Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RARβ and tumor regression

Robert Gallicchio, Alessio Paone, Muller Fabbri, Nicola Zanesi, Federica Calore, Luciano Cascione, Mario Acunzo, Antonella Stoppani, Andrea Tubaro, Francesca Lovat, Pierluigi Gasparini, Paolo Fadda, Hansjürgen Alder, Stefano Volinia, Antonio Filippini, Elio Ziparo, Anna Riccioli, and Carlo M. Croce.

Toll-like receptors (TLRs) are a family of transmembrane receptors that recognize conserved ligands of microbial origin called pathogen-associated molecular patterns and provide the first line of defense against pathogen-induced inflammatory responses (1, 2). In addition, TLRs play an important role in tissue repair and tissue injury-induced inflammation (1, 2). The role of TLRs in cancer is still being debated; their pro- or anticancer effect depends on the TLR stimulated and the tumor type (3). We and others previously demonstrated that activation of Toll-like receptor 3 (TLR3) by the specific agonist polyinosinic:polycytidylic acid [Poly(I:C)] exerts an antitumoral effect by inducing apoptosis (4, 5) and recruiting an antitumoral immune response (6–8). In Western countries, prostate and breast cancers are the most common malignant tumors in men and women, respectively (9, 10). MicroRNAs (miRNAs) are small noncoding RNAs with gene regulatory function, and their dysregulation has been demonstrated in all human malignancies (11). Recently, several studies have shown that miRNAs have a role in regulating immune response and inflammation (12). In particular, TLR signaling can modulate miRNA expression (13). Here we demonstrate that TLR3 stimulation induces up-regulation of four miRNAs (hsa-miR-29b, -29c, -148b, and -152) on DU145 and TRAMP-C1 prostate cancer cells, and the demonstration that TLR3 stimulation induces up-regulation of the miR-148b/152 family in dendritic cells, and this affects cytokine production. To determine whether Poly(I:C) treatment can induce changes in the miRNomes of prostate cancer cells, we used DU145, which is the only validated human prostate cancer cell line that expresses high levels of TLR3 but does not undergo a strong apoptotic effect upon Poly(I:C) stimulation (4, 6). By performing a NanoString assay on Poly(I:C)-treated cells, we found that miR-29b, -29c, -148b, and -152 were among the most significantly up-regulated miRNAs at different time points (Fig. 1). We validated these results by performing quantitative real-time PCR (qRT-PCR) (Fig. SL4). Consistent with the NanoString data, all four miRNAs were significantly up-regulated by Poly(I:C) treatment. To validate these data on other cellular models, we used TRAMP and MDA-MB-231 cells, two cell lines previously described to express TLR3 and to be resistant to Poly(I:C)-induced apoptosis (8, 24). We treated these cell lines with Poly(I:C) and observed increased expression of all four miRNAs of interest at different times (Fig. SL4). The observed rapid up-regulation of the miRNAs...
of interest suggests a direct mechanism of signal transduction. More complex mechanisms involving the secretion of IFN-β, known to often be part of the response to TLR3 stimulation, may be excluded because, for example in breast cancer, this molecule is secreted 18 h after the stimulation (5).

To confirm the direct involvement of TLR3 in the Poly(I:C)-induced miRNA up-regulation, we transfected DU145, TRAMP, and MDA-MB-231 cells with an empty vector, used as a control, or with a vector encoding a dominant-negative (DN) form of TLR3. In the presence of Poly(I:C), TLR3-DN treatment abolished the up-regulation of miR-29b, -29c, -148b, and -152, suggesting that miRNA up-regulation induced by Poly(I:C) is strictly TLR3 dependent (Fig. S1B).

**Effect of Poly(I:C)-Induced miRNAs on DNMTs.** Because miR-148b and -152 are known to target DNMT1 (14), whereas the miR-29 family targets all DNMTs (15, 16), we hypothesized that Poly(I:C) treatment would inhibit DNMT activity by inducing these miRNAs. Therefore, we treated DU145, TRAMP, and MDA-MB-231 cells with Poly(I:C) for 24 or 48 h and assayed DNMT activity. Poly(I:C) treatment caused a strong reduction of DNMT activity (49%, 31%, and 37%, respectively, for DU145, TRAMP, and MDA-MB-231 cells), albeit with different timing (Fig. S2). In fact, the effect on DNMT activity is faster in DU145 and MDA-MB-231 (after 24 h of treatment) than in TRAMP cells (after 48 h of treatment). Then, we transfected DU145, TRAMP, and MDA-

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**Fig. 1.** MiRNA up-regulation after Poly(I:C) treatment in DU145 cells. DU145 prostate cancer cells were treated with 25 μg/mL Poly(I:C) from 1 to 6 h, the RNA was extracted, and NanoString assay was performed. Untreated cells were used as a control. The heat map shows the results of NanoString for miRNA-29b, -29c, -148b, and -152 at the indicated time points.

**Fig. 2.** Effect of Poly(I:C)-induced miRNAs on DNMT expression in prostate and breast cancer cells. DU145, TRAMP, or MDA-MB-231 cells were transfected with the Renilla luciferase expression construct pRL-TK and one of the following constructs: the luciferase construct PGL3-DNMT1WT-3′-UTR-luc (DNMT1) or PGL3-DNMT1MUT-3′-UTR-luc (DNMT1 mut), PGL3-DNMT3AWT-3′-UTR-luc (DNMT3A) or PGL3-DNMT3 AMUT-3′-UTR-luc (DNMT3A mut), PGL3-DNMT3BWT-3′-UTR-luc (DNMT3B) or PGL3-DNMT3BMUT-3′-UTR-luc (DNMT3B mut). In the "mut" constructs, the mutations were introduced in DNMT target sites for a specific miRNA. Twenty-four hours after transfection, the cells were treated with 25 μg/mL Poly(I:C) for 18, 24, or 48 h before the luciferase assay was performed. All data represent mean and SD from four determinations from three independent experiments. *P < 0.05; **P < 0.01. a.u., arbitrary units.
MB-231 cells with plasmids containing the 3′-UTR of DNMT1, -3A, or -3B genes cloned downstream of the firefly luciferase gene (14, 15). Cells were treated with Poly(I:C), and a luciferase reporter assay was performed. Poly(I:C) treatment significantly reduced the luciferase reporter activity in the cells transfected with each plasmid, and this effect was reversed by si-specific mutagenesis of the miRNA binding sites on DNMTs (Fig. 2 and Fig. S3).

RARβ Demethylation After Poly(I:C) Treatment. To investigate the functional implications of Poly(I:C) treatment on our model, we treated DU145 cells with Poly(I:C) for 48 h and performed a Methyl-Profiler analysis designed specifically for prostate cancer. Among the 24 genes analyzed, RARβ was the most significantly demethylated (Fig. 3A). Interestingly, the promoter of this gene is one of the most commonly hypermethylated in prostate and breast cancer patients and cell lines (17, 19, 25). The results were validated by methylation-specific PCR (MSP) and Pyrosequencing assay. Using 5-aza-cytidine (5-AZA) as a positive control, we observed that Poly(I:C) induced a partial demethylation of RARβ promoter after 48 h of treatment (Fig. 3B and C). Poly(I:C) treatment of DU145 cells also resulted in increased levels of RARβ protein, with an expression peak detected after 48 h (Fig. 3D). We also performed MSP assay on TRAMP and MDA-MB-231 cells and showed that Poly(I:C) could induce a partial demethylation of RARβ promoter on both cell lines (Fig. 3E and Fig. S4). Moreover, we demonstrated that RARβ protein was reexpressed in both cell lines after 48 h of Poly(I:C) treatment (Fig. 3F).

Effects of miRNA and of DNMT Modulation of RARβ Expression. To demonstrate that the expression of RARβ was correlated with the overexpression of Poly(I:C)-induced miRNAs, we transfected DU145, TRAMP, and MDA-MB-231 cells with antisense molecules for miR-29b, -29c, -148b, and -152 individually or in combination (miR-29b/29c, miR-148b/152), and we treated cells with Poly(I:C) for 48 h. Poly(I:C)-induced RARβ expression was blocked in the presence of all miRNA antisense molecules, except miR-29b in MDA-MB-231 cells (Fig. 4). These data suggest that all four miRNAs are directly involved in RARβ reexpression modulation, although with different efficiency. Moreover, DU145, TRAMP, and MDA-MB-231 cells were transfected with antisense molecules for miR-147 and -574-5p (miRNAs not involved in DNMT targeting) as negative controls and for miR-29c as a positive control, then the cells were treated with Poly(I:C) (or its solvent as a control). No induction of RARβ expression occurred in any of the analyzed conditions without Poly(I:C) treatment (Fig. S5A). Conversely, after Poly(I:C) treatment, we observed RARβ expression in cells transfected with scrambled and antisense molecules for miR-147 and -574-5p, but not in the cells transfected with the antisense molecule for miR-29c, excluding off-target effects of the involved miRNAs. We also transfected DU145, TRAMP, and MDA-MB-231 cells with each of the specific miRNA mimics alone or in the previously described combination, although with different efficiency, the transfection of each of the four miRNAs induces partial reexpression of RARβ in all three cell lines, except miR-29b in MDA-MB-231 cells (Fig. S5B). It may be postulated that Poly(I:C)-induced up-regulation of miR-29β might not be necessary for DNMT targeting in MDA-MB-231 cells, because miR-29β previously was reported to be up-regulated in this cell line (26). The combination of miR-29b/29c is the most efficient to induce RARβ reexpression in DU145 and TRAMP cells, whereas for MDA-MB-231 cells, the best result is obtained using the miR-148b/152 combination.

To investigate the robustness of the epigenetic control of RARβ in prostate and breast cancer, we transfected DU145, TRAMP, and MDA-MB-231 cells with the specific siRNA for DNMT1, -3A, or -3B alone; with a combination of these; or with an siRNA for the DNMT silencing by using qRT-PCR (Fig. 5A). Although with different efficiency, the transfection of each the four miRNAs is directly involved in RARβ expression. We also transfected DU145, TRAMP, and MDA-MB-231 cells with the antisense molecules for miR-147 and -574-5p (miRNAs not involved in DNMT targeting) as negative controls and for miR-29c as a positive control, then the cells were treated with Poly(I:C) (or its solvent as a control). No induction of RARβ expression occurred in any of the analyzed conditions without Poly(I:C) treatment (Fig. S5A). Conversely, after Poly(I:C) treatment, we observed RARβ expression in cells transfected with scrambled and antisense molecules for miR-147 and -574-5p, but not in the cells transfected with the antisense molecule for miR-29c, excluding off-target effects of the involved miRNAs. We also transfected DU145, TRAMP, and MDA-MB-231 cells with each of the specific miRNA mimics alone or in the previously described combination, although with different efficiency, the transfection of each of the four miRNAs induces partial reexpression of RARβ in all three cell lines, except miR-29b in MDA-MB-231 cells (Fig. S5B). It may be postulated that Poly(I:C)-induced up-regulation of miR-29b might not be necessary for DNMT targeting in MDA-MB-231 cells, because miR-29β previously was reported to be up-regulated in this cell line (26). The combination of miR-29b/29c is the most efficient to induce RARβ reexpression in DU145 and TRAMP cells, whereas for MDA-MB-231 cells, the best result is obtained using the miR-148b/152 combination.

To investigate the robustness of the epigenetic control of RARβ in prostate and breast cancer, we transfected DU145, TRAMP, and MDA-MB-231 cells with the specific siRNA for DNMT1, -3A, or -3B alone; with a combination of these; or with an siRNA for the methylation-unrelated proteins Rab27-a, -27-b, or Rab27-b as negative controls. After checking for the efficiency and specificity of the DNMT silencing by using qRT-PCR (Fig. 5A), we observed reexpression of RARβ 48 h after treatment with all three DNMT siRNAs (Fig. 5B) but not with the Rab27-a, -27-b, or control siRNA. To confirm these data, we performed a rescue experiment (Fig. S5C). We transfected DU145, TRAMP, and MDA-MB-231 cells with a plasmid encoding for each specific DNMT, with an
siRNA against the DNMT, or with a combination of the plasmid and the siRNA for the same DNMT. Our data show that DNMT down-regulation induces RARβ expression. In the cells transfected with the combination of each DNMT plus the relative siRNA, we observed a reduced expression level of RARβ with respect to the cells transfected with the siRNA alone. Overall, these data indicate that all three main DNMTs are involved in the epigenetic control of RARβ expression.

**Apoptotic Effect of Poly(I:C)/Retinoid Combined Treatment.** Because retinoids are natural ligands of RARβ, we further investigated the effects of retinoids in DU145, TRAMP, and MDA-MB-231 cells by reexpressing RARβ after Poly(I:C) treatment. 9-cis-RA is an activator of both RAR and retinoic x receptor (RXR), but the affinity for the first group is at least 20 times higher than for the second (27). Although not as specific for RAR as 13-cis-RA or all trans retinoic acid (ATRA), 9-cis-RA has been demonstrated to have higher affinity for RARβ (27, 28). All cell lines were treated with Poly(I:C) for 48 h, then a time course of 9-cis-RA was performed (Fig. S6A). Cell cycle analysis showed that after 48 h of Poly(I:C) stimulation, there was a significant increase in the sub-G1 apoptotic population only after subsequent stimulation for 72 h with 9-cis-RA. A strong apoptotic peak was induced only in the cells treated with the combination of Poly(I:C) and 9-cis-RA and not in the cells treated with Poly(I:C) or 9-cis-RA alone (Fig. 6A–C). To confirm that the observed apoptotic induction was the result of RARβ activation, an RNA interference against RARβ was used to transfect DU145, TRAMP, and MDA-MB-231 (whereas a scrambled sequence and an RNA interference against Rab27-a and another one against Rab27-b were used as negative controls); then after 24 h, all the cells were treated as previously described. Cell cycle analysis (Fig. S6B) showed that the apoptotic induction was lower in the cells transfected with RNA interference against RARβ than in those transfected with the negative controls, confirming that the apoptotic signal was induced through this receptor. Moreover, to validate the data obtained with cell cycle analysis, we performed a colony assay treating DU145, TRAMP, and MDA-MB-231 cells with Poly(I:C) and 9-cis-RA singularly or in combination. The combination of the two drugs induced a strong reduction in colony number (Fig. S6C). We also found that the Poly(I:C)/9-cis-RA combination induced poly ADP ribose polymerase (PARP) precursor cleavage, mediated by caspase cascade activation. Interestingly, only the combined Poly(I:C)/9-cis-RA treatment, but not the single agents of this combination, could induce PARP. 

**Fig. 5.** RARβ protein expression is DNMT dependent. (A) DU145, TRAMP, or MDA-MB-231 cells were transfected with antisense molecules for DNMT1, -3A, or -3B (200 nM each); a combination of the three siRNAs (200 nM each); or a scrambled molecule (600 nM). Forty-eight hours after transfection, RNA was extracted and qRT-PCR was performed to verify the efficiency of siRNA transfection. Data represent the mean of triplicate samples from three independent experiments and are expressed as mean ± SD. *P < 0.05; **P < 0.01. a.u., arbitrary units. (B) Whole-cell lysates from cells transfected as in A plus cells transfected with Rab27-a and Rab27-b siRNA used as negative controls were used to perform a Western blot analysis using a polyclonal Ab against RARβ. *P < 0.05. All data represent typical experiments that were repeated three times with similar results.

**Fig. 6.** Apoptotic effect on DU145, TRAMP, and MDA-MB-231 cells after stimulation of RARβ induced by Poly(I:C). DU145, TRAMP, or MDA-MB-231 cells were pretreated with 25 μg/mL Poly(I:C) for 48 h, then with 10 μM 9-cis-RA for 72 h. Cells untreated or stimulated with 9-cis-RA alone were used as a control. The cells then were detached and subjected to propidium iodide PI staining. M1 is the percentage of apoptotic cells (A–C). (D) Western blotting of PARP precursor and PARP cleaved forms of DU145, TRAMP, and MDA-MB-231 cells treated with 25 μg/mL Poly(I:C) for 48 h or with 10 μM 9-cis-RA for 72 h alone, or pretreated with 25 μg/mL Poly(I:C) for 48 h and subsequently with 10 μM 9-cis-RA for 72 h. Untreated cells were used as a control. β-Actin was used as loading control. Data represent typical experiments that were repeated three times with similar results. *P < 0.05; **P < 0.01. a.u., arbitrary units.
precursor cleavage and cleaved PARP appearance (Fig. 6D). Overall, these data indicate that pretreatment with Poly(I:C) induces reexpression of a functionally active RARβ; however, this is not sufficient per se to induce cell cycle arrest or apoptosis, as reported for different cell types, although it restores DU145, TRAMP, and MDA-MB-231 cell sensitivity to treatment with RARβ agonist 9-cis-RA.

**Poly(I:C)/9-cis-RA Effects in Vivo.** To investigate the in vivo relevance of our in vitro experiments, we injected DU145 cells s.c. in the backs of 20 nude mice. Two weeks after the injection, we randomly divided the mice into four groups (n = 5 per group). All groups were treated as shown in Table S1.

In a first experiment, mice were treated for a total two cycles, with a week of rest between treatments. In the second experiment, we eliminated the week of rest between treatment cycles. In both cases, starting from the second week of treatment, we observed a significant reduction in tumor growth only in group 4 [mice treated with the Poly(I:C)/9-cis-RA combination] (Fig. 7A–C). We and others previously reported that Poly(I:C) stimulation may induce an indirect antitumor effect, activating a strong inflammatory response with the recruitment of immune cells acting against the tumor (6, 29). To test the involvement of the immune system in the mechanism described, we injected TRAMP cells in syngeneic C57 black 6 immunocompetent mice. Two weeks after the injection, the mice were divided randomly and treated as described for the experiment in Fig. 7B. After 2 wk of treatment, we observed a reduction in tumor growth in Poly(I:C)-treated mice compared with PBS-treated mice; however, these data were not statistically significant. We also observed a significant reduction in tumor mass in mice treated with the Poly(I:C)/9-cis-RA combination (Fig. 7D and E). We also analyzed ex vivo the xenograft and syngeneic tumors (Fig. 7F, *Left and Right*, respectively) and observed that Poly(I:C) treatment induced up-regulation of miR-29b, -29c, -148b, and -152, and RARβ up-regulation (Fig. 7G, *Upper and Lower*, respectively), confirming that TLR3 agonist Poly(I:C) induces reexpression of the epigenetically silenced RARβ through an miRNA-mediated mechanism and restores sensitivity of cancer cells to retinooids.

**Quantification of miR-29b, -29c, -148b, and -152 in Prostate and Breast Human Cancer Samples.** To quantify the basal expression level of miR-29b, -29c, -148b, and -152 in human primary prostate and breast cancer patients, we used qRT-PCR on RNA extracted from both normal and tumoral samples, whose clinical characteristics are listed in Fig. S7A and B. We observed strong down-regulation of all four miRNAs of interest in the prostate tumor vs. the normal counterpart (Fig. S7C; P < 0.01, Wilcoxon test). No correlation was observed with the Gleason score. Although not statistically significant using the Wilcoxon test, in breast cancer patients we observed down-regulation for all four miRNAs of interest, with median values of 0.79, 0.50, 0.83, and 0.62 for miR-29b, -29c, -148b, and -152, respectively (Fig. S7D). Also, in this case, no correlation.
was observed with the tumor grade. A recent paper demonstrated that in breast cancer, down-regulation of the miR-29 family induces dedifferentiation of the cells and increases cancer stem cell population, which favors tumor progression (30). Moreover, previously published data from our laboratory demonstrates that the miR-29 family is down-regulated in invasive breast cancer (30, 31). Overall, these data strongly suggest that in prostate and breast cancer, down-regulation of these miRNA levels might be an important condition for the onset of the pathology, and treatment with Poly(I:C) might be beneficial in reversing this condition. In summary, here we describe a mechanism of action through which Poly(I:C) exerts an antitumor effect on prostate and breast cancer cell lines by modulating the expression of miRNAs, thereby restoring retinoid sensitivity by reexpressing functionally active. The evidence provided by this study suggests that Poly(I:C) might be used effectively in combination with retinoids, opening possible therapeutic avenues for treating prostate and breast cancer. In addition, the demethylating response to Poly(I:C) might allow stratification of prostate and breast cancer patients based on their responsiveness to retinoid therapy.

Methods

A detailed description of the materials and methods used in this study may be found in SI Methods.

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