Effects of Promyelocytic Leukemia Protein on Virus-Host Balance

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The cellular promyelocytic leukemia protein (PML) associates with the proteins of several viruses and in some cases reduces viral propagation in cell culture. To examine the role of PML in vivo, we compared immune responses and virus loads of PML-deficient and control mice infected with lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV). PML−/− mice exhibited accelerated primary footpad swelling reactions to very-low-dose LCMV, higher swelling peaks upon high-dose inoculation, and higher viral loads in the early phase of systemic LCMV infection. T-cell-mediated hepatitis and consequent mortality upon infection with a hepatotrophic LCMV strain required 10- to 100-times-lower inocula despite normal cytotoxic T-lymphocyte reactivity in PML−/− mice. Furthermore, PML deficiency rendered mice 10 times more susceptible to lethal immunopathology upon intracerebral LCMV inoculation. Accordingly, 10-times-lower VSV inocula elicited specific neutralizing-antibody responses, a replication-based effect not observed with inactivated virus or after immunization with recombinant VSV glycoprotein. These in vivo observations corroborated our results showing more virus production in PML−/− fibroblasts. Thus, PML is a contributor to innate immunity, defining host susceptibility to viral infections and to immunopathology.

The promyelocytic leukemia protein (PML) was first described as the product of a gene that fuses to the retinoic acid receptor α gene in chromosomal translocation t(15;17), resulting in acute promyelocytic leukemia (10, 20, 30).

Structural analysis of the PML protein revealed several domains involved in protein-protein (RING finger, coiled coil domain) or protein-DNA (cysteine-rich B boxes) interactions (30, 39, 59; P. S. Freemont, I. M. Hanson, and J. Trowsdale, Letter, Cell 64:483–484, 1991). In accord with these structural properties, subsequent studies suggested that PML acts as a growth and transformation suppressor (45, 55), cell cycle regulator (28, 42), mediator of apoptosis (69), and regulator of transcription (2, 24, 34, 48, 67, 73), translation (12), and hematopoietic differentiation (68). In agreement with these in vitro studies, PML−/− mice have been shown to be prone to tumorigenesis upon exposure to different carcinogens, although the incidence of spontaneous tumors did not differ from that for controls (68). On the other hand, a murine plasmacytoma expressing a mutated PML molecule suppressed cytopathic T-lymphocyte (CTL)-dependent rejection, apparently due to loss of expression of molecules involved in major histocompatibility complex (MHC) class I presentation (72).

Immunofluorescence and electron microscopy studies have revealed a dynamic distribution of PML: in normal fibroblasts PML has a speckled nuclear distribution, and it is associated with multiprotein complexes described as nuclear bodies, nucleolar domain 10, Kr bodies, or PML oncogenic domains (26).

To a lesser extent, PML bodies are also present in the cytoplasm of normal cells and are dynamically exchanged between the nucleus and cytoplasm (64). In acute promyelocytic leukemia (19, 39, 43) as well as after several viral infections in vitro (herpes simplex virus type 1, human cytomegalovirus, adenovirus type 5, human immunodeficiency virus, and lymphocytic choriomeningitis virus [LCMV]) (9, 23, 29, 41, 66), this normal distribution is changed, resulting in a diffuse nuclear or cytoplasmic distribution of PML. By means of immunofluorescence and coimmunoprecipitation, PML has been shown to colocalize with the Z proteins of the Arenaviridae, LCMV and Lassa fever virus, offering a possible explanation for the coincidence of LCMV infection and PML redistribution (9). Other viruses such as Epstein-Barr virus or simian virus 40 also exhibited direct interaction with PML within intact nuclear bodies (38, 65). To complement these findings, PML overexpression induced partial resistance to influenza virus and vesicular stomatitis virus (VSV) in vitro: 100-fold-less virus production along with reduced mRNA and protein synthesis (15) indicated a strong contribution of PML to cellular antiviral defense strategies. A less dramatic effect was also observed when production of LCMV was analyzed in cultures of PML−/− and PML+/- primary embryonic fibroblasts (21). Taken together with the interferon (IFN) inducibility of PML (14, 21, 44), these in vitro observations suggest a participation in IFN-mediated antiviral effects. In cell culture, PML expression also potentiates IFN-γ effects on viral transcription (21), but the impact of PML on the course and outcome of viral infection in vivo still remains elusive. Neither has a potential influence of...
PML on immune responses, either directly or by modulating the virus-host balance, has been investigated so far.

We have therefore analyzed virus-specific cellular and humoral immune responses in mice deficient in PML protein (PML<sup>−/−</sup>) mice) by using a cytotoxic virus, VSV, and a noncytopathic virus, LCMV. LCMV was used because different strains of LCMV due to different multiplication capacities (49, 52) and another strain in particular for its ability to cause liver disease (75). The acute phase of a primary LCMV infection, lasting approximately 2 to 3 weeks, is immunologically almost exclusively controlled by CTLs (11, 74). Intracerebral (i.c.) inoculation leads to development of fetal choriomeningitis in immunocompetent mice but not in T-cell-deficient nude mice (16, 22, 36, 46). This well-characterized CTL-mediated immunopathology offers a good model in which to assess the beneficial or detrimental effects of host resistance. In contrast, VSV, a highly cytopathogenic RNA virus, was used to study the effects of PML on B-cell responses. Intravenous (i.v.) infection of mice leads to disease only if very limited replication secondarily spreads to the central nervous system, a process that can be prevented only by an early neutralizing antibody response (6). Immunoglobulin M (IgM) levels up to day 4 after infection are T-cell independent, while a subsequent switch to IgG is strictly dependent on CD4<sup>+</sup> T helper cells (4). Our results demonstrate an inhibitory effect of PML on virus infections in vivo and in vitro; PML reduces the extent of infection at an early stage, thereby indirectly downregulating subsequent specific immune responses to low-dose viral exposure and reducing immunopathological disease upon intermediate- to high-dose infections.

**Materials and Methods**

**Mice.** PML-deficient mice were generated on a 129Sv background as described previously (66). Age- and sex-matched 129Sv control mice were purchased from the Institute for Laboratory Animals (Veterinary Hospital, Zurich, Switzerland). Experiments were performed in a conventional mouse house facility, and mice were used at the ages of 6 to 15 weeks. After i.e. infection with LCMV (see Fig. 4), mice were assessed for clinical symptoms of choriomeningitis every 12 h. Animals exhibiting clear signs of disease (usually after day 5) (half-closed eyes, seizures, and coarse tremors of head and extremities after they were suspended by the tail) were euthanatized in accordance with Swiss laws for animal protection. Mice from the experiment for which results are shown in Fig. 3 were assessed every 24 h for signs of terminal liver disease, apparent between day 7 to 10 by ruffled fur, severe excesosis with enophthalmus, and a precomatose state. Only upon appearance of these severe signs of terminal disease were the mice sacrificed as required by local animal protection laws.

**Viruses, recombinant viral antigens, and virus detection.** VSV Indiana (VSV-IND; Mudd-Summers isolate) was originally obtained from D. Kodakovsky (University of Geneva, Geneva, Switzerland). For virus stock production, BHK21 cells were infected at a multiplicity of infection (MOI) of 0.01. After 2 h of incubation at room temperature, the initial inoculum was discarded and replaced with fresh medium. The virus was harvested after 22 h of incubation at 37°C from the second supernatant. For UV inactivation, a small volume of a high-titer VSV preparation was exposed as a thin layer in a petri dish to a UV lamp (15 W, Philips) for 3 min at a distance of 8 cm (3). The recombinant baculovirus expressing the glycoprotein of VSV (VSV-G) was a gift from D. H. L. Bishop, Philips. The recombinant baculovirus preparation was exposed as a thin layer in a petri dish to a UV lamp (15 W, Philips) for 3 min at a distance of 8 cm (3). The recombinant baculovirus expressing the glycoprotein of VSV (VSV-G) was a gift from D. H. L. Bishop, Philips.

**Antibody detection.** VSV neutralizing antibodies were detected by a neutralization assay as described previously (77). Briefly, single-cell suspensions were prepared from the spleens of mice at day 8 after LCMV infection and were used at a concentration of 5 × 10<sup>6</sup> cells/ml in 96-well plates coated with LCMV WE, LCMV-DOC, or LCMV-ARM. Infections for virus stock production were performed at an MOI of 0.01 for 48 h. The viruses, culture supernatants, and infectious viruses in mouse spleens were titrated on MC57G cell monolayer cultures, and titers were evaluated by an immunological focus-forming assay (8). Viral titers are expressed as PFU per gram of spleen or milliliter of cell culture supernatant.

**Determination of serum ALT enzyme concentrations.** Assays for concentrations of alanine aminotransferase (ALT) in plasma were carried out in the Department of Clinical Chemistry at the University Hospital Zurich, by using photometric assays on a Hitachi 747 autoanalyzer.

**Assessment of the primary footpad swelling reaction.** The indicated amounts of LCMV-ARM or LCMV-DOC were injected in a volume of 30 μl of balanced salt solution into both hind footpads. The footpad thickness was measured with a spring-loaded calliper (Kroeplein, Schluchtern, Hesse, Germany) (54). The mean thickness of both hind footpads of an individual mouse at a particular time point was taken for further evaluation.

**CTL assay and peptides.** Virus-specific cytotoxic T cells were assayed as described previously (77). Briefly, single-cell suspensions were prepared from the spleens of mice at day 8 after LCMV infection and were used directly in a primary in vitro 51Cr release assay. Target cells were MC57G cells coated with GP33 (10−6 M) and uncoated control cells. The immunodominant H-2D<sup>b</sup> binding LCMV peptide gp 33-41 (GP33) was purchased from NeoSystem Laboratoire (Strasbourg, France).

**Antibody detection.** VSV neutralizing antibodies were detected by a neutralization assay as described previously (57). IgG was determined by its resistance to reduction with 2-mercaptoethanol. The difference between total immunoglobulin M and IgG represents IgM. Total immunoglobulin titers of two or more titer steps above IgG were considered IgM.

**Generation of mouse primary embryonic fibroblasts.** Embryos from PML<sup>−/−</sup> or control mice at 14 days of gestation were digested with a trypsin-EDTA solution at 37°C for 30 min. Fibroblasts in suspension were separated by a centrifugation step and were grown in 150-cm<sup>2</sup> tissue culture flasks at 37°C under a 5% CO<sub>2</sub> atmosphere.

**Statistical analysis.** Differences between groups in the time of onset of the footpad swelling reaction (see Fig. 1A) and differences between virus titers in the spleens of PML<sup>−/−</sup> or 129Sv control mice (see Fig. 2) were determined by the nonparametric Mann-Whitney test. Differences between groups in the extent of footpad swelling over the entire time span of an experiment were assessed by repeated measures analysis of variance using one or two factors (mouse genotype [Fig. 1A] or mouse genotype and experiment [Fig. 1A through C]). Figure 1A, B, and C show data from only one representative experiment, while four (panels A and C) and two (panel B) similar sets of experiments were used for statistical analysis, respectively. For analysis of intragroup differences in virus production (Table 1), the respective virus yields obtained from PML<sup>−/−</sup> mouse embryonic fibroblasts (MEF) supernatants (P1) were compared at six different MOIs (for VSV, 5 × 10<sup>−3</sup> to 5; for LCMV, 5 × 10<sup>−3</sup> to 5 × 10<sup>−2</sup>) with control supernatants from PML<sup>−/−</sup> MEFs (P0), matched for the dose and virus. For each pair of data (P0 and P1), the ratio (P1/P0) was calculated and tested for values greater than zero by using the nonparametric signed-rank Wilcoxon test. For evaluation of LCMV plaque size, 50 randomly chosen plaques per group were measured and analyzed in a two-sided Student t test. Percentages of lethality occurring in PML<sup>−/−</sup> and PML<sup>−/−</sup> mice in the experiment for which results are shown in Fig. 4 were compared by using the Fischer exact test.

**RESULTS**

**Accelerated and increased primary footpad swelling reaction in PML<sup>−/−</sup> mice.** We used the well-established primary footpad swelling reaction after subcutaneous intrafootpad (i.f.) inoculation of LCMV as an overall readout for virus-specific CTL and T helper cell responses in PML-deficient mice (54). Low-dose i.f. infection requires local propagation before...
spreading to secondary lymphoid organs leads to induction of LCMV-specific CTL and T helper cells. Activated T cells then migrate to the infected footpad and mediate tissue destruction and swelling of the footpad. Higher doses directly drained via lymphatic vessels result in immediate T-cell induction and earlier swelling reactions. Therefore, monitoring of the swelling reaction during a period of 15 days recapitulates the ongoing in vivo CTL and T helper response. In this system, the correlation of maximal virus replication and the swelling reaction of the footpad gives further insight into the extent of the local infection (54). The use of a large dose range of LCMV-DOC, a virus strain exhibiting rapid propagation and distribution kinetics, allowed the analysis of a wide spectrum of virus loads generated (49, 51). Both PML$^{-/-}$ and PML$^{+/+}$ mice were infected i.f. with 3 PFU (Fig. 1A), 3,000 PFU (Fig. 1B), or $3 \times 10^5$ PFU (Fig. 1C), and the footpad swelling reaction was monitored. After low-dose infection, control mice developed a response peaking at day 9 after inoculation whereas PML$^{-/-}$ mice reached the highest swelling level around days 7 to 8, significantly earlier than controls ($P < 0.001$). On the other hand, infection with $3 \times 10^5$ PFU of LCMV-DOC evoked a swelling reaction of similar kinetics but greater extent ($P < 0.0001$) in PML$^{-/-}$ mice (Fig. 1C). Interestingly, infection with 3,000 PFU elicited swelling reactions that were not significantly different in PML$^{-/-}$ and PML$^{+/+}$ mice ($P = 0.462$). These results suggested that during the course of a low-dose infection, virus loads necessary for induction of specific cellular immune responses are generated later, when PML protein is present (Fig. 1A). Intermediate doses did not reveal a phenotype of PML-deficient mice (Fig. 1B; see also Discussion); the observation of less tissue destruction by virus-specific CTLs after high-dose infection of PML$^{-/-}$ mice was most likely due to less-widespread infection and less subsequent immunopathology (Fig. 1C). Similar observations were also made with LCMV-ARM and LCMV-WE.

**Increased virus titers in organs of PML$^{-/-}$ mice early after LCMV-infection.** To determine whether virus amplification was accelerated in vivo in the absence of PML, we analyzed early viral titers in the spleen, an organ that parallels virus replication in most major organs (47). PML$^{-/-}$ or control mice were infected i.v. with low doses of LCMV-ARM (20 or 200 PFU) or an intermediate dose of LCMV-WE ($10^4$ PFU), and viral titers were measured in the spleen as described in Materials and Methods. After 48 h, when virus was first detectable (Fig. 2, LCMV-ARM), or after 24 h, when amplification of the higher viral inoculum could first be detected (Fig. 2, LCMV-WE, and data not shown), mice were sacrificed and viral titers were determined. Five of seven PML$^{-/-}$ mice infected with 20 PFU of LCMV-ARM produced detectable amounts of virus within 48 h after infection, while no virus was found in the spleens of similarly infected control mice (Fig. 2). When mice were infected with 200 PFU of LCMV-ARM, only five of seven control mice exhibited detectable viral titers (Fig. 2). All PML-deficient mice had titers clearly above the detection level, with a median value more than 50 times higher than that of the control group (Fig. 2, center). Similarly, when mice were infected i.v. with the hepatotropic LCMV-WE at $10^8$ PFU, 10- to 50-times-higher viral titers were found in the spleens of PML$^{-/-}$ mice 24 h after infection (Fig. 2). In all these experiments, viral titers in PML-deficient mice were significantly higher than those in control mice ($P = 0.026$ for LCMV-ARM at 20 PFU; $P < 0.001$ for LCMV-ARM at 200 PFU and LCMV-WE at $10^4$ PFU).

Along with the findings of accelerated or more-extensive primary footpad swelling reactions in PML-deficient mice, these results confirmed that PML either suppresses or slows down virus multiplication, depending on the infectious dose used.

**PML-deficient mice are more susceptible to cytotoxic T-cell-mediated hepatitis in response to LCMV-WE infection.** Cytotoxic CD8$^+$ T-cell-mediated hepatitis in mice caused by infection with the hepatotropic LCMV-WE (35, 37, 46, 71) correlates with elevated levels of ALT in the serum (75). To determine whether PML influenced this immunopathologic disease, which depends on critical parameters of virus-host balance such as infectious dose and immune competence of the host (75), PML$^{-/-}$ and control mice were infected i.v. with LCMV-WE in 10-fold dilution steps ranging from $10^2$ to $10^6$ PFU. Serum samples were taken at the indicated time points and analyzed for ALT activity as described in Materials and Methods.

**FIG. 1.** Accelerated or higher primary footpad swelling reactions in PML$^{-/-}$ mice. PML$^{-/-}$ (○) and control (wild-type [wt]) (□) mice were immunized on day 0 with 3 PFU (A), $3 \times 10^3$ PFU (B), or $3 \times 10^5$ PFU (C) of LCMV-DOC. Footpad swelling was monitored as described in Materials and Methods. Each symbol represents a group of three to four mice. One experiment representative of two to four is shown for each respective dose. Values are means for three to four mice per group ± standard deviations. Asterisks indicate a $P$ value of <0.001 (C) or <0.005 (A).
Methods. Mice exhibiting signs of severe disease were sacrificed. Infection with $10^2$ PFU of LCMV-WE i.v. resulted only in a moderate, temporary increase in ALT levels in either genotype (Fig. 3A). Exposure to $10^3$ (Fig. 3B) or $10^4$ (Fig. 3C) PFU of LCMV-WE i.v. caused terminal immunopathology-induced hepatitis: five of six PML$^{-/-}$ mice displayed extreme ALT levels, while one mouse had to be sacrificed in the period between two measurements. Sera from control mice exhibited background ALT activity after infection with $10^3$ PFU (Fig. 3B) and only moderately increased levels after infection with $10^4$ PFU (Fig. 3C). However, at $10^5$ PFU, both PML$^{-/-}$ and control mice exhibited similar, typical ALT time courses peaking around days 6 to 8 after infection (75) and decreasing thereafter to normal levels around days 10 to 14. The threshold for relevant liver tissue destruction as determined by ALT levels was 10- to 100-fold lower in the absence of PML. This was apparently due to more-extensive viral propagation (compare Fig. 2, right panel) and consequent CTL-mediated lysis of a higher number of infected hepatocytes. At the highest dose of infection, the two experimental groups exhibited similar kinetics of liver pathology, and both survived. At first sight this result appears contradictory, since PML$^{-/-}$ mice died from lower infectious doses, but this outcome may readily be explained by the different levels of balance between LCMV and the host’s immune system (53) (see Discussion). In control mice infected with high doses ($10^4$ to $10^5$ PFU), ALT levels went up but were resolved without any lethality. This difference in the outcome of infection was not due to differences in T-cell responsiveness between PML$^{-/-}$ and control mice, as will be shown (see Fig. 4B). All mice surviving the observation period of 15 days had cleared the virus (data not shown).

PML-deficient mice are more susceptible to lethal CTL-mediated immunopathology after i.c. inoculation with LCMV. The role of PML in virus-host relationships was further tested with the well-established model of i.c. infection with LCMV, which causes a CTL-mediated choriomeningitis (16). PML$^{-/-}$ and control mice were infected i.c. with LCMV-ARM in 10-fold dilution steps ranging from $10^3$ to 10 PFU. Mice were monitored twice a day for signs of disease until day 34 after infection. Clinical symptoms of choriomeningitis were observed between days 6 and 9, and the respective mice were sacrificed. With the lowest doses of $10^3$ and $10^2$ PFU, all mice survived regardless of genotype, while all mice receiving a dose of 1 or 10 PFU eventually died. Inoculation with $10^{-1}$ PFU of LCMV-ARM led to lethal choriomeningitis in 12 of 13 PML$^{-/-}$ mice but only in 1 of 13 control mice ($P < 0.001$) in a total of four separate experiments (Fig. 4A). Comparable observations were made with i.c. LCMV-WE infection (data not shown).

Since a productive i.c. infection leads to a specific CTL response and lethal immunopathology, these data indicated that the in vivo threshold for a productive infection with LCMV was decreased by a factor of about 10 in the absence of...
PML. The lethal i.c. inoculum in PML/+/ mice, 1 PFU, was identical to the minimal unit of virus needed to infect the MC57G cell monolayers (PML/+/) used to quantify virus stocks (see Materials and Methods). This indicates that 1 PFU contains at least 10 potentially infectious viral particles, although this is not detectable in PML-competent animals or tissue cultures. A direct effect of PML on CTL responsiveness as the underlying reason for the above results seemed unlikely, because PML/-/ and control mice generated comparable CTL responses on day 8 after i.v. infection with 200 PFU of LCMV-WE (Fig. 4B) or LCMV-ARM (data not shown).

Enhanced antibody responses to low doses of VSV in PML/-/ mice are due to higher viral replication rather than to enhanced responsiveness of PML/-/ mice. The influence of PML on virus-specific humoral immune responses was assessed in the well-established murine VSV infection model (6). We infected PML/-/ mice and 129Sv control mice i.v. with different doses of replicating VSV-IND (Fig. 5A). At the indicated time points, serum samples were collected, and neutralizing antibody titers were determined as described in Materials and Methods. After infection with 10^5 PFU of VSV, PML/-/ and control mice generated comparable neutralizing IgM and IgG titers (Fig. 5Aa). Infection with 10^3 PFU led to comparable IgM responses independent of the mouse genotype, but IgG titers were increased in PML/-/ mice compared to control mice (Fig. 5Ab). After inoculation with 10^5 PFU of VSV, specific neutralizing IgM and IgG could be detected only in PML/-/ mice, while control mice remained unresponsive (Fig. 5Ac). This effect could have been either the consequence of a higher humoral immune responsiveness of PML/-/ mice or the result of higher antigen levels due to better virus multiplication in PML/-/ mice. Due to very poor extraneuronal replication in the murine host, VSV stays below detectable levels under the experimental conditions used here (i.v. infection with 10^5 to 10^6 PFU). Infectious VSV can be measured only for the first 24 to 48 h upon inoculation of 2 × 10^6 or more PFU i.v. and does not allow discrimination between the initial inoculum and progeny virus particles (A. F. Ochsenbein, unpublished data). Accordingly, we had to use the intensity of the immune response as a sensitive but only indirect readout of viral antigen loads to differentiate between those two possible mechanisms for the above observations: PML/-/ and control mice were immunized with different doses of UV-inactivated and therefore nonreplicating VSV. Independent of the mouse genotype, 5 × 10^7 PFU equivalents of UV-inactivated VSV led to equal neutralizing IgM and IgG titers (Fig. 5Ba). An intermediate dose (5 × 10^5 PFU of UV-inactivated VSV [Fig. 5Bb]) still evoked comparably high IgM levels in all mice, while only one of three mice in every group exhibited a T-cell-dependent isotype switch to IgG. With 5 × 10^5 PFU equivalents, only T-cell-independent IgM could be detected in PML-competent and -deficient mice (Fig. 5Bc). To additionally quantify antibody responses to inert antigens, we immunized PML/-/ and control mice with 20 μg of recombinant VSV-G (Fig. 5C). Serum samples were collected at the indicated time points, and VSV-specific neutralizing IgM and IgG responses were determined. PML/-/ and PML/+ mice exhibited identical VSV neutralizing-antibody responses after immunization with VSV-G (Fig. 5C), corroborating that B cells and CD4+ T helper cells respond equally well in PML/-/ and control mice (Fig. 5Ba through c and 5C). Therefore, the higher IgM and IgG levels of PML/-/ mice are very likely the result of better virus propagation and not the result of differences in immune responsiveness (Fig. 5Ab and c).
Replication of VSV and LCMV in PML<sup>−/−</sup> MEFs. The above experiments indicated enhanced propagation of VSV and LCMV in PML<sup>−/−</sup> mice. We therefore investigated PML-dependent effects on virus production in vitro. MEFs were generated from PML<sup>−/−</sup> and control mice as described in Materials and Methods. The two cell lines exhibited comparable cell sizes and cell cycle kinetics (data not shown). A total of 5 × 10<sup>5</sup> MEFs of either genotype per well were seeded and grown overnight in 24-well tissue culture plates. Thereafter, VSV infection was performed by using decreasing MOIs ranging from 5 to 5 × 10<sup>−3</sup>. Twenty-four hours later, the supernatants of eight individual cultures infected at the same MOI were collected and pooled; VSV production was determined by a standard VSV plaque assay as described in Materials and Methods. The two cell lines exhibited comparable cell sizes and cell cycle kinetics (data not shown). A total of 5 × 10<sup>5</sup> MEFs of either genotype per well were seeded and grown overnight in 24-well tissue culture plates. Thereafter, VSV infection was performed by using decreasing MOIs ranging from 5 to 5 × 10<sup>−3</sup>. Twenty-four hours later, the supernatants of eight individual cultures infected at the same MOI were collected and pooled; VSV production was determined by a standard VSV plaque assay as described in Materials and Methods. Virus yields obtained from supernatants of PML<sup>−/−</sup> MEFs infected at an MOI of 5 to 5 × 10<sup>−3</sup> exceeded those from controls by a factor of 1.9 to 9 (P = 0.014) (Table 1). A similar experimental setup was used to establish LCMV production in vitro: 5 × 10<sup>5</sup> MEFs per well of the PML<sup>−/−</sup> or control genotype were seeded in 24-well plates, infected simultaneously with LCMV-WE at MOIs ranging from 5 × 10<sup>−5</sup> to 5 × 10<sup>−3</sup>, and cultured for 48 h. Amounts of infectious viral progeny were determined in the culture supernatants by using a standard LCMV plaque assay (see Materials and Methods). At MOIs from 5 × 10<sup>−5</sup> to 5 × 10<sup>−3</sup>, PML<sup>−/−</sup> fibroblasts produced 2 to >10 times more virus than control fibroblasts (P = 0.014) (Table 1). To further address PML effects on LCMV replication in tissue culture, we analyzed infectious focus formation in PML<sup>−/−</sup> and control MEFs by plating 2.8 × 10<sup>5</sup> MEFs simultaneously with LCMV-WE or LCMV-ARM (MOI = 5 × 10<sup>−4</sup>). Four to five hours later, we supplemented the cultures with an overlay of methylcellulose and medium. At 48 h after inoculation, infected cells were visualized by an intracellular LCMV-specific stain. The diameter of plaques formed by LCMV-WE on PML<sup>−/−</sup> fibroblasts was 802 ± 120 μm, and that on PML<sup>−/−</sup> fibroblasts was 448 ± 90 μm. With LCMV-ARM we measured 732 ± 170 μm (PML<sup>−/−</sup>) and 374 ± 90 μm (PML<sup>+/+</sup>) for the respective plaque diameters. Therefore, plaques formed on PML<sup>−/−</sup> fibroblasts had approximately twice the diameter of plaques in control cells (P < 0.0001 for LCMV-WE; P < 0.0001 for LCMV-ARM) (data correspond to one experiment representative of two).

**DISCUSSION**

In cell culture, PML expression has negative effects on the propagation of certain RNA viruses (VSV, influenza virus, LCMV) (15, 21; also this report); however, it was not clear that these effects could impact viral disease. Our results show distinct antiviral effects of PML in vivo. Inoculations with limiting doses of VSV i.v. and LCMV i.c. suggest that PML can, under certain conditions, suppress productive viral infection in vivo to below the minimal levels required for induction of a specific immune response (Fig. 4A and 5Ac). This was observed for control animals where lethal immunopathology was prevented (Fig. 4A) or a specific immune response was absent (Fig. 5Ac). Ten- to 100-fold-lower doses of hepatotropic LCMV-WE administered i.v. caused terminal T-cell-mediated immunopathology in PML-deficient animals (Fig. 3), and infection with high-dose LCMV-DOC resulted in stronger T-cell-mediated swelling of the footpad (Fig. 1C). These observations, together with larger LCMV plaque sizes in PML<sup>−/−</sup> fibroblasts, increased virus production, and a lower minimal MOI required for infection in PML<sup>−/−</sup> cell culture, suggest a mechanism of higher viral burden with secondary effects on antigen-driven immune responses.

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**FIG. 5.** VSV neutralizing antibody responses of PML<sup>−/−</sup> or 129Sv control mice. PML<sup>−/−</sup> (circles) and control (wild-type [wt]) (triangles) mice were inoculated i.v. on day 0 with either live VSV (A) at 10<sup>4</sup> (a), 10<sup>3</sup> (b), or 10<sup>0</sup> (c) PFU, UV-inactivated VSV (B) at 5 × 10<sup>4</sup> (a), 5 × 10<sup>3</sup> (b), or 5 × 10<sup>2</sup> (c) PFU equivalents, or VSV-G (20 μg) (C). Serum samples were taken at the indicated time points, and neutralizing IgM (open symbols) and IgG (solid symbols) titers were determined by a neutralization assay. Each symbol represents an individual mouse. Results from one representative experiment of two are shown.
The virus dose range within which in vivo effects of PML could be observed varies with the experimental setup. LCMV footpad inoculation revealed a clear PML-dependent phenotype at low and very high virus doses; VSV immunization did so at low doses. In PML-deficient animals, LCMV-WE-mediated terminal immunopathologic hepatitis was observed at 10^5 to 10^6 PFU given i.v.—a dose controlled by normal mice without serious signs of clinical disease. Higher and lower doses of virus elicited a course of infection similar to that in wild-type mice (Fig. 3). Different explanations that are not mutually exclusive may apply. We favor the idea that the specific immune system has evolved to fight intermediate doses of pathogens, with low doses being partially (Fig. 1A) or completely (Fig. 3A) controlled by innate defenses (56) such as the PML factor alpha mechanisms used to control hepatitis B virus (HBV) infection (31–33). High-dose infection with viruses is probably very rare in nature but can occur during transplacental transmission, neonatal infections during birth, blood transfusions, or organ transplants, or occasionally upon encounter with arthropod-borne diseases. Further, our experiments with high-dose LCMV given i.f. and particularly with intermediate doses of LCMV-WE given i.v. may imitate infections with noncytolytic viruses such as HBV, HCV, and possibly human immunodeficiency virus in humans, not primarily with respect to the initial inoculum (except for HCV when it is acquired by transfusion) but with regard to the number of infected cells critically determining immunopathology.

A study in which recurrent model tumors emerged as a consequence of a dominant-negative mutation of PML suggested that PML might be required for expression of functional MHC class I-peptide complexes (see the introduction) (72). Our data indicate that MHC class I and II expression and peptide processing as monitored by the resulting T-cell response and immunoglobulin class switch in vivo and in vitro, as well as class I and II levels assessed by fluorescence-activated cell sorter analysis, were normal when disruption of exon 2 (this study) was used to completely eliminate PML expression (68).

As an IFN-inducible gene, PML could mediate antiviral effects through a variety of intracellular pathways: PML is constitutively expressed, unlike other mediators of antiviral IFN activity such as Mx proteins, P1/eIF-2 protein kinase, and 2',5'-oligoadenylate synthetase (1, 61, 62). This would allow for immediate antiviral activity of this molecule, an effect which could be potentiated upon upregulation through IFN. Alternatively, PML could indirectly participate in the still poorly defined IFN-mediated upregulation of known or yet unknown IFN-regulated proteins with antiviral activity. Future studies with extensive and detailed molecular work will be needed to dissect this large array of potentially redundant and parallel

### TABLE 1. Enhanced virus production in the absence of PML in vitro

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<th>MEF genotypea</th>
<th>MOI</th>
<th>Virus productionb (PFU/ml)</th>
<th>MOI</th>
<th>Virus productionb (PFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PML+/−</td>
<td>5</td>
<td>6.5 × 10^6</td>
<td>5 × 10^−2</td>
<td>8 × 10^4</td>
</tr>
<tr>
<td>PML+/−</td>
<td>5</td>
<td>2.5 × 10^6</td>
<td>5 × 10^−2</td>
<td>2 × 10^4</td>
</tr>
<tr>
<td>PML+/−</td>
<td>0.5</td>
<td>2.3 × 10^5</td>
<td>5 × 10^−3</td>
<td>3.6 × 10^6, 1.6 × 10^6, 4.5 × 10^6</td>
</tr>
<tr>
<td>PML+/−</td>
<td>0.5</td>
<td>2.4 × 10^4</td>
<td>5 × 10^−3</td>
<td>4 × 10^5, 5 × 10^5, 5.4 × 10^5</td>
</tr>
<tr>
<td>PML+/−</td>
<td>5 × 10^−2</td>
<td>6.5 × 10^5</td>
<td>1 × 10^−3</td>
<td>2 × 10^7</td>
</tr>
<tr>
<td>PML+/−</td>
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<td>1.2 × 10^6</td>
<td>1 × 10^−3</td>
<td>9.5 × 10^6</td>
</tr>
<tr>
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<td>1 × 10^−2</td>
<td>4.5 × 10^5, 4.7 × 10^6</td>
<td>5 × 10^−4</td>
<td>1.5 × 10^6</td>
</tr>
<tr>
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<td>7 × 10^5, 7.1 × 10^6</td>
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</tr>
<tr>
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<td>8 × 10^5</td>
<td>5 × 10^−5</td>
<td>4.4 × 10^5</td>
</tr>
<tr>
<td>PML+/−</td>
<td>5 × 10^−3</td>
<td>4.2 × 10^5</td>
<td>5 × 10^−5</td>
<td>4 × 10^4</td>
</tr>
</tbody>
</table>

a A total of 5 × 10^5 PML−/− or PML+/+ control MEFs for each experimental condition were inoculated with 2.5 × 10^5, 2.5 × 10^5, 5 × 10^5, or 2.5 × 10^6 PFU of VSV or with 2.5 × 10^5, 2.5 × 10^5, 5 × 10^5, 250, or 25 PFU of LCMV-WE corresponding to the indicated MOIs.

b Each value represents viral titers determined after 24 (VSV) or 48 (LCMV) h by transferring pooled supernatants of eight individual cultures onto Vero (VSV) or MC57G (LCMV) cell monolayers.


