mice. (A) $CD4^+$ T cells were isolated from the spleens of KI and WT mice 5, 8 and 15 days p.i., and stimulated with anti-CD3 antibody for 48 h. The levels of IL-4, IFN-γ, IL-10, and TGF-β in the culture supernatants were measured by ELISA. The value for each group represents the mean ± the standard deviation of three mice, and the data shown are representative of two different experiments. ** $P < 0.01$ (significant differences based on Student's *t* test). (B) Total RNA was extracted from lymph nodes of MV-infected KI and WT mice at indicated time points and analyzed for the expression of IL-10 mRNA by quantitative real-time RT-PCR. Data were normalized to the corresponding level of β-actin mRNA expression. The value at each time point represents the mean of three mice, and the data shown are representative of three different experiments. (C) DCs prepared from WT and KI mice were left uninfected (-) or infected with MV at an MOI of 1. The cells were cultured for 24 h, and the levels of IL-12 and IL-10 in the culture supernatants were measured by ELISA. The infected cells were also cultured for the indicated time periods, and the expression of IL-12p40 and IL-10 mRNAs were analyzed by quantitative real-time RT-PCR. The data shown are representative of three independent experiments.

Figure 5. Possible mechanisms of MV-induced immunological alterations in KI mice. (A–C) Numbers of total mononuclear cells (A), T cells (B) and B cells (C) in various organs from MV-infected mice. Single cell suspensions were prepared from the spleens, lungs, livers and inguinal lymph nodes of MV-infected WT (white bar) and KI (black bar)

mice at 5 days p.i. Total mononuclear cell numbers were counted, and T and B cell numbers were calculated from the proportions of $Thy1.2^+$ and $B220^+$ positive cells as examined by flow cytometry, respectively. The value for each organ represents the mean ± the standard deviation of three WT or KI mice, and the data shown are representative of two different experiments. ** $P < 0.01$, * $P < 0.05$ (significant differences based on Student's *t* test). (D) The lymph node cells were prepared from WT and KI mice at indicated times after MV infection, stained with Annexin V and propidium iodide and analyzed by flow cytometry. Proportions of Annexin V-positive, propidium iodide-negative cells are shown. The value at each time point represents the mean ± the standard deviation of four mice, and the data shown are representative of three different experiments. ** $P < 0.01$ (significant difference based on Student's *t* test). (E) Cells were prepared from the spleens and inguinal lymph nodes of WT (white bar) and KI (black bar) mice at indicated time points after MV infection, and the proportions of $CD4^+FoxP3^+$ cells among total splenocytes and lymph node cells were determined by flow cytometry. The value at each time point represents the mean ± the standard deviation of three mice, and the data shown are representative of two different experiments.

Figure 6. The effect of anti-IL-10R antibody on MV-induced immunological alterations in KI mice. (A) KI mice were sensitized with DNFB and infected with MV (+) or left uninfected (-) 24 h after sensitization. The mice were given anti-IL-10R MAb (+) or control rat IgG (-) at 3 days p.i., and challenged with DNFB 5 days p.i. Ear swelling was measured

48 h after challenge. Five to six mice were analyzed for each group, and the data shown are representative of four independent experiments. ** $P < 0.01$ (significant differences based on a *Student t* test). (B and C) Splenocytes were prepared 5 days p.i. from MV-infected or uninfected KI mice which had been given either anti-IL-10R MAb or control rat IgG at 0 and 3 days p.i. The numbers of total, $B220^+$ and $Thy1.2^+$ cells were determined as described in Fig. 1B (B). $CD4^+$ T cells were isolated from the splenocytes prepared as above, stimulated in plates coated with anti-CD3 antibody for 48 h, and pulsed with ^{3}H-thymidine for the last 6 h. ^{3}H-thymidine incorporation was measured in triplicate (C). The value for each group represents the mean ± the standard deviation of three mice, and the data shown are representative of three different experiments.

Figure 1

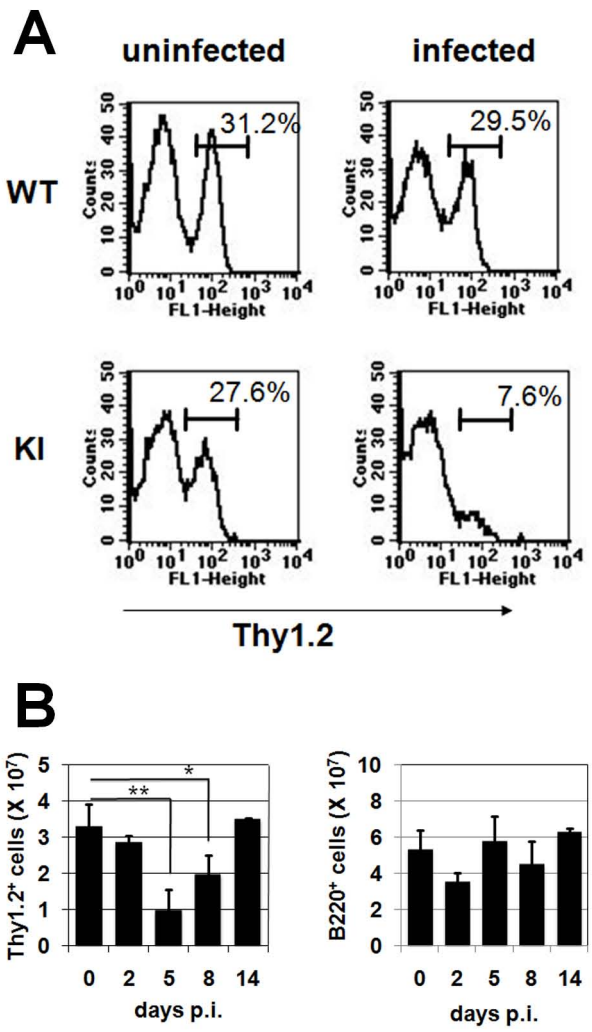

A
uninfected
infected
WT
KI
31.2%
29.5%
27.6%
7.6%
Counts
FL1-Height
Thy1.2
B
Thy1.2+ cells (X 10^7)
B220+ cells (X 10^7)
days p.i.
*
**

Figure 2

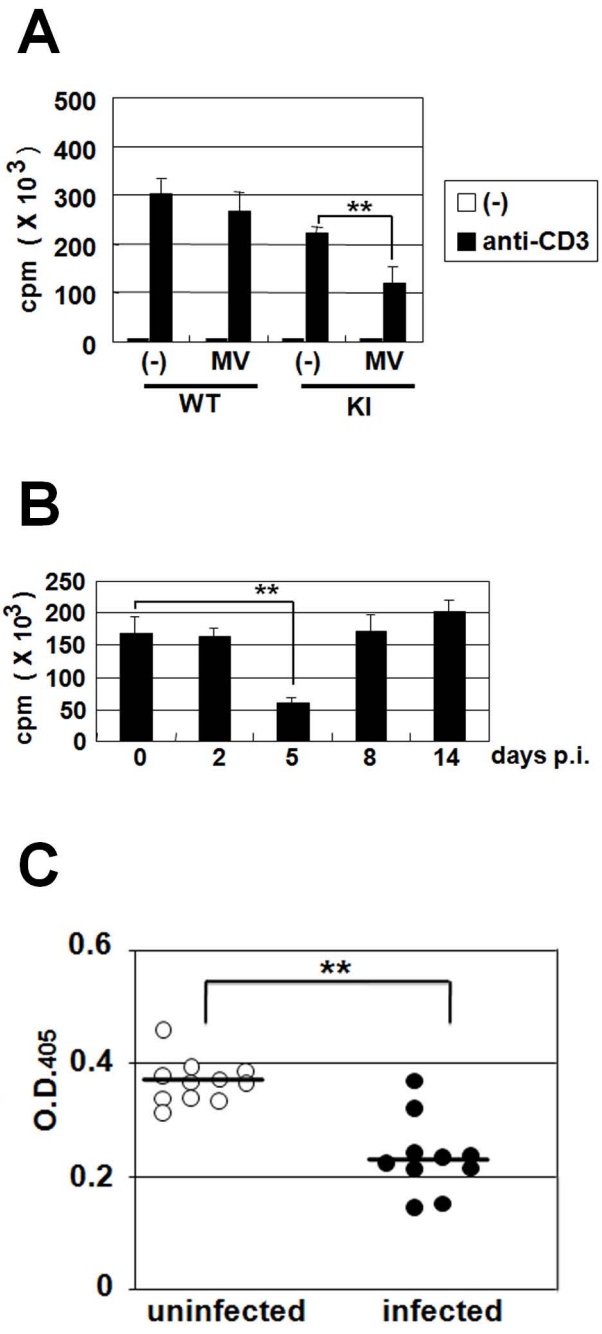

A
500
400
300
200
100
0
cpm (X 10^3)
**
(-)
anti-CD3
(-)
MV
(-)
MV
WT
KI
B
250
200
150
100
50
0
cpm (X 10^3)
**
0
2
5
8
14
days p.i.
C
0.6
0.4
0.2
0
O.D.405
**
uninfected
infected

Figure 3

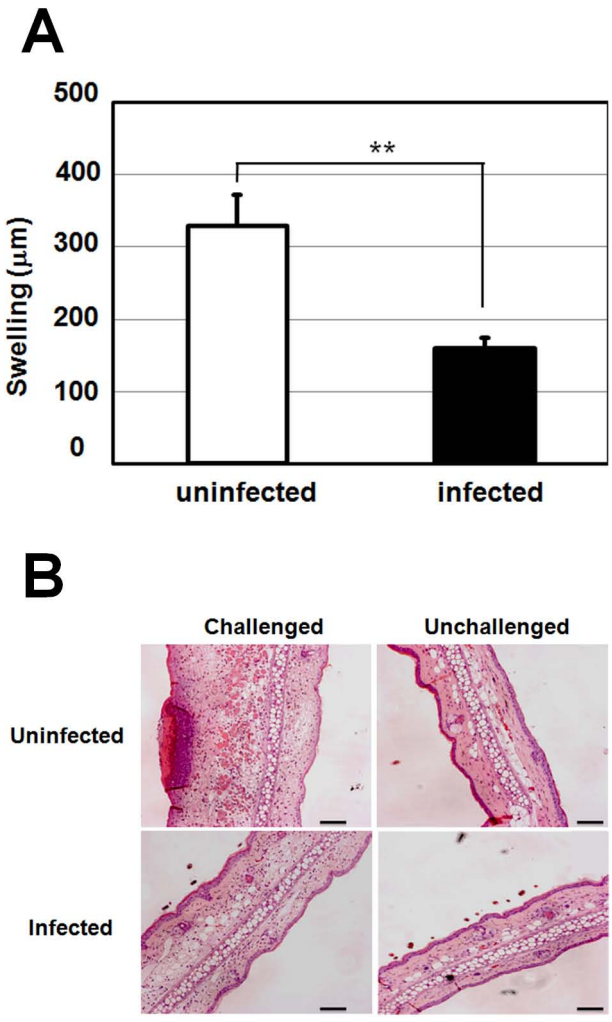

A
500
400
300
200
100
0
Swelling (μm)
**
uninfected
infected
B
Challenged
Unchallenged
Uninfected
Infected

Figure 4

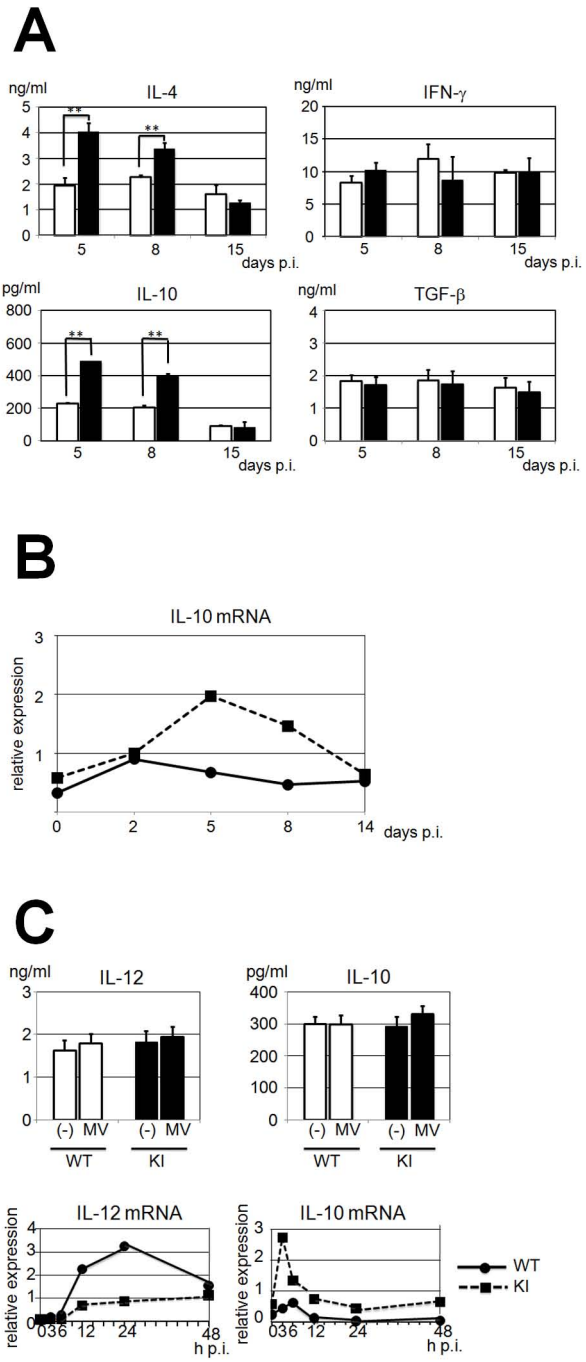

A
ng/ml
IL-4
**
**
5
8
15
days p.i.
ng/ml
IFN-γ
pg/ml
IL-10
ng/ml
TGF-β
B
IL-10 mRNA
relative expression
0
2
5
8
14
days p.i.
C
ng/ml
IL-12
pg/ml
IL-10
(-) MV
WT
KI
IL-12 mRNA
IL-10 mRNA
relative expression
h p.i.
WT
KI

Figure 5

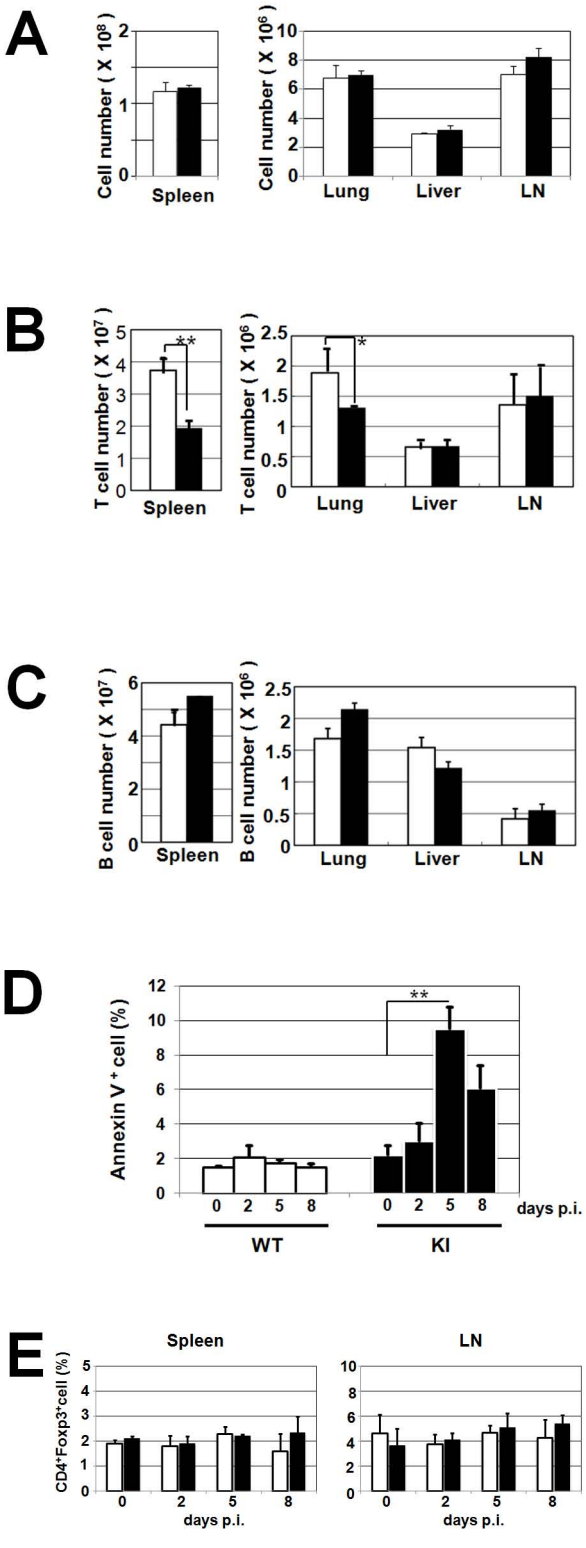
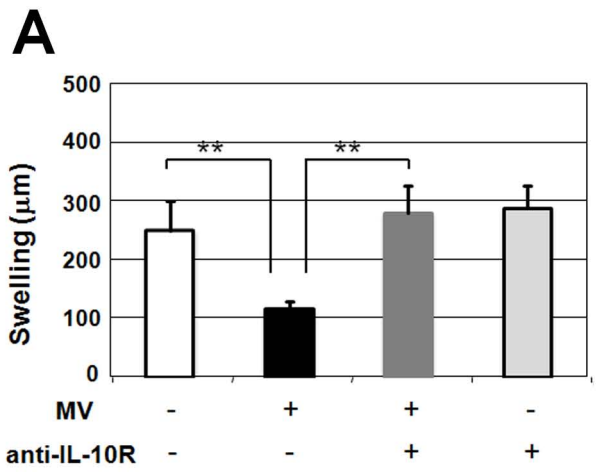
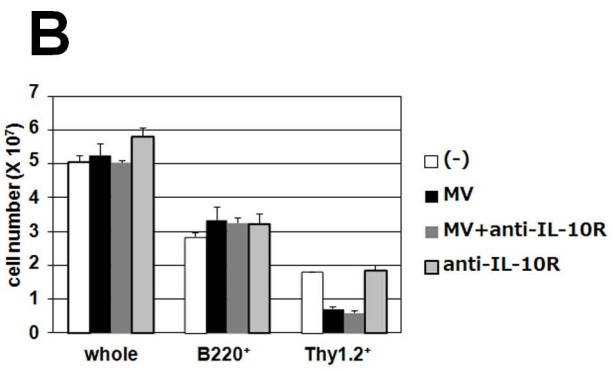
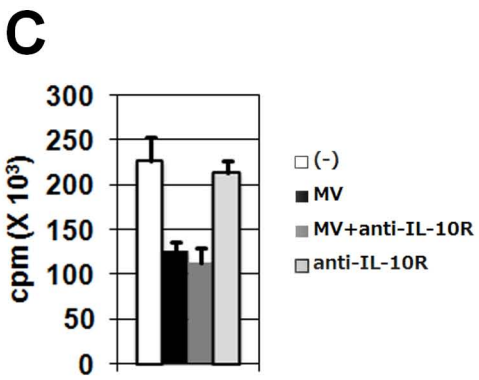

A
Cell number (X 10^6)
Spleen
Cell number (X 10^6)
Lung
Liver
LN
B
T cell number (X 10^7)
**
Spleen
T cell number (X 10^6)
*
Lung
Liver
LN
C
B cell number (X 10^7)
Spleen
B cell number (X 10^6)
Lung
Liver
LN
D
Annexin V+ cell (%)
**
0 2 5 8
days p.i.
WT
KI
E
Spleen
CD4+Foxp3+cell (%)
0 2 5 8
days p.i.
LN
0 2 5 8
days p.i.

Figure 6


A
500
400
300
200
100
0
Swelling (μm)
**
**
MV - + + -
anti-IL-10R - - + +


B
7
6
5
4
3
2
1
0
cell number (X 10⁷)
whole
B220⁺
Thy1.2⁺
(-)
MV
MV+anti-IL-10R
anti-IL-10R


C
300
250
200
150
100
50
0
cpm (X 10³)
(-)
MV
MV+anti-IL-10R
anti-IL-10R