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Activation of Melanoma Differentiation-Associated Gene 5 Causes Rapid Involution of the Thymus

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In the course of infection, the detection of pathogen-associated molecular patterns by specialized pattern recognition receptors in the host leads to activation of the innate immune system. Whereas the subsequent induction of adaptive immune responses in secondary lymphoid organs is well described, little is known about the effects of pathogen-associated molecular pattern-induced activation on primary lymphoid organs. Here we show that activation of innate immunity through the virus-sensing melanoma differentiation-associated gene 5 (MDA-5) receptor causes a rapid involution of the thymus. We observed a strong decrease in thymic cellularity associated with characteristic alterations in thymic subpopulations and microanatomy. In contrast, immune stimulation with potent TLR agonists did not lead to thymic involution or induce changes in thymic subpopulations, demonstrating that thymic pathology is not a general consequence of innate immune activation. We determined that suppression of thymocyte proliferation and enhanced apoptosis are the essential cellular mechanisms involved in the decrease in thymic size upon MDA-5 activation. Further, thymic involution critically depended on type I IFN. Strikingly however, no direct action of type I IFN on thymocytes was required, given that the decrease in thymic size was still observed in mice with a selective deletion of the type I IFN receptor on T cells. All changes observed were self-limiting, given that cessation of MDA-5 activation led to a rapid recovery of thymic size. We show for the first time that the in vivo activation of the virus-sensing MDA-5 receptor leads to a rapid and reversible involution of the thymus. The Journal of Immunology, 2009, 182: 6044–6050.
periphery (17–19). An important fraction of self-reactive T cells is deleted in the thymus by apoptosis, and thymic cellularity is maintained by vigorous proliferation of immature thymocytes. Viral infections are in some cases associated with in vivo alterations of thymic function; thymic atrophy and a reduced T cell output are seen in HIV-infected patients (20) and have also been described in a mouse model of reovirus infection (21). The mechanisms involved remain, however, unclear. We describe here for the first time that in vivo activation of the virus-sensing MDA-5 receptor causes involution of the thymus.

Materials and Methods

Mice

Female C57BL/6 and BALB/c mice were purchased from Harlan-Winkelmann. Experiments were done on C57BL/6 mice unless indicated otherwise. Type I IFN receptor-deficient mice (IFNAR−/−) were backcrossed 20 times on the C57BL/6 background (22). CD4cre+/− IFNARbw mice, CD19cre+/− IFNARbw mice, and MDA-5+/− mice have been previously described (23, 24). Mice were at least 8 wk of age at the onset of experiments. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany).

Ligands for MDA-5 and TLRs

Polyinosinic acid-polycytidylic acid (poly(I:C); InvivoGen) was applied i.p. (250 μg in 250 μl of distilled water). The fully phosphorylated-modified CpG oligonucleotide 1826 (5′-TCCATGACGTTCCTGGATT-3′; Coley Pharmaceutical Group), LPS (Sigma-Aldrich), and R848 (Alexis Biochemicals) were injected s.c. into the flank in 200 μl of PBS (100, 5, and 20 μg, respectively).

Flow cytometry

Single-cell suspensions were stained with anti-CD3-Pacific Blue or PerCP, anti-CD4-PE-Cy7, anti-CD8-allophycocyanin-Alexa750, anti-CD25-PE and anti-CD44-allophycocyanin (all BD Biosciences). Events were measured on a FACScanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Histology

Specimens were fixed in formalin before embedding in paraﬁn blocks. The resulting tissue sections were stained with H&E. For immunofluorescence analysis, 5-μm frozen cryosections were fixed in acetone before staining with 10% goat serum. Rat anti-mouse CD4 (Biolegend) and rat anti-mouse CD8 (BD Biosciences) were used as primary Abs. Because both Abs are derived from the same species, we used a protocol based on sequential application of primary Abs and detection with Fab fragments. First, we applied the anti-CD8 Ab followed by detection with biotinylated goat-anti-rat IgG Fab fragments; these Fab fragments fully saturate the first primary Ab and thus prevent binding of subsequently applied anti-rat secondary Abs (25). After detection with a Cy2-conjugated streptavidin, the second primary Ab (anti-CD4) was applied followed by detection with rhodamine red X-conjugated mouse anti-IgG. To prevent cross-reactions of the goat anti-rat Fab fragments to endogenous mouse IgG, tissues were generally blocked with (Fab) goat anti-mouse IgG before staining. Images were obtained using a fluorescence microscope (Axioskop 2000; Carl Zeiss) and processed using Adobe Photoshop for adjustment of contrast and size.

Intrathymic FITC injection

Intrathymic injections of FITC were performed as described (26). Poly(I:C) was applied at days 1 and 3 after FITC injection, and organs were isolated at day 4 to assess thymocyte emigration to the periphery.

In vivo BrdU proliferation assay

Mice received three i.p. injections of 2 mg of BrdU in PBS (Sigma-Aldrich) at 6-h intervals. Thymus and spleen were isolated 6 h after the last injection of BrdU. After surface staining, single-cell suspensions were fixed and permeabilized using a ready-mixed kit from Ebioscience. BrdU incorporation was detected using FastImmune Anti-BrdU FITC with DNase according to the manufacturer’s instructions (BD Biosciences), and FITC− cells were quantified by flow cytometry.

Detection of apoptotic cells

An Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) was used for detection of apoptotic cells. After surface staining, single-cell suspensions of thymus and spleen were washed twice with PBS before resuspension in the provided buffer and incubation with annexin V and propidium iodide. Cells were subsequently analyzed by flow cytometry.

Statistical analysis

All data are presented as mean ± SEM and were analyzed as appropriate by the unpaired Student t test or the ANOVA test. Statistical analysis was performed using SPSS software.

Results

Stimulation of innate immunity through MDA-5 causes involution of the thymus

To investigate whether in vivo activation of innate immunity affects the thymus, we treated mice with the dsRNA molecule poly(I:C). Poly(I:C) stimulates the immune system through two different pathways mediated by activation of either the endosomal TLR3 or the cytoplasmic helicases RIG-I and MDA-5 (4–6, 24). Adult mice were injected twice with poly(I:C) at 3-day intervals, and organs were examined 24 h after the second injection. We observed a substantial decrease in the volume of the thymus (Fig. 1A) reﬂected by a >3-fold reduction in thymic weight (Fig. 1B). In contrast, no decrease in weight was observed for the spleen (Fig. 1B) and the peripheral lymph nodes (data not shown), demonstrating that this effect was selective for the thymus. The rapid involution was due to a strong reduction in cellularity, whereby the average number of thymocytes dropped from >130 × 106 cells to <3 × 106 cells (Fig. 1C). To determine which pattern recognition receptor was involved in poly(I:C)-induced thymic involution, we examined thymic pathology in mice deﬁcient for MDA-5. This cytoplasmic helicase plays a crucial role in the recognition of many common viruses by the innate immune system (10). In striking contrast to wild-type mice, poly(I:C) treatment of MDA-5−/− mice did not affect the macroscopic aspect of the thymus and only slightly decreased thymic weight (Fig. 1D), indicating an essential role for this receptor in thymic pathology. To investigate whether decrease of thymic size is a general consequence of innate immune activation, we treated mice with ligands for different TLRs. Although application of the TLR7 ligand R848 led to a moderate decrease in thymic weight, neither stimulation with the TLR4-activating LPS nor stimulation with the TLR9 ligand CpG DNA resulted in a significant decrease of thymic size (Fig. 1E). All three TLR ligands are potent stimulators of innate immunity that efﬁciently induce production of proinﬂammatory cytokines and lymphocyte activation in vivo (7). Thus, in vivo immune activation through the cytoplasmic helicase MDA-5, but not through TLRs in general, rapidly leads to involution of the thymus.

MDA-5-induced thymic suppression is characterized by a decrease in CD4+ CD8− double-positive (DP) thymocytes

The stages of T cell maturation within the thymus are characterized by the differential expression of surface markers. Immature progenitor cells are double-negative (DN) for both CD4 and CD8 upon immigration and can be further differentiated into four stages of maturation: the progenitor cells first express CD44 (DN1 cells), then become consecutively CD44+CD25− (DN2), CD44+CD25− (DN3), and finally CD44+CD25+ (DN4). DN cells subsequently acquire double positivity for CD4 and CD8 in the thymic cortex and finally lose the expression of either CD4 or CD8 to leave the thymus as mature, single-positive T lymphocytes (17, 27). After treatment with poly(I:C), we observed a signiﬁcant drop in the cell count for all major thymic populations (Fig. 2A). In contrast, the loss in cellularity was prevented to a large extent in MDA-5-deﬁcient mice after treatment with poly(I:C) in all thymic subpopulations including the DN subsets (Fig. 2B). Proportional analysis demonstrated a strong decrease within the fraction of DP cells,
MDA-5-induced immune activation leads to a reduction of the thymic cortex and altered histomorphology

Histologically, the thymus is divided into a highly cellular cortex with strong proliferative activity and a medulla defined by a coarse reticulum with lower lymphocyte density (27). To assess changes in thymic microanatomy upon in vivo stimulation of innate immunity, organs from poly(I:C)-treated mice were examined by histology. Although both thymic cortex and medulla were markedly decreased in volume, the reduction of the cortex was clearly more pronounced, resulting in a decreased ratio of cortical to medullary space (Fig. 3A). Furthermore, the corticomedullary border was blurred, resulting in a disorganized aspect of thymic microanatomy. Fluorescent double staining for CD4 (red) and CD8 (green) demonstrated a decrease in the DP-cell fraction in the cortex detected by a reduced merged (yellow) color signal (Fig. 3B). To

dropping from >80% to <40% of all thymocytes, whereas a relative increase was seen within the single-positive cells (Fig. 2C). These changes were nearly entirely absent in MDA-5-deficient mice stimulated with poly(I:C), confirming the essential role of the MDA-5 activation pathway for the suppressive effect of poly(I:C) on the thymus (Fig. 2C). Stimulation with poly(I:C) further led to a relative increase in the DN1 compartment that was accompanied by a drop in the DN3 fraction (Fig. 2D). Thus, the overall decrease in thymic cellularity by stimulation. Mice were treated with poly(I:C) as described in Fig. 1. Thymocytes were counted and analyzed by flow cytometry. A. Absolute cell numbers for thymocyte subpopulations, means of 5 mice ± SEM. B, Poly(I:C) (pI:C)-induced change in cell numbers (ratio of untreated to poly(I:C) treated) in the indicated thymocyte subpopulations of wild-type (WT; n = 4) and MDA-5-deficient (n = 5) mice. C, Proportions of thymocyte subpopulations in wild-type (n = 4) and MDA-5-deficient (n = 5) mice; means ± SEM are indicated as bars. D, Means of the proportions within DN thymocyte subsets (DN1, CD44+CD25−; DN2, CD44+CD25+; DN3, CD44−CD25− and DN4, CD44−CD25+); n = 5 mice for each group ± SEM. E, Fraction of DP thymocytes of BALB/c mice 8 h after the last of two injections (days 0 and 3) of poly(I:C), R848 (ligand for TLR7), LPS (ligand for TLR4), and CpG (ligand for TLR9) for n = 5 mice per group. *p < 0.05; ** p < 0.01; ***p < 0.001; n.s., not significant; comparison with untreated unless indicated by brackets.
assess the importance of MDA-5-mediated signaling in histomorphological changes, thymi from MDA-5-deficient mice treated with poly(I:C) were examined. In contrast to wild-type mice, no histological alterations were observed in MDA-5-deficient mice (Fig. 3 C), confirming the essential role of MDA-5 for poly(I:C)-induced thymic pathology.

Involution is caused by suppression of proliferation and enhanced apoptosis of thymocytes

The rapid thymocyte loss upon MDA-5 activation could be due either to enhanced emigration of thymocytes or to increased apoptosis of developing T lymphocytes. In addition, poly(I:C)-triggered antiproliferative effects on thymocytes could contribute to thymic involution. To explore the mechanisms involved, we first measured T cell emigration out of the thymus after treatment with poly(I:C). FITC was injected directly into the thymus (26), and peripheral lymphoid organs were analyzed 4 days later for the presence of FITC-positive recent thymic emigrants. Two applications of poly(I:C) did not increase the fraction of emigrated lymphocytes detected in the peripheral lymphoid organs (Fig. 4). Instead, lymphocyte emigration was slightly reduced, albeit not significantly, after treatment with poly(I:C).

To investigate whether innate immune activation affects T cell proliferation in the thymus, we quantified proliferating cells by measuring the in vivo incorporation of BrdU. DP thymocytes that represent the most abundant population in the thymus showed the highest proliferative activity in untreated mice (Fig. 5 A). Upon immunostimulation with poly(I:C), we observed a marked suppression of proliferation for DP cells and for single-positive CD8 cells (Fig. 5 A). The DN and CD4 subpopulations showed low baseline proliferation that was not significantly affected by poly(I:C) (data not shown). The level of proliferation in DP and CD8 cells tended to recover as early as 48 h after the last application of poly(I:C). In the spleen, we observed conversely an increase in the number of proliferating CD8 T cells and B cells upon poly(I:C) treatment, indicating that suppression of cellular proliferation is specific for the thymus (Fig. 5 A).

To evaluate the role of apoptosis in thymic involution, we assessed the percentage of apoptotic cells within the thymocyte subpopulations upon treatment with poly(I:C). Early and late apoptosis was significantly enhanced in DP and DN thymocytes, respectively (Fig. 5 B). No changes in apoptosis were observed for T cells in the spleen (data not shown). Taken together, our results show that thymic involution following MDA-5 stimulation results from a marked decrease in thymocyte proliferation associated with increased rates of apoptosis.

MDA-5-mediated thymic suppression depends on the type I IFN receptor

Antiproliferative and proapoptotic effects are characteristic features of type I IFN activity. Recent studies in fetal thymic organ
cultures suggested that type I IFN inhibits thymocyte development in vitro (28). To examine a possible involvement of type I IFN in MDA-5-mediated thymic reduction, we treated mice deficient for IFNAR with poly(I:C). In contrast to the decrease in thymic weight seen in wild-type animals, we observed no significant decrease in IFNAR-deficient mice (Fig. 6, top). Further, the characteristic loss of DP thymocytes was completely absent in these mice. Because the receptor for type I IFN is expressed by a broad spectrum of cells in the organism (29), we investigated whether thymic involution is mediated through direct action of type I IFN on developing T cells. We used CD4cre+/−/IFNARflox/flox-transgenic mice in which the IFNAR is selectively deleted on all T cells. Loss of thymic weight and cellularity in these mice were comparable with those of wild-type animals (Fig. 6, middle). To examine whether B cells, representing an important fraction of immune cells in the mouse, may be involved in type I IFN-mediated thymic involution, CD19cre+/−/IFNARflox/flox mice that lack IFNAR expression on B cells, were injected with poly(I:C). Here again, as in wild-type mice, thymic weight and the fraction of DP cells were strongly reduced (Fig. 6, bottom). These data demonstrate that thymic involution is not mediated by the direct action of type I IFN on T cells or B cells.

Suppression of thymic cellularity occurs rapidly and is self-limiting

Typically, the secretion of type I IFN is an early and short-lasting event (2–48 h) upon viral infection or stimulation by synthetic...
ligands (30). Because thymic suppression is dependent on type I IFN, we examined whether poly(I:C)-mediated involution is reversible upon cessation of treatment by determining thymus weight at different times after a single application of poly(I:C). A significant reduction was observed as early as 24 h after stimulation (Fig. 7A). Suppression was less pronounced than after two injections of poly(I:C) (Fig. 7, A and B). After 3 days, an increase in thymus weight was detectable, and weight returned to initial levels 1 wk after stimulation (Fig. 7A). Recovery was delayed after two consecutive applications of poly(I:C) and did not reach initial levels 10 days after the second injection (Fig. 7B). All histological alterations observed during thymic involution were reversible, and the ratio of cortical to medullary tissue was restored 10 days after the last injection (Fig. 7C). These results demonstrate that MDA-5-induced suppression of the thymus is self-limiting and organ integrity is restored within 10 days after cessation of immune stimulation.

**Discussion**

Our results demonstrate that stimulation of innate immunity by in vivo activation of MDA-5 leads to involution of the thymus. Furthermore, we show that immune activation by a panel of potent TLR agonists neither caused involution of the thymus nor induced changes within the relative fractions of thymic subpopulations. Indeed, the only significant TLR-mediated effect observed was a mild decrease in thymic weight induced by two applications of the potent TLR7 ligand R848. These novel findings expand on recent results describing blockade of thymic output by stimulation with the dsRNA molecule poly(I:C) (31). In addition, our data support the concept that MDA-5, rather than TLR3 or RIG-I, is involved during the in vivo recognition of synthetic long dsRNA. Previous studies examining the relative importance of these receptors and their downstream signaling pathways for immune stimulation by poly(I:C) have focused on the induction of proinflammatory cytokines (4, 5, 24). Here we describe thymic pathology as a novel functional readout that reaffirms the importance of MDA-5 as an in vivo mediator of poly(I:C) activity. Taken together, our results show that in vivo activation of innate immunity through the cytoplasmic helicase MDA-5, but not through TLRs in general, leads to involution of the thymus.

Stimulation of pattern recognition receptors such as MDA-5, RIG-I, TLR3, or TLR7 with synthetic ligands mirrors the immune activation induced by infections with RNA viruses (2, 4). Infection of mice with reovirus, an RNA virus that activates innate immunity via RIG-I and MDA-5 (32), has been associated with atrophy of the thymus (21). In humans, a massive involution of the thymus was observed in children who died from acute infection with measles virus, a ssRNA virus that induces type I IFN via the MDA-5 receptor (33–35). Infection with HIV also leads to thymic damage and results in a decreased overall emigration of thymocytes that is reversible upon antiviral treatment (20, 36). Furthermore, a decrease in thymic cellularity has also been described in a model of T cell-restricted overexpression of lymphotoxins, proteins known to be induced upon viral infection (37). Taken together, these observations and our results suggest that viral infections may represent a natural trigger for rapid and reversible involution of the thymus. Furthermore, our results suggest that thymic involution may occur selectively upon infection with viruses known to activate innate immunity via MDA-5.

In our study, the synthetic dsRNA poly(I:C) causes thymic involution. This molecule is known as potent inducer of type I IFN (7), and we delineate here a key role for this cytokine in thymic suppression. It has been shown that plasmacytoid dendritic cells, the professional type I IFN producers, are abundantly present in the thymus. Isolated thymic plasmacytoid dendritic cells produce large amounts of type I IFNs upon viral stimulation and suppress the development of CD34⁺CD11c⁺ thymic progenitor cells in coculture assays (38, 39). Inhibition of thymic development by IFN-α has also been shown in newborn mice, where treatment with an active IFN-α/IFN-β hybrid molecule was associated with decreased cellularity of bone marrow and thymus (40). Additionally, type I IFN induced by poly(I:C) is reported to suppress fetal thymic organ cultures in vitro (28) and to block output of thymocytes in vivo (31). Here we demonstrate that type I IFN does not act directly on T cells, given that mice in which the IFNAR deficiency is restricted to T cells (CD4-Cre⁺/⁻ IFNARflx/flx) show a decrease in thymic cellularity similar to that of wild-type mice upon stimulation with poly(I:C). Similarly, we observed no direct effects of type I IFN on B cells. This suggests that the site of action of type I IFN is on nonlymphoid cells that, once stimulated, suppress T cell development in the thymus. Among non-immune cells residing in the thymus, thymic epithelial cells (TEC) represent a population that plays an essential role in thymocyte development and proliferation (41, 42). It has been reported that type I IFN, by directly acting on TECs, induces phenotypical and functional alterations of these cells in vitro that may impair thymocyte proliferation (43). It has further been shown that TECs themselves have the ability to produce type I IFN in response to transfection with poly(I:C) in vitro (43). It is thus possible that TECs can respond to in vivo stimulation by poly(I:C) and produce type I IFN that could essentially contribute to the MDA-5-induced thymic involution. Because MDA-5 is expressed ubiquitously in all lymphoid and various nonlymphoid tissues (44), systemic production of type I IFN upon poly(I:C) treatment may additionally affect TEC function and, consequently, thymocyte development. Finally, we cannot exclude that the effects seen in DN thymocyte subset may result from a direct action by type I IFN: in CD4-Cre⁺/⁻ IFNARflx/flx mice, the IFNAR is irreversibly deleted upon first expression of the CD4 Ag during T cell maturation, so that T lymphocyte precursors in the DN stage remain susceptible to type I IFN-mediated effects.

Activation of RIG-I-like helicases and TLRs has evolved as a promising strategy to activate the immune system for therapeutic purposes. We and others have shown that the ligand for TLR9, CpG, can be successfully used as an adjuvant for vaccination and CpG oligonucleotides are in clinical trials for the therapy of infectious and malignant diseases (45–48). Further, TLR7 agonists support Ag-specific T and B cell activation (49) and are effectively used against cutaneous malignant or premalignant lesions (50). Stimulation of the RIG-I receptor using triphosphate RNA or of MDA-5 with poly(I:C) mediates potent antitumoral effects in mice (51, 52). Finally, type I IFN itself is used for the treatment of cancer and chronic viral infections as hepatitis (16, 53). All of these molecules could affect thymic cellularity in humans, in particular potent inducers of type I IFN such as ligands for MDA-5 or RIG-I. Thus, the impact on thymic function should be considered during therapeutic immune activation, specifically in the case of chronic treatment regimens.

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**References**


