Ro60 Requires Y3 RNA for Cell Surface Exposure and Inflammation Associated with Cardiac Manifestations of Neonatal Lupus

Joanne H. Reed, Soyeong Sim, Sandra L. Wolin, Robert M. Clancy and Jill P. Buyon

*J Immunol* 2013; 191:110-116; Prepublished online 22 May 2013;
doi: 10.4049/jimmunol.1202849
http://www.jimmunol.org/content/191/1/110

References This article cites 29 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/191/1/110.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Ro60 Requires Y3 RNA for Cell Surface Exposure and Inflammation Associated with Cardiac Manifestations of Neonatal Lupus

Joanne H. Reed,* Soyeong Sim,† Sandra L. Wolin,† Robert M. Clancy,* and Jill P. Buyon*

Cardiac neonatal lupus (NL) is presumed to arise from maternal autoantibody targeting an intracellular ribonucleoprotein, Ro60, which binds noncoding Y RNA and only becomes accessible to autoantibodies during apoptosis. Despite the importance of Ro60 trafficking in the development of cardiac NL, the mechanism underlying cell surface exposure is unknown. To evaluate the influence of Y RNA on the subcellular location of Ro60 during apoptosis and activation of macrophages, stable Ro60 knockout murine fibroblasts expressing wild-type or mutated FLAG-Ro60 were assessed. FLAG<sub>3</sub>-Ro60(K170A R174A) binds Y RNA, whereas FLAG<sub>3</sub>-Ro60(H187S) does not bind Y RNA; fibroblasts expressing these constructs showed equivalent intracellular expression of Ro60. In contrast, apoptotic fibroblasts containing FLAG<sub>3</sub>-Ro60(K170A R174A) were bound by anti-Ro60, whereas FLAG<sub>3</sub>-Ro60(H187S) was not surface expressed. RNA interference of mY3 RNA in wild-type fibroblasts inhibited surface translocation of Ro60 during apoptosis, whereas depletion of mY1 RNA did not affect Ro60 exposure. Furthermore, Ro60 was not exposed following overexpression of mY1 in the mY3-depleted fibroblasts. In an in vitro model of anti-Ro60-mediated injury, Y RNA was shown to be an obligate factor for TLR-dependent activation of macrophages challenged with anti-Ro60–opsonized apoptotic fibroblasts. Murine Y3 RNA is a necessary factor to support the surface translocation of Ro60, which is pivotal to the formation of immune complexes on apoptotic cells and a TLR-dependent proinflammatory cascade. Accordingly, the Y3 RNA moiety of the Ro60 ribonucleoprotein imparts a critical role in the pathogenicity of maternal anti-Ro60 autoantibodies.


This work was supported by an American Heart Association postdoctoral fellowship (to J.H.R.), Australian National Health and Medical Research Council Overseas Postdoctoral Research Grant 595989 (to J.H.R.), an Arthritis Foundation postdoctoral fellowship (to J.H.R.), National Institutes of Health Grant R01 GM073863 (to S.L.W.), a grant from the Lupus Research Institute (to S.L.W.), and by National Institutes of Health Merit Award R37 AR042455 (to J.P.B.).

Received for publication October 11, 2012. Accepted for publication April 23, 2013.

Abbreviations used in this article: β2GPI, β2-glycoprotein I; MFI, mean fluorescence intensity; NL, neonatal lupus; PI, propidium iodide; siRNA, small interfering RNA.
Despite the relevance of Ro60 translocation to the initiation of tissue injury in cardiac NL, the mechanisms underlying cell surface exposure during apoptosis are unknown. Given the potential for Y RNA to modulate the subcellular location of Ro60 and initiate inflammatory responses in macrophages, in this study we explore the requirement of Y RNA for Ro60 translocation and subsequent activation of TLR signaling. Cells expressing Ro60 mutants in which single amino acid substitutions influence the binding of associated RNA were used, together with RNA interference and overexpression of specific Y RNA subsets, to address the dependency of Y RNA in the translocation of Ro60 during apoptosis.

**Materials and Methods**

**Antibodies**

Affinity-purified anti-Ro60 or anti-La Abs were isolated from the serum of a mother with a child with cardiac NL (anti-Ro60, n = 5; anti-La, n = 3) by affinity column chromatography using recombinant Ro60 or La coupled to Affigel10 (Bio-Rad) as previously described (6). Eluted Abs were tested for specificity by ELISA and immunoblot (4, 6). All mothers were enrolled in the Research Registry for Neonatal Lupus and signed informed consent approved by the New York University School of Medicine Institutional Review Board for the use of their sera. Control IgG was isolated from the sera of healthy adults (n = 3) using a protein A-IgG isolation kit (Pierce, Rockford, IL) according to the manufacturer’s recommendations. IgG fractions were treated with Detoxi-Gel and LPS concentration was <1 endotoxin unit/ml as assessed by a Limulus amebocyte lysate endotoxin quantification assay (Lanza).

**Cells**

The wild-type and Ro60 knockout murine fibroblasts have been described (16), as has the generation of stable Ro60 knockout fibroblast lines expressing wild-type FLAG3-Ro60, and FLAG3-Ro60 containing either the single mutation H1875S or the two point mutations K170A and R174A (16). The H1875S point mutation uniquely bind Y RNA, whereas K170A R174A mutations do not affect the Ro60 Y RNA interaction. All fibroblasts were cultured in DMEM (Invitrogen) containing 10% FBS and 2 mmol/lL-glutamine.

Human macrophages derived from PBMCs were obtained from random healthy donors (New York Blood Center, New York, NY) by centrifugation on Ficoll-Hypaque gradients and positive selection using anti-CD14 microbeads (Miltenyi Biotec). Isolated CD14+ cells were cultured in Teflon beakers (RPMI 1640/10% FBS) for 7 d in the presence of GM-CSF (19, 20). The human monocytic cell line THP-1 (21) was differentiated with 0.2 µM PMA as described (21). To confirm knockdown, RNA was prepared from cells treated with secondary Ab only. Anti-Ro60 binding was normalized to expression relative to untreated cells. The siRNA-treated cells and 5S rRNA as described (16). Y RNA expression was calculated by the manufacturer’s recommendations. Extracellular expression of Ro60 was assessed on nonfixed, nonpermeable, early apoptotic fibroblasts gated as annexin V+, PI−. Cells (permeabilized or apoptotic) were incubated with 5 µl annexin V-allophycocyanin (BD Biosciences) and 5 µmol/lPI (Invitrogen) in annexin V binding buffer (10 mmol/lHEPES, 140 mmol/lNaCl, 2.5 mmol/lCaCl2 [pH 7.4]) were added for 15 min at room temperature. 

Flow cytometry for evaluation of Ro60 expression

**Evaluating intracellular Ro60 expression by immunoblot**

Fibroblasts were lysed in TBS containing 1% Nonidet P-40 and protease inhibitors (Roche) for 30 min on ice. Lysates were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes and blocked with 5% nonfat skim milk powder in PBS overnight at 4°C. After three washes (PBS/0.1% Tween 20), membranes were probed with either human anti-Ro60 Ab (1 µg/ml) or anti-FLAG mAb (0.5 µg/ml) for 1 h at room temperature. Membranes were stained with IRDye 800CW anti-human or anti-mouse IgG conjugates and analyzed on the Odyssey infrared imager (LI-COR Biosciences).

**D cocultures of apoptotic fibroblasts and macrophages**

For coculture experiments, PBMC-derived macrophages or PMA-differentiated THP-1 cells (2 × 10^6/well) were seeded in 24-well tissue culture plates. After washing twice with HBSS, cells were primed with 50 pg/ml IFN-γ in serum-free RPMI 1640 for 6 h. Apoptotic murine fibroblasts incubated with control IgG or anti-Ro60 Ab for 45 min at room temperature were then washed twice and added to IFN-γ–primed macrophages or THP-1 cells for 18 h. To confirm dependence on TLR7 signaling, 10 µg/ml TLR7 inhibitor IRS661 (22), a gift from Dr. Franck Barrat (Dyavax Technologies, Berkeley, CA), was added to macrophages 30 min prior to the addition of apoptotic fibroblasts. In other experiments, the protective properties of the plasma protein β2-glycoprotein I (β2GPI), previously shown to compete with anti-Ro60 Ab for binding to apoptotic cells, were evaluated. β2GPI (1−50 µg/ml) was coincubated with anti-Ro60 Ab and apoptotic fibroblasts prior to the addition to macrophages. Conditioned media from the culturing conditions were centrifuged at 14,000 rpm for 3 min to remove cell debris. The readout of TLR signaling was the release of the inflammatory cytokine TNF-α measured by ELISA (R&D Systems) according to the manufacturer’s instructions.

**Statistical analysis**

For flow cytometry experiments, the binding of affinity-purified Ab was expressed as the mean fluorescence intensity (MFI) minus the baseline MFI of cells treated with secondary Ab only. Anti-Ro60 binding was normalized by dividing the MFI of siRNA treated fibroblasts by the MFI of untreated fibroblasts ×100. The percentage inhibition of TNF-α secretion was evaluated by dividing the TNF-α concentration in the presence of IRS661 or β2GPI by the TNF-α concentration in the absence of IRS661 or β2GPI. Differences were compared by one-way ANOVA or repeated measures ANOVA with Bonferroni post hoc analysis where appropriate. A p value < 0.05 was considered statistically significant.

**Results**

Intracellular expression of Ro60 is equivalent in knockout fibroblasts stably expressing varied FLAG3-Ro60 constructs

As our experimental system, we used murine Ro60 knockout fibroblasts that stably express epitope-tagged wild-type Ro60 (FLAG3-Ro60) and two mutant forms of Ro60, FLAG3-Ro60

Induction of apoptosis in murine fibroblasts

Apoptosis was induced by treating fibroblasts with 0.1 µg/ml murine IFN-γ for 24 h. Cells were then seeded onto tissue culture dishes coated with poly (2-hydroxyethyl methacrylate), which disrupts adhesion to the surface and hence stimulatory signals received during growth in the presence of IFN-γ (0.1 µg/ml), TNF-α (5 ng/ml), and cycloheximide (100 µg/ml) for 4−18 h (5). Apoptosis was confirmed by flow cytometric analysis of phosphatidylserine exposure (annexin V binding). The integrity of apoptotic cell membrane was monitored by propidium iodide (PI).

Flow cytometry for evaluation of Ro60 expression

Intracellular expression of Ro60 was evaluated on cultured fibroblasts using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s recommendations. Extracellular expression of Ro60 was assessed on nonfixed, nonpermeable, early apoptotic fibroblasts gated as annexin V−, PI+. Cells (permeabilized or apoptotic) were incubated with 5 µg/ml affinity-purified Abs for 45 min at room temperature. After incubation cells were washed twice (PBS/1% BSA/0.02% sodium azide) and stained with anti-human IgG-FITC (1:200) for 30 min. For apoptotic cells, 5 µl annexin V-allophycocyanin (BD Biosciences) and 5 µmol/l PI (Invitrogen) in annexin V binding buffer (10 mmol/lHEPES, 140 mmol/lNaCl, 2.5 mmol/lCaCl2 [pH 7.4]) were added for 15 min at room temperature. Binding was assessed on a LSRII flow cytometer (BD Biosciences).

Knockdown of mY1 and mY3 RNAs and Northern blot

siRNA targeting mY1 or mY3 were designed as described (16). Non-targeting siRNA no. 1 (Dharmacon, Lafayette, CO) was used as a control. Wild-type murine fibroblasts (1 × 10^5) in growth medium were transfected with 400 pmol siRNA mixed with 37.5 pmol Lipofectamine 2000 (Invitrogen) in 75-cm² flasks. After 48 h, cells were harvested in HBSS (Invitrogen). To confirm knockdown, RNA was prepared from ∼2 × 10^5 cells and subjected to Northern blot with oligonucleotide probes to detect mY1, mY3 RNA, and 5S rRNA as described (16). Y RNA expression was calculated by the following equation: (quantification of pixels for mY1 or mY3 immunoblot quantification of pixels for 5S (RNA) × 100). Y RNA levels were presented as a ratio of expression relative to untreated cells. The siRNA-treated cells were permeabilized or rendered apoptotic to evaluate intracellular or extracellular expression of Ro60, respectively, as described below.

**Overexpression of mY1 and mY3 RNAs**

To overexpress Y RNAs, the genes and flanking regions were amplified from mouse genomic DNA and cloned into the EcoRI and BamHI sites of pBluescriptII KS+. The mY3 clone has sequences of 225 nt downstream. Wild-type fibroblasts in growth medium were cotransfected using Lipofectamine 2000 with 1 µg plasmid and 400 pmol siRNA. After 48 h, cells were harvested in HBSS and Y RNA overexpression and/or knockdown was confirmed by Northern blotting (16). Intracellular and extracellular expression of Ro60 was evaluated as described below.
pressing FLAG3-Ro60 constructs by evaluating binding of anti-broblast cell lines bound control IgG (Fig. 1A). Intracellular content, both FLAG3-Ro60(K170A R174A) and FLAG3-Ro60(H187S) showed equivalent reactivity with anti-FLAG mAb bound to permeabilized fibroblasts. Fibroblasts expressing FLAG3-Ro60(K170A R174A) (binds Y RNA) were bound by anti-Ro60 Ab but not control IgG (MFI of 254 ± 79 versus 27 ± 15, p < 0.05). Whereas cell surface expression of this Ro60 mutant was significantly less than that of wild-type murine fibroblasts (MFI of 659 ± 74, p < 0.05 versus FLAG3-Ro60(K170A R174A)), expression was equivalent to wild-type FLAG3-Ro60 mutant (MFI of 274 ± 92, p = NS versus FLAG3-Ro60(H170A R174A)). In contrast, apoptotic fibroblasts expressing FLAG3-Ro60(H1785) were not bound by anti-Ro60 Ab (MFI of 30 ± 20 versus FLAG3-Ro60(K170A R174A), p < 0.05; versus wild-type, p < 0.0005; versus FLAG3-Ro60, p < 0.05) and showed an equivalent MFI to Ro60 knockout apoptotic cells (MFI of 19 ± 17, p = NS versus FLAG3-Ro60(H1785)) and that obtained when incubating with control IgG (Fig. 2B). Late apoptotic fibroblasts showed similar binding profiles as early apoptotic for each fibroblast line (wild-type, MFI of 1824 ± 53; FLAG3-Ro60, MFI of 53 ± 35; FLAG3-Ro60, MFI of 795 ± 243; FLAG3-Ro60(K170A R174A), MFI of 873 ± 292; FLAG3-Ro60(H1785), MFI of 178 ± 30) (Fig. 2C). La, previously shown to be expressed on late apoptotic cells (24), was also evaluated on apoptotic murine fibroblasts to determine whether Ro60/Y RNA composition (or its absence) affects La expression. Affinity-purified anti-La Ab showed equivalent binding to all murine fibroblasts independent of the expression of Ro60/Y RNA (Fig. 1D).

**Surface translocation of Ro60 during apoptosis varies in fibroblasts expressing mutated FLAG3-Ro60 constructs**

Having established equivalent intracellular expression of Ro60 in fibroblasts expressing FLAG3 constructs, cell surface expression of Ro60 was then evaluated. Murine fibroblasts were rendered apoptotic, and binding of anti-Ro60 Ab was measured by flow cytometry. Apoptotic cells were gated according to annexin V and PI staining where early apoptotic cells were annexin V+ and PI− (i.e., maintained membrane integrity). Late apoptotic cells were annexin V+ and PI+ (Fig. 2A). Early apoptotic fibroblasts expressing FLAG3-Ro60(K170A R174A) (binds Y RNA) were bound by anti-Ro60 Ab but not control IgG (MFI of 254 ± 79 versus 27 ± 15, p < 0.05). Whereas cell surface expression of this Ro60 mutant was significantly less than that of wild-type murine fibroblasts (MFI of 659 ± 74, p < 0.05 versus FLAG3-Ro60 (K170A R174A)), expression was equivalent to wild-type FLAG3-Ro60(H187S); knockout murine fibroblasts did not react with anti-FLAG mAb (MFI of 19 ± 11 and 6 ± 3, respectively, p = NS for all comparisons). As expected, wild-type and Ro60 knockout fibroblasts did not react with anti-FLAG mAb (MFI of 1824 ± 531, p = NS for all comparisons). Previous characterization of these cell lines demonstrated that although Y RNAs are reduced ~30-fold in the Ro60 knockout fibroblasts and FLAG3-Ro60(H1785) cells (16, 23), these RNAs are present at near wild-type levels in the FLAG3-Ro60 and FLAG3-Ro60(K170A R174A) cells (16). Initial experiments were done to assure intracellular binding of human affinity-purified anti-Ro60 Ab to murine wild-type and mutated Ro60 and to compare intracellular levels of Ro60 across the different fibroblast cell lines. Permeabilized wild-type murine fibroblasts were bound by human anti-Ro60 but not control IgG (MFI of 4614 ± 413 versus 145 ± 60, p < 0.0005), substantiating crossreactivity between murine Ro60 and human anti-Ro60 Ab. The specificity of the anti-Ro60 Ab was confirmed by the absence of binding to permeabilized Ro60 knockout fibroblasts (MFI of 33 ± 33). Despite differences in Y RNA content, both FLAG3-Ro60(K170A R174A) and FLAG3-Ro60(H1785) cells showed equivalent intracellular binding of anti-Ro60 (MFI of 1664 ± 355 and 1471 ± 699, p = NS). In cells stably expressing wild-type FLAG3-Ro60, intracellular expression of Ro60 was similar to that observed with FLAG3-Ro60(K170A R174A) and FLAG3-Ro60(H1785) mutant cells (MFI of 2280 ± 539, p = NS). None of the permeabilized fibroblast cell lines bound control IgG (Fig. 1A).

Intracellular expression of Ro60 was also compared among fibroblasts expressing FLAG3-Ro60 constructs by evaluating binding of anti-FLAG mAb after permeabilization. Fibroblasts expressing FLAG3-Ro60, FLAG3-Ro60(K170A R174A), and FLAG3-Ro60(H1785) showed equivalent reactivity with anti-FLAG mAb (MFI of 1800 ± 158, 1886 ± 156, and 1681 ± 38, respectively, p = NS for all comparisons). As expected, wild-type and Ro60 knockout fibroblasts did not react with anti-FLAG mAb (MFI of 19 ± 11 and 6 ± 3, respectively) (Fig. 1B). Ro60 and FLAG expression was confirmed by immunoblot with human anti-Ro60 Ab or anti-FLAG mAb (Fig. 1C). Intracellular expression of La, an autoantigen that also interacts with Y RNA, was evaluated on the murine fibroblast lines to determine whether Ro60/Y RNA composition (or its absence) affects La expression. Affinity-purified anti-La Ab showed equivalent binding to all murine fibroblasts independent of the expression of Ro60/Y RNA (Fig. 1D).
Cell surface translocation of Ro60 during apoptosis is dependent on mY3 RNA

Because the Ro60 mutations FLAG3-Ro60(K170A R174A) and FLAG3-Ro60(H187S) differ in their ability to bind Y RNA, it was hypothesized that the absence of Y RNA bound to FLAG3-Ro60 (H187S) accounts for the reduced expression of this mutant Ro60 on the surface of apoptotic cells. To test whether Y RNA is a requirement for translocation of Ro60 to the cell surface, both murine Y RNA subsets, mY1 and mY3, were depleted in wild-type fibroblasts and surface expression of Ro60 was evaluated. Northern blot revealed that RNA interference depleted the appropriate mY1 or mY3 RNA from wild-type fibroblasts whereas SS rRNA, used as a loading control, was not altered by siRNA transfection (Fig. 3A, 3C). To determine whether intracellular expression of Ro60 was affected by the depletion of Y RNA, anti-Ro60 binding to permeabilized cells was evaluated by flow cytometry. Intracellular Ro60 levels were comparable among wild-type fibroblasts transfected with control (98 ± 7%), mY1 (95 ± 10%), mY3 (93 ± 10%), or both mY1 and mY3 (94 ± 8%) siRNA where the binding of anti-Ro60 Ab to untreated fibroblasts is 100%. In contrast, when siRNA transfected fibroblasts were rendered apoptotic, translocation of Ro60 was significantly reduced in fibroblasts depleted of mY3 RNA compared with control siRNA-treated cells (12 ± 7% versus 101 ± 25%, respectively, p < 0.005). However, depletion of mY1 RNA from wild-type fibroblasts did not affect the translocation of Ro60 (102 ± 25%, p = NS versus control siRNA, p < 0.005 versus mY3 siRNA-treated cells). The simultaneous depletion of mY1 and mY3 RNA from wild-type fibroblasts impaired the translocation of Ro60 (4 ± 3%) to the same extent as that of mY3 RNA depletion alone (p = NS versus mY3 siRNA-treated cells) (Fig. 3E).

If mY3 is more abundant than mY1, knockdown of mY1 may have a negligible effect on Ro60/Y RNA complexes because the remaining mY3 RNA may be sufficient to sequester Ro60 in the cytoplasm. However, in wild-type fibroblasts cotransfected with mY1 plasmids and mY3 siRNA to simultaneously overexpress mY1 while depleting mY3, anti-Ro60 binding to the surface of apoptotic fibroblasts was similar to that observed when the cells were treated with mY3 siRNA alone (Fig. 3F). Northern blotting confirmed that wild-type fibroblasts transfected with mY1 plasmids overexpressed mY1 RNA relative to untreated cells (Fig. 3B, 3D). Interestingly, overexpression of mY1 was associated with decreased levels of mY3, suggesting that mY1 and mY3 RNAs compete for binding to Ro60. Consistent with competition for Ro60 binding, the overexpression of mY1 RNA was further increased when mY3 siRNA was cotransfected with mY1 plasmids compared with control siRNA (Fig. 3B, 3D). However, whereas Ro60 required mY3 RNA for surface translocation, the levels of mY3 did not influence the amount of surface-expressed Ro60, indicating that the lower levels of mY3 remaining after mY1 overexpression were adequate for Ro60 surface translocation. Moreover, overexpression of mY3 RNA (by ~2-fold) did not enhance anti-Ro60 binding to apoptotic fibroblasts relative to untreated apoptotic fibroblasts (Fig. 3F).

Apoptotic fibroblast immune complexes containing Y RNA stimulate TLR7-dependent TNF-α secretion by macrophages

Given the varied expression of the mutated forms of Ro60 on the surface of apoptotic cells and the requirement of Y RNA for Ro60 translocation, the functional consequences of surface-bound Ro60/Y RNA-anti-Ro60 complexes were subsequently addressed in coculture experiments. Human PBMC-derived macrophages cultured with apoptotic fibroblasts expressing FLAG3-Ro60(K170A R174A) preincubated with anti-Ro60 secreted significantly higher levels of TNF-α compared with macrophages incubated with FLAG3-Ro60(H187S) apoptotic fibroblasts similarly preincubated with anti-Ro60 (85 ± 27 versus 34 ± 2 pg/ml, respectively, p < 0.05). Macrophages cocultured with wild-type and FLAG3-Ro60 apoptotic fibroblasts bound by anti-Ro60 Ab showed similar TNF-α readouts compared with FLAG3-Ro60(K170A R174A) apoptotic fibroblasts (wild-type, 109 ± 24 pg/ml; FLAG3-Ro60, 106 ± 25 pg/ml; p = NS for all comparisons). In contrast, macrophages cultured with Ro60 knockout apoptotic fibroblasts preincubated with anti-Ro60 showed reduced levels of TNF-α (22 ± 5 pg/ml). Preincubation of apoptotic fibroblasts with control IgG prior to culturing with macrophages did not enhance TNF-α secretion (Fig. 4A). Coculture experiments with PMA-differentiated macrophages demonstrated that TNF-α secretion was enhanced only by apoptotic fibroblasts expressing full-length Ro60 that is capable of surface translocation (Fig. 4B).
THP-1 cells showed a similar pattern of TNF-α secretion as PBMC-derived macrophages (Fig. 4B). Treatment of THP-1 cells with anti-Ro60–opsonized apoptotic wild-type fibroblasts stimulated TNF-α release compared with challenge with Ro60 knockout fibroblasts preincubated with anti-Ro60 (85 ± 26 versus 25 ± 2, \( p < 0.05 \)), an effect also observed with challenge using FLAG3-Ro60(K170A R174A) but not FLAG3-Ro60(H187S) (80 ± 13 versus 43 ± 12 pg/ml) (Fig. 4B). TNF-α secretion by THP-1 cells cocultured with wild-type, FLAG3-Ro60, or FLAG3-Ro60(K170A R174A) apoptotic fibroblasts, all opsonized with anti-Ro60 Ab, was inhibited by 43 ± 6 (\( p < 0.005 \)), 43 ± 7 (\( p < 0.05 \)), and 47 ± 3% (\( p < 0.005 \)), respectively, in the presence of the TLR7 inhibitor IRS661 (Fig. 4C). To further evaluate the requirement of Ro60 accessibility to Ab for TLR-mediated inflammation, we determined whether blocking Ro60 on apoptotic cells would reduce TNF-α secretion in coculture experiments. Plasma protein, β2GPI, previously shown to bind Ro60 on the surface of apoptotic cells and block the binding of anti-Ro60 Ab (25, 26), was added to apoptotic wild-type fibroblasts prior to anti-Ro60 Ab. The fibroblasts were then cultured with PMA-differentiated THP-1 cells. Human native β2GPI bound late apoptotic fibroblasts (data not shown). The addition of β2GPI to apoptotic fibroblasts prior to anti-Ro60 Ab and coculturing with THP-1 cells resulted in a dose-dependent inhibition of TNF-α secretion (range of inhibition 20–70% with 1–50 μg/ml β2GPI compared with coculture experiments without β2GPI) (Fig. 4D).

**Discussion**

Exaggerated apoptosis has been proposed as a pathologic link between anti-Ro60 Abs and injury in cardiac NL and provides a mechanism for facilitating accessibility of an otherwise intracellular autoantigen to circulating maternal Abs (5). To test the hypothesis that Y RNA is critical for the translocation of Ro60 to the apoptotic cell surface, initial experiments exploited murine fibroblasts stably expressing mutations of Ro60 that affect Y RNA binding. Two experimental approaches supported the dependence of Y RNA for surface translocation of Ro60: absence of anti-Ro60 Ab binding to apoptotic fibroblasts expressing a Ro60 point mutation (H187S), which prevents Y RNA binding, and wild-type murine fibroblasts depleted of mY3 RNA. Moreover, the translocation of Ro60 during apoptosis and subsequent opsonization by anti-Ro60 Ab is highlighted as a key mediator for the initiation of inflammation.
and perpetuation of inflammation via TLRs, as cells expressing FLAG3-Ro60(H187S) do not activate macrophages because this mutated protein is not exposed on the cell surface. In contrast, FLAG3-Ro60(K170A R174A) and FLAG3-Ro60 (both bind Y RNA) translocate to the cell surface during apoptosis and activate macrophages in a TLR-dependent manner. These results support the hypothesis that Y RNA plays a critical role in the pathogenesis of fetal cardiac injury by altering the subcellular location of Ro60.

Translocation of Ro60 to the apoptotic cell surface was dependent on mY3 RNA but not mY1 RNA, as depletion of mY3 RNA in murine astrocytoma cells resulted in nuclear accumulation of Ro60. In contrast, Ro60 was retained in the cytoplasm after mY1 RNA knockdown (16). Initially we postulated that mY1 RNA, by being less abundant than mY3, was not sufficient to sequester Ro60 in the cytoplasm (for subsequent transport to the cell surface during apoptosis) in mY3-depleted fibroblasts. However, increased expression of mY1 RNA did not overcome the absence of Ro60 translocation upon mY3 depletion. Several non–mutually exclusive explanations are plausible to account for the differential effects of mY3 and mY1 RNA knockdown. For the latter, retention of a fragment of the 5’ mY1 RNA stem following knockdown may be sufficient to mask the Ro60 nuclear localization signal (16). Another possibility is that cell surface translocation is restricted to Ro60 associated with mY3 RNA, and may explain, in part, why levels of surface-exposed Ro60 tend to be lower than intracellular levels. The molecular explanation notwithstanding, the differences observed in Ro60 translocation after mY1 and mY3 depletion would imply distinct roles for the Y RNA subsets, a concept that was previously considered when particular subsets of Y RNAs associated with specific proteins (17, 27). For example, the zipcode binding protein 1 was recently reported to associate with Ro60/mY3 RNA complexes for nuclear export but not Ro60/mY1 RNA complexes (18). It is possible that a protein that interacts exclusively with Ro60/mY3 RNA complexes may chaperone the Ag to the cell surface during apoptosis.

The participation of Y RNA in cellular trafficking appears to be specific to Ro60, as intracellular expression and cell surface exposure of La was not affected in apoptotic cells with significantly reduced levels of Y RNA (Ro60 knockout or FLAG3-Ro60(H187S)). In support of these data, a previous study found that the association of Y RNA with La may be partially lost during apoptosis, as immunoprecipitation of apoptotic cell lysates with anti-Ro60 Ab yielded more Y RNA than did immunoprecipitation with anti-La Ab (28). Moreover, La predominantly binds Y RNA in the nucleus, whereas during apoptosis, La undergoes caspase-dependent cleavage of the COOH-terminal nuclear localization signal and accumulates in the cytoplasm (29). Taken together, these data imply that the mechanism of La translocation during apoptosis is independent of Y RNA.

Perhaps paradoxical to the findings of this study, apoptosis has been demonstrated to induce a caspase-dependent truncation of Y RNAs. However, the resulting 22- to 36-nt degradation products correspond to the most highly conserved region of the Y RNA,
116 Ro60 REQUIRES Y RNA FOR EXPOSURE DURING APOPTOSIS

which remains bound to Ro60 (28). Thus, it is predicted that these truncated products would still mask the Ro60 nuclear localization signal, allowing cell surface translocation of cytotoxic Ro60/mY3 RNA complexes. The truncated Y RNA remaining after apoptosis should not only facilitate surface translocation but also be sufficient to engage TLRs during macrophage FcgammaRI-mediated uptake of anti-Ro60–opsinized apoptotic cells (30).

In summary, a single point mutation of Ro60 that blocks Y RNA binding and siRNA-mediated knockdown of mY3 RNA attenuates a permissive signal that is required for surface accessibility of the Ro60 Ag to bind extracellular Ab and form immune complexes capable of inciting a TLR-dependent proinflammatory cascade. Accordingly, the mY3 RNA moiety of the Ro60 ribonucleoprotein imparts a critical role to the pathogenicity of anti-Ro60 Ab. The molecular dissection of the components involved in the pathogenesis of cardiac NL, including translocation of Ro60 during apoptosis, Ro60-associated RNA, and binding of anti-Ro60 Ab to apoptotic cells and potential protective factors such as beta2m, may lead to novel therapeutic approaches.

Acknowledgments
We thank the Flow Cytometry and Cell Sorting Center at the New York University Langone Medical Center for technical assistance with flow cytometry experiments.

Disclosures
The authors have no financial conflicts of interest.

References

Downloaded from http://www.jimmunol.org/ by guest on August 3, 2017