Cutting Edge: Polyinosinic:Polycytidylic Acid Boosts the Generation of Memory CD8 T Cells through Melanoma Differentiation-Associated Protein 5 Expressed in Stromal Cells

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Cutting Edge: Polyinosinic:Polycytidylic Acid Boosts the Generation of Memory CD8 T Cells through Melanoma Differentiation-Associated Protein 5 Expressed in Stromal Cells

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Polyinosinic:polycytidylic acid (poly I:C), a synthetic analog of double-stranded viral RNA, serves as a potent adjuvant for vaccination against soluble proteins, pathogens, and tumors. Poly I:C is sensed by both TLR3 in the endosomes and melanoma differentiation-associated protein 5 (MDA5) in the cytoplasm. Although it is known that TLR3 is required for cross-priming of CD8 T cells specific for viral Ags, the role of MDA5 in inducing CD8 T cell responses is still unclear. In this study, we demonstrate that in mice lacking MDA5, the majority of CD8 T cells do not survive after primary immunization with poly I:C and Ag, impairing memory response to subsequent Ag challenge. Furthermore, bone marrow chimera experiments revealed that MDA5 expression in radiation-resistant stromal cells, but not in radiation-sensitive hematopoietic cells, is essential for establishing CD8 T cell memory. We conclude that MDA5 and TLR3 mediate substantially distinct yet complementary functions during poly I:C-mediated activation of Ag-specific CD8 T cell responses. The Journal of Immunology, 2010, 184: 2751–2755.

Polyinosinic:polycytidylic acid (poly I:C) is a synthetic analog of viral dsRNA that activates immune responses through two dsRNA sensors, melanoma differentiation-associated protein 5 (MDA5) and TLR3. MDA5 detects poly I:C that penetrates into the cytosol, whereas TLR3 senses poly I:C that has been internalized by endocytosis (1, 2). Upon detection of poly I:C, MDA5 transmits signals through the adaptor IFN-β promoter stimulator 1 (IPS1), whereas TLR3 signals through the adaptor Toll/IL-1 resistance domain-containing adaptor-inducing IFN-β (TRIF). Both of these adaptors initiate downstream signaling pathways that lead to activation of a similar array of transcription factors, including IFN regulatory factor (IRF) 3, IRF7, IRF1, and NF-κB. These factors induce the expression of genes encoding type I IFNs (i.e., IFN-α and IFN-β), proinflammatory cytokines, and cell surface molecules involved in Ag presentation (1).

Because poly I:C is potentially an effective vaccine adjuvant (3, 4), considerable attention has recently focused on the mechanisms by which poly I:C boosts Ag-specific responses. Two recent studies demonstrated that poly I:C induces durable and protective CD4 T cell immunity when used as adjuvant for vaccines that target dendritic cells (DCs) (5, 6). In these settings, poly I:C boosted DC activation and CD4 T cell responses by promoting the systemic release of type I IFN by both stromal and hematopoietic cells. Type I IFN responses to poly I:C were primarily dependent on MDA5, although TLR3 also contributed to type I IFN secretion by DCs (5, 6).

The mechanisms by which poly I:C boosts CD8 T cell responses, particularly the relative importance of MDA5 and TLR3 and the types of cells activated (DCs and/or other accessory cells), are not well understood. An original study demonstrated that poly I:C stimulates TLR3 in CD8α+ DCs, which consequently acquire the ability to cross-prime CD8 T cells (7). Subsequently, it was shown that poly I:C enhances differentiation of effector CD8 T cells through a TLR3-independent pathway that is completely dependent on IFN-α/β (8). Corroborating this, a recent study showed that IPS1 and TRIF signaling pathways are both necessary for effective Ag-specific CD8 T cell responses (9), thereby implicating both TLR3 and MDA5 in this process. Whether MDA5 synergizes with TLR3 in CD8α+ DC to promote cross-priming of CD8 T cells or augments CD8 T cell responses through alternative mechanisms has not been addressed.

In this report, we investigated the relative contributions of MDA5 and TLR3 to poly I:C-mediated generation of Ag-specific CD8 T cells using MDA5−/−, TLR3−/−, and MDA5−/−×TLR3−/− double knockout (DKO) mice. We found that although TLR3 is essential for primary CD8 T cell responses, MDA5 is required for CD8 T cell memory.

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Abbreviations used in this paper: DC, dendritic cell; DKO, double knockout; HV, herpesvirus; IPS1, IFN-β promoter stimulator 1; IRF, IFN regulatory factor; MDA5, melanoma differentiation-associated protein 5; poly I:C, polyinosinic-polycytidylic acid; TRIF, Toll/IL-1 resistance domain-containing adaptor-inducing IFN-β; WT, wild-type.

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responses. Adoptive transfer of Ag-specific CD8 T cells demonstrated that MDA5 acts extrinsically to CD8 T cells by promoting a microenvironment that supports the survival of Ag-activated T cells and their conversion from effector to memory CD8 T cells. By generating reciprocal bone marrow chimeras between wild-type (WT) and MDA5−/− mice, we found that MDA5 acts primarily through radio-resistant stromal cells rather than radio-sensitive hematopoietic cells. Mechanistically, we provide evidence that MDA5 is essential for induction of the antipapoptotic molecule Bcl-3 in activated CD8 T cells, because upregulation of Bcl-3 is dependent on the direct exposure of CD8 T cells to systemic IFN-α released by radio-resistant cells in response to poly I:C. We conclude that MDA5 and TLR3 mediate substantially distinct yet complementary functions during poly I-C-mediated activation of Ag-specific CD8 T cell responses.

Materials and Methods

Mice

MDA5−/− (10), TLR3−/− (11), and DKO (5) mice were all on the C57BL/6 background (confirmed by genome-wide simple sequence length polymorphism typing at 10-cM intervals for the MDA5−/− mice by the Rheumatology Speed Congenics Core Laboratory at Washington University School of Medicine, St. Louis, MO).

Immunizations and viral challenge

Mice were immunized with 500 μg soluble OVA (Sigma-Aldrich) and 50 μg poly I:C (Invivogen, San Diego, CA) i.p. For secondary challenge, immunized mice were administered the same Ag-poly I:C mixture i.p. 5 wk after the initial immunization. Alternatively, secondary challenge was performed by infecting mice with 10⁶ PFU γ herpesvirus (HV)-OVA virus i.p. Spleens were harvested 7 d postinfection as previously described (12). Briefly, spleens were homogenized with silicon beads, serially diluted, and plated onto National Institutes of Health 3T12 cells. Viral plaques were determined 7 d postinfection.

Adoptive transfers

A total of 1 × 10⁶ purified OT-1 CD8 T cells (99% purity) were injected i.v.; soluble OVA or OVA plus poly I:C was given i.v. 24 h post cell transfer as previously described (13).

Bone marrow chimeras

WT or MDA5−/− mice were lethally irradiated with 1000 rads. T cell- and B cell-depleted bone marrow cells (3 × 10⁶) were infused i.v. 24 h postirradiation. Mice were treated with antibiotics for 6 d postirradiation. To assess reconstitution of the hematopoietic compartment, we determined percentages of CD3+, CD4+, CD8+, CD19+, CD11c+, Gr-1+, and NK1.1+ cells in the spleen. To assess chimerism, we determined the relative abundance of WT and mutated MDA5 alleles in genomic DNA obtained from tails (recipient) and spleen cells (donor) by PCR.

Flow cytometry and ELISA

Splenic cells were stained with H2-Kb/SIINFEKL tetramers-FTTC (Beckman Coulter, Fullerton, CA) and CD8-APC (BD Pharmingen, San Diego, CA) and analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). For intracellular staining, cells were first fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin, then stained with anti-IFN-γ APC (BD Pharmingen). Bcl-3-APC (Cell Signaling Technology, Beverly, MA), or purified Bcl-3 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) plus APC labeled secondary Ab. Additional stainings were performed using Abs for granzyyme B, perforin, CD3, CD4, CD19, CD11c, Gr-1, and NK1.1 (BD Pharmingen). To measure systemic IFN-α, serum was collected 24 h postimmunization and analyzed using an IFN-α ELISA kit (PBL Interferon Source, Piscataway, NJ).

Immunoblot

CD8 T cells were purified from OT-1 mice using Miltenyi mouse CD8 T cell negative selection beads (Miltenyi Biotec, Auburn, CA). Purified cells were cultured in the presence of 1000 U/ml IFN-β with or without IL-2. Bcl-3 was detected using a polyclonal Bcl-3 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Results and Discussion

Poly I:C boosts cross-priming of naive CD8 T cells independently of MDA5

Because TLR3 promotes cross-presentation of viral Ags to CD8 T cells (7), we first investigated whether MDA5 is also involved in this process. WT, MDA5−/−, TLR3−/−, and MDA5−/− × TLR3−/− DKO mice were immunized with soluble OVA and poly I:C. Spleens were harvested 7 d postimmunization and the frequency of OVA257–264-specific CD8 T cells was assessed by flow cytometry using H2-Kb-SIINFEKL tetramers. Additionally, IFN-γ–producing OVA257–264-specific CD8 T cells were examined by stimulating spleenocytes in vitro with SIINFEKL peptide. In agreement with Schulz et al. (7), TLR3−/− mice were unable to mount a robust CD8 T cell response against OVA (Fig. 1A, Supplemental Fig. 1A). Similarly, essentially no OVA-specific CD8 T cells were detected in DKO mice. In contrast, lack of MDA5 did not affect cross-priming of CD8 T cells, as both the percentage and absolute number of OVA257–264-specific CD8 T cells were comparable to those observed in WT mice. The frequency of IFN-γ+ CD8 T cells was partially reduced in the MDA5−/− mice (Fig. 1B), but the intracellular content

**FIGURE 1.** Primary CD8 T cell response in TLR3−/−, MDA5−/−, DKO, and WT mice. WT, MDA5−/−, TLR3−/−, and DKO mice were immunized with OVA and poly I:C. Spleen cells were examined by flow cytometry for the frequency of H2-Kb-SIINFEKL tetramer+ cells within CD8 T cells 7 d postimmunization (A). Numbers within the plots represent mean percentage ± SE of tetramer+ CD8 T cells. B, Splenic cells were also stimulated in vitro with SIINFEKL peptide (1 μg/ml) in the presence of brefeldin A (5 μg/ml) for 6 h, then stained for intracellular IFN-γ and analyzed by flow cytometry. Plots of IFN-γ intracellular content of CD8 T cells are representative of three different experiments.
of granzyme B or perforin in OVA257–264-specific CD8 T cells was similar in WT and MD5−/− mice (Supplemental Fig. 1B). These results suggest that MD5 is dispensable for CD8 T cell priming, but is partly required to enhance effector function.

Poly I:C enhances CD8 T cell memory response through MD5

We next asked whether MD5 is required for CD8 T cell memory responses. WT, MD5−/−, TLR3−/−, and DKO mice were primed with OVA and poly I:C. Five weeks after the primary immunization, no OVA257–264-specific CD8 T cells could be detected in the peripheral blood (data not shown). At this point, we challenged mice with OVA plus poly I:C and, after 5 d, harvested spleens and assessed the numbers of OVA257–264-specific CD8 T cells as well as the frequency of IFN-γ+ CD8 T cells following in vitro restimulation with the SIINFEKL peptide. Both the frequency and absolute numbers of OVA257–264-specific and IFN-γ+ CD8 T cells were markedly reduced in MD5−/− mice as compared with WT mice (Fig. 2A, 2B). As expected based on the defect in primary CD8 T cell responses, memory CD8 T cells and IFN-γ+ CD8 T cells were also significantly reduced in TLR3−/− mice and essentially absent in DKO mice (Fig. 2A, 2B). The relative numbers of OVA257–264-specific and IFN-γ+ CD8 T cells in the peripheral blood of MD5−/−, TLR3−/−, and DKO mice paralleled those observed in the spleens (data not shown).

To see whether MD5 is required to establish a CD8 T cell memory response that protects from viral challenge, we vaccinated WT and MD5−/− mice with poly I:C and OVA and then challenged them 5 wk after with a γHV engineered to express OVA (12). MD5−/− mice showed significant reduction of tetramer+ CD8 T cells and increase of viral titers in the spleen as compared with WT mice (Fig. 2C, 2D). Altogether, these results demonstrate that MD5 is essential for establishing Ag-specific memory CD8 T cells when poly I:C is used as an adjuvant.

Poly I:C boosts memory CD8 T cell responses by promoting the survival of activated T cells via T cell-extrinsic mechanisms

To determine whether MD5 promotes CD8 T cell memory responses through T cell-intrinsic or -extrinsic mechanisms, we purified CD8 T cells that recognize the OVA257–264 peptide from OT-I TCR transgenic mice and transferred them into WT, MD5−/−, TLR3−/−, or DKO mice. Twenty-four hours posttransfer, mice were immunized i.v. with OVA with or without poly I:C. OVA257–264-specific CD8 T cells in the peripheral blood were monitored over a time course of 12 d using H2-Kb/SIINFEKL tetramers. Consistent with previous studies of effector and memory CD8 T cell differentiation (14), OT-I T cells transferred into WT mice immunized with OVA and poly I:C initially expanded and peaked on day 4 posttransfer, then declined during a contraction phase after which the numbers of OT-I T cells, which were presumably differentiating into memory cells, remained relatively stable at least up to day 12 (Fig. 3A). OT-I T cells that were transferred into mice receiving OVA without poly I:C disappeared rapidly after an initial expansion. OT-I CD8 T cells transferred into TLR3−/− mice that received both Ag and poly I:C initially expanded, but then rapidly declined to baseline levels similar to those observed in WT mice that had received Ag alone (Fig. 3A). This was presumably due to lack of CD8× DC activation and cross-priming of T cells (7). OT-I CD8 T cells transferred to MD5−/− mice immunized with both Ag and poly I:C expanded, peaked, and contracted in a manner almost indistinguishable from those transferred to WT mice primed with OVA and poly I:C. However, the percentage of OT-I CD8 T cells continued to decline after day 8 up to day 12 in the MD5−/− mice (Fig. 3A). This result suggested that MD5 may promote the survival of activated CD8 T cells that outlive the contraction phase, facilitating their differentiation into memory cells.

To corroborate this hypothesis, we immunized WT, MD5−/−, TLR3−/−, and DKO mice with OVA plus poly I:C and assessed the frequency and total numbers of OVA257–264-specific CD8 T cells at day 10 postimmunization, when, in our experimental setting, the contraction phase was essentially complete. In contrast to what we observed on day 7 postimmunization (Fig. 1), the frequency and total numbers of OVA257–264-specific CD8 T cells were notably reduced in the spleens of MD5−/− mice in comparison with WT mice (Fig. 3B). This supports the idea that lack of MD5 may prohibit survival of Ag-specific CD8 T cells after the initial expansion and contraction phases, which may affect subsequent generation of the CD8 T cell memory pool.

We also investigated whether TLR3 plays a role in the secondary CD8 T cell response, independently of its function...
in the initial cross-priming of CD8 T cells. For this purpose, we first activated OT-I CD8 T cells in vitro using IL-2 and Ag-pulsed cells. We then transferred these cells into WT, MDA5−/−, and TLR3−/− mice. We challenged mice with OVA plus poly I:C 4 wk postinjection and then measured the numbers of tetramer+ CD8 T cells in the spleen. Although both MDA5−/− and TLR3−/− showed minor reductions of total tetramer+ CD8 T cells as compared with WT mice, such differences were not statistically significant (Fig. 3B). We conclude that neither sensor is required for CD8 T cell recall responses once a pool of activated/memory CD8 T cells has been generated.

Poly I:C enhances memory CD8 T cells through MDA5 expression in stromal cells

MDA5 is expressed in both hematopoietic and stromal cells and is strongly upregulated by type I IFN (1). To determine whether preservation of OVA257–264-specific memory CD8 T cells required MDA5 function in the radio-sensitive hematopoietic or radio-resistant stromal compartment, we generated reciprocal bone marrow chimeras between WT and MDA5−/− mice. Reconstituted bone marrow chimeras were immunized with OVA plus poly I:C and challenged 5 wk later with the same mixture; the frequency of OVA257–264-specific CD8 T cells in the spleen was determined 5 d after the recall challenge. Both the frequency and total numbers of OVA257–264-specific CD8 T cells were significantly reduced in MDA5−/− mice reconstituted with WT bone marrow as compared with WT mice reconstituted with MDA5−/− bone marrow (Fig. 4A). We conclude that survival of activated CD8 T cells and subsequent generation of memory CD8 T cells in response to Ag plus poly I:C requires MDA5 function in the radio-resistant compartment, which includes stromal cells and, possibly, some radio-resistant hematopoietic cells, such as certain macrophage subsets.

Induction of Bcl-3 expression in CD8 T cells postimmunization with Ag and poly I:C is MDA5-dependent

Type I IFNs are essential for keeping activated T cells alive after Ag stimulation (15–20). Moreover, when used as adjuvants in immunizations with soluble proteins or peptides, LPS and poly I:C promote the survival of activated T cells through induction of the Bcl-2 family member proteins Bcl-3 and Bcl-xL (21, 22). Given this, we asked whether MDA5 is essential for type I IFN responses and/or expression of antiapoptotic molecules in CD8 T cells following immunization with poly I:C and OVA. In agreement with previous studies (10, 23), we found that immunization with poly I:C and OVA induced a marked increase of IFN-α in the serum of WT mice that

FIGURE 3. Impact of MDA5 and TLR3 deficiencies during different phases of CD8 T cell response. A, Kinetics of OT-I CD8 T cell expansion and contraction after primary Ag challenge. OT-I CD8 T cells were adoptively transferred into WT, MDA5−/−, TLR3−/−, and DKO mice. OVA or OVA plus poly I:C was injected 24 h posttransfer. Peripheral blood OT-I CD8 T cells were monitored for 12 d using H2-Kb/SIINFEKL tetramers. Absolute numbers of splenic OVA257–264-specific CD8 T cells in WT, MDA5−/−, TLR3−/−, and DKO mice 10 d after primary immunization with OVA and poly I:C. B, Total numbers of H2-Kb/SIINFEKL tetramer+ CD8 T cells in mice adoptively transferred with activated OT-I cells and then challenged with OVA plus poly I:C. OT-I T cells were adoptively transferred into WT mice and then challenged with OVA plus poly I:C. C, Total numbers of H2-Kb/SIINFEKL tetramer+ CD8 T cells in mice adoptively transferred with activated OT-I cells and then challenged with OVA plus poly I:C. OT-I T cells were adoptively transferred into WT, MDA5−/−, and TLR3−/− mice. Four weeks later, one group of mice was challenged with OVA and poly I:C, another group was left untreated. Tetramer+ cells were measured 5 d postchallenge and unchallenged mice. ***p < 0.001; *p < 0.05 (Student t test).

FIGURE 4. Role of stromal MDA5 in secondary CD8 T cell responses and MDA5-dependency of systemic IFN-α response and CD8 T cell expression of Bcl-3. A and B, Bone marrow cells from WT mice were injected into lethally irradiated MDA5−/− mice and vice versa. Chimeric mice were immunized with OVA or OVA plus poly I:C, and frequency (A) and absolute numbers (B) of splenic H2-Kb/SIINFEKL tetramer+ CD8 T cells were examined 5 d postchallenge. C, Systemic IFN-α response to poly I:C in WT, MDA5−/−, TLR3−/−, and DKO mice. Serum was collected from mice immunized with OVA and poly I:C 24 h postinjection. IFN-α levels were determined by ELISA. D, Bcl-3 and Bcl-xL expression in Ag-specific CD8 T cells. Expression of Bcl-xL and Bcl-3 in H2-Kb/SIINFEKL tetramer+ CD8 T cells was examined by flow cytometry 10 d postimmunization with OVA and poly I:C mixture. Black lines = Bcl-3 or Bcl-xL in WT mice; gray lines = Bcl-3 or Bcl-xL in MDA5−/− mice; gray filled profiles = control Ab (similar in all mice). E, Direct exposure of CD8 T cells to type I IFN (IFN-β) induces Bcl-3 expression. Purified WT OT-I CD8 T cells were incubated with recombinant IFN-β and/or IL-2, which induces CD8 T cell survival and therefore is used as a positive control. Cells were lysed and analyzed by immunoblot for the expression of Bcl-3. **p < 0.01 (Student t test).
was completely MDA5-dependent (Fig. 4B). Furthermore, we assessed Bcl-3 and Bcl-xL expression in H2-Kb/SIINFEKL tetramer+ CD8 T cells from WT and MDA5−/− mice 10 d postinfection with OVA and poly I:C. This analysis could not be performed in TLR3−/− mice because of the very low numbers of tetramer+ CD8 T cells. Remarkably, Bcl-3 was expressed in Ag-specific CD8 T cells from WT mice but not in those from MDA5−/− mice (Fig. 4C). No expression of Bcl-xL was found in tetramer+ CD8 T cells from either WT or MDA5−/− mice (Fig. 4C). Thus, poly I:C-induced Bcl-3 expression in activated CD8 T cells is MDA5-dependent. Finally, because type I IFNs were shown to act directly on CD8 T cells to prolong their survival during Ag-driven clonal expansion (15, 17, 19), we asked whether exposure of CD8 T cells to type I IFN could induce Bcl-3 expression. Immunoblot analysis of CD8 T cells incubated in vitro with type I IFN demonstrated that this is, indeed, the case (Fig. 4D). Together, these results indicate that poly I:C induces an MDA5-dependent release of systemic IFN-α, which can act directly on CD8 T cells inducing Bcl-3 expression.

In summary, this study delineates a striking dichotomy in the mechanisms by which poly I:C boosts CD8 T cell responses to soluble Ags. Poly I:C enhances memory CD8 T cell responses predominantly through MDA5. Our results are consistent with a model in which poly I:C activates MDA5 in the stromal compartment, resulting in a systemic increase of IFN-α. Exposure of Ag-activated CD8 T cells to IFN-α induces the expression of Bcl-3, which promotes their survival after the initial phases of proliferation and contraction, facilitating the generation of memory CD8 T cells. Poly I:C also boosts primary CD8 T cell responses to soluble Ag. However, in agreement with previous studies (7), this effect is mainly TLR3-dependent. Although MDA5 is not required for generation and expansion of Ag-specific CD8 T cells during the primary response, it does have a partial role in the acquisition of CD8 T cell effector functions, such as IFN-γ secretion. Most likely, this effect is also secondary to the MDA5-dependent increase of systemic IFN-α, which has been shown to induce IFN-γ through engagement of the type I IFN receptor and subsequent activation of STAT4 (24). Because CD4 T cells provide help to CD8 T cell responses, and a recent study showed that poly I:C-induced type I IFN enhances CD4 T cell responses (5), the adjuvant effect of poly I:C on CD8 T cell response might be partly mediated via CD4 T cells. This hypothesis will require further investigation. We conclude that the adjuvant effect of poly I:C on CD8 T cell responses requires both MDA5 and TLR3, which act in distinct cell compartments and during different phases of CD8 T cell activation, providing complementary mechanisms for optimal adaptive CD8 T cell responses.

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Disclosures

The authors have no financial conflicts of interest.

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