Extracellular mRNA Induces Dendritic Cell Activation by Stimulating Tumor Necrosis Factor-α Secretion and Signaling through a Nucleotide Receptor*

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We previously demonstrated that dendritic cell (DC) pulsing with antigen-encoded mRNA resulted in the loading of both major histocompatibility complex class I and II antigen presentation pathways and the delivery of an activation signal. Coculture of mRNA-pulsed DC with T cells led to the induction of a potent primary immune response. DC, in addition to recognizing foreign antigens through pattern recognition receptors, also must respond to altered self, transformed, or intracellularly infected cells. This occurs through cell surface receptors that recognize products of inflammation and cell death. In this report, we characterize two signaling pathways utilized by extracellular mRNA to activate DC. In addition, a novel ligand, poly(A), is identified that mediates signaling through a receptor that can be inhibited by pertussis toxin and suramin and can be desensitized by ATP and ADP, suggesting a P2Y type nucleotide receptor. The role of this signaling activity in vaccine design and the potential effect of mRNA released by damaged cells in the induction of immune responsiveness is discussed.

Dendritic cells (DC) are the sentinel cells of the adaptive immune system and function in the induction of primary and memory T cell immune responses (1, 2). They mainly populate tissues that interface with the environment and acquire antigenic peptides via molecular patterns, contained within microbial lipids, carbohydrates, and nucleic acid. This pattern recognition occurs through a set of germ-line encoded receptors, which are exemplified by the Toll-like receptor (TLR) family (4–6). Immature DC, upon receiving an activation signal, undergo phenotypical and functional changes, including: 1) decreased antigen acquisition with a coordinated increase in presentation of MHC-peptide complexes on the cell surface; 2) increased stability of MHC class II-peptide complexes; 3) increased expression of surface molecules that aid and promote T cell activation; and 4) a changed pattern of release of chemokines and cytokines leading to attraction of T cells, promotion of T cell activation, and direction of their ultimate phenotype (Th0, Th1, Th2, or T regulatory (Treg)) (5, 8, 9); and 5) a shift in the repertoire of chemokine receptor expression that allows and directs DC migration to lymphoid organs (10, 11).

Identified categories of DC activators include: 1) conserved constituents of bacteria (lipopolysaccharide (LPS), cell wall lipoproteins, flagellar proteins, and DNA), 2) host cell-derived molecules released during cell injury and death (proinflammatory cytokines and nucleotides), and 3) intermediates of viral replication (double-stranded RNA (dsRNA)) (12), and 4) molecules on activated CD4 T cells (CD40 ligand (CD40L)). The signaling by each DC activator leads to the transformation of a DC from antigen acquisition to antigen presentation but produces a DC that differs in the type of immune response it induces (13). DCs treated with TNF-α, a pleiotropic stimulus of DC activation, and prostaglandin E produce a low level of IL-12 and induce a mixed population of Th0 and Th1 T cells. DCs exposed to dsRNA secrete high levels of IL-12 and IFN-α leading to a strong Th1 response. DCs exposed to apoptotic cells or malaria-infected red blood cells do not secrete IL-12 but produce IL-10 (14, 15) leading to the induction of Treg cells (16).

Nucleotide receptors are comprised of two families, a metabotropic family (P2Y) that belong to the 7-transmembrane, G-protein coupled receptor (GPCR) superfamily, and a pore-forming, cation-selective family (P2X). Human DCs express mRNA for P2Y1, -2, -4, -6, -11 and P2X1, -2, -4, -5, -7 nucleotide receptors (17). Signaling through each family by selected nucleotides induces different aspects of DC activation. ATP, which can be released by damaged cells and has been demonstrated to synergize with TNF-α in the activation of DCs (18, 19), acts through P2Y11 receptor signaling. This signaling pathway leads to the generation of cAMP (19). The P2X7 receptor is important in cytokine secretion in human DC (20) and antigen presentation in murine DC (21). In addition to DC activation, it has also been observed that signaling through nucleotide receptors by ATP leads to DC apoptosis (22) and...
aberrant DC activation. DC pretreated with low, non-toxic doses of ATP, produced lower amounts of IL-1α, IL-1β, TNF-α, IL-6, and IL-12 after subsequent stimulation with LPS or CD40L (23).

The targeting of DC for antigen delivery in vivo and in vitro represents an important approach in vaccine research. The first step in immune responsiveness induced by vaccines is the delivery of antigen in a form that the DC can acquire, process, and present to CD4+ T, CD8+ T, and/or B cells. With this delivery of antigen, the vaccine also must deliver an activation signal to the DC. This is often done by the inclusion of adjuvants, such as mycobacterium to complete Freund’s adjuvant, or with components of the antigen delivery system, such as CpG motifs in DNA vaccines (reviewed in Ref. 24). We previously reported that pulsing DC with mRNA encoding antigen led to loading of both CD4+ and CD8+ T cell antigen presentation pathways, delivery of an activation signal to DC, and the induction of potent antigen-specific T cell activation (25). In this report, we determined the mechanisms whereby mRNA activates DC.

**EXPERIMENTAL PROCEDURES**

*In Vitro RNA Transcription—* Transcription was performed on Gag- and luciferase-encoding plasmid templates linearized downstream from a stretch of dA∞ using the T7 message machine kit (Ambion, Austin, TX) as described previously (25). Purification of the transcripts was performed by DNase I digestion followed by LiCl precipitation and 70% EtOH washing. Additional poly(A) tail was added to the transcripts with yeast poly(A) polymerase (Amer sham Biosciences, Inc., Piscataway, NJ), and the mRNA was again purified. All Gag- or luciferase-encoding mRNA contained a poly(A) tail unless otherwise noted. Assays for LPS in RNA preparations using the Lumilux Amebocyte lyse-erase-encoding mRNA contained a poly(A) tail unless otherwise noted. Samples were stored in siliconized tubes at −20°C until use.

*Cell Culture—* HL60, U937, and 293T cells (ATCC, Rockville, MD) and P2Y11 nucleotide receptor stably transfected Chinese hamster ovary and 1231N1 cells (26) were propagated in Dulbecco’s modified Eagle’s medium supplemented with glutamine (Invitrogen, Rockville, MD) and 10% FCS (HyClone, Ogden, UT). Leukapheresis samples were obtained from HIV-infected volunteers through an institutional review board-approved protocol. Peripheral blood mononuclear cells (PBMC) were purified by Ficoll-Hypaque density gradient centrifugation. PBMC were cryopreserved in RPMI 1640 (Invitrogen) with 50% FCS and 10% FCS (HyClone, Ogden, UT). Leukapheresis samples were measured in supernatants by sandwich ELISA. Cultures were performed in duplicate and measured in duplicate.

*Statistical Analysis—* Student’s t tests were performed using Microsoft Excel software.

**RESULTS**

*mRNA Induces DC Activation—* DC activation is induced by classes of stimuli through specific receptors (TLR, TNF family, nucleotide, proline, and signaling cascades. Although each of these classes of agents induces activation and maturation of DC, the function of the resulting DC regarding the type of immune response induced differs. We previously demonstrated that HIV Gag-encoding mRNA activates DC as measured by expression of the DC maturation marker CD83 and antigen-specific T cell activation (25). To better define the phenotype of mRNA activated DC, we studied the following markers of DC activation: 1) MHC class II and costimulatory molecules (CD80, CD86) necessary for T cell activation; 2) chemokine receptors (CCR5, CCR6, CXCR4) that traffic DC to peripheral tissues and lymphoid organs; 3) functional uptake of extracellular antigen, and 4) cytokines (IL-12, IL-8, IFN-α, TNF-α) released by DC that regulate and shape the resulting T cell response. mRNA in comparison to the standard DC activation agents, LPS, TNF-α + PGE3, dsRNA, and CD40L, demon-
strated the greatest increase in surface proteins associated with T cell activation, CD80, CD86, and MHC classes I and II, and lesser regulation of chemokine receptor expression (Fig. 1 and Table I). Both Gag- and luciferase-encoding mRNA-containing poly(A) tails were used, and no difference in activation of DC was observed. Poly(A) minimally increased CD83 and induced a smaller subset of markers of DC activation while poly(U) did not induce markers of DC activation (Fig. 1 and Table I).

The cytokines produced by activated DC during interactions with T cells determines, in part, the phenotype of the resulting T cell response. IL-12, IFN-α, TNF-α, and IL-8 are differentially produced in response to DC activation stimuli. IL-12 in humans acts on T cells to induce them to produce IFN-γ (Th1 response). IFN-α acts on DCs to down-regulate IL-12 and increase IL-10 production (16). The lack of IL-12 and the production of IL-10 favor Th2 and Treg T cells. IL-8 acts to attract inflammatory cells and potentiates the local immune response (31). DC cytokine production induced by the different forms of activation demonstrated that mRNA led to moderate levels of IL-12 production compared with the very high levels observed with CD40L and the low amounts observed with TNF-α + PGE3 stimulation (Fig. 2 and Table I). Gag encoding mRNA induced moderate levels of IFN-α and TNF-α, whereas poly(A) did not increase IFN-α or TNF-α production but induced IL-12 suggesting that poly(A) represented a subset of mRNA activation of DC.

Activated DCs are superior to immature DC in inducing T cell activation. A system that measures the ability of DC maturation agents to increase DC-induced T cell activation was employed. Unactivated T cells were cocultured with immature or activated DC in the presence of suboptimal concentrations of superantigen for 6 h and then analyzed for the early activation marker CD69. mRNA mediated activation of DC resulted in the induction of CD69 on T cells at similar levels observed for other potent DC-activating agents (Fig. 3 and Table II). Studies examining the cytokines made by T cells activated by DC demonstrated that immature DC induced both IL-4- and IFN-γ-secreting T cells (Fig. 4). This assay measured the percentage of cells induced to make IL-4 or IFN-γ by DC stimulation and differs from other studies that polyclonally stimulate T cells after coculture with DCs, which determine the potential to produce a cytokine induced by the DC (12, 32). LPS induced predominantly IFN-γ-producing T cells with a small population of IL-4 producing CD4+ T cells. Poly(I)·poly(C) activation of DC resulted in CD4+ T cells that produced almost entirely IFN-γ.

Fig. 1. RNA activates DC as measured by the up-regulation of T cell activation molecules and modulation of chemokine receptors. Dotted lines, immature; thin solid lines, LPS (1.0 μg/ml) treated; and heavy solid lines, Gag-encoding mRNA pulsed DC were analyzed for expression of CD80, CD86, MHC classes I and II, CXCR4, and CCR5 after 24 h. Data presented are from one DC preparation and are representative of at least five preparations from at least five different donors.

Table I

| Rank order change of DC activation markers by stimuli |
|------------------------------------------------------|
| The changes in DC activation markers induced by each stimul after 24–48 h of treatment of immature DC were quantitated (mean fluorescence by flow cytometry for surface molecule expression and amount in supernatant by ELISA for cytokines) and rank-ordered with 1 being change compared to immature (no treatment). Rank order placement of each activator was determined if a statistically different result was observed in comparing at least five separate experiments between activators. An equal rank was given if no statistically significant difference was observed between activators. |

| DC activators | ↑CD83 | ↑HLA-ABC | ↑HLA-DR | ↑CD80 | ↑CD86 | ↑CD25 | ↑CCR5 | ↑CCR6 | ↑CXCR4 | ↑Endocytosis | ↑IL-12 | ↑IFN-α | ↑TNF-α | ↑IL-8 |
|---------------|-------|----------|---------|-------|-------|-------|-------|-------|-------|-------------|-------|--------|--------|-------|
| TNF + PGE3    | 4     | 4        | 5       | 3     | 3     | 3     | 3     | 3     | 2     | 4           | 4     | 2      | 1      | ND    |
| LPS           | 5     | 4        | 5       | 3     | 3     | 3     | 3     | 4     | 2     | 4           | 4     | 5      | 2      | 3     |
| CD40L         | 4     | 4        | 5       | 3     | 3     | 3     | 3     | 4     | 2     | 4           | 4     | 5      | 2      | 3     |
| Poly(I)·poly(C)| 4     | 3        | 4       | 3     | 3     | 3     | 4     | 4     | 2     | 4           | 4     | 3      | 4      | 2     |
| Gag-encoding mRNA poly(A) | 3   | 5       | 6       | 4     | 4     | 4     | 3     | 2     | 3     | 4           | 4     | 3      | 2      | 3     |
| Poly(A)       | 2     | 2        | 3       | 2     | 2     | 1     | 2     | 1     | 2     | 3           | 3     | 3      | 1      | 2     |
| Poly(U)       | 1     | 1        | 2       | 1     | 1     | 1     | 1     | 1     | 1     | 2           | 1     | 1      | 1      | 1     |
| None          | 1     | 1        | 1       | 1     | 1     | 1     | 1     | 1     | 1     | 1           | 1     | 1      | 1      | 1     |

* ND, not done.
but consistently had a lower percentage of total T cells making IFN-γ compared with other stimuli. mRNA-treated DC activated CD4+ T cells to produce mainly IFN-γ, similar to poly(I)-poly(C) and CD40L (Fig. 4 and data not shown).

RNA Induces a Calcium Flux in DC—We observed that poly(A) partially activated DC whereas mRNA fully induced DC maturation and that poly(A) did not whereas mRNA induced TNF-α secretion. It was recently demonstrated that ATP or TNF-α treatment of DC induced low level expression of the DC maturation marker CD83. The addition of both TNF-α and ATP resulted in a synergistic activation of DC (18). A similar synergy was also observed when TNF-α and poly(A) were used to activate DC (data not shown). ATP signals cells through nucleotide receptors that are divided into two families, one G-protein linked (P2Y) and the second through selective cation pore formation (P2X). Poly(A) at similar molar AMP equivalents as ATP stimulated a calcium flux in Fura-2-loaded DC (Fig. 5). Dose-response analysis demonstrated that 1.0 μM AMP equivalents of poly(A) could flux calcium in DC. In vitro transcribed mRNA encoding the HIV Gag protein and containing a 50-nucleotide or longer poly(A) tail also fluxed Ca2+ in DC, whereas mRNA lacking a poly(A) tail did not (Fig. 5). The P2X receptors flux extracellular calcium through plasma membrane pores, and their signaling is inhibited by removal of extracellular Ca2+ with EGTA. RNA signaling of DC was not inhibited by the absence of extracellular calcium, suggesting that it did not signal through P2X receptors (Fig. 5). These data suggested that poly(A) signaled through P2Y receptors present on DC whose ligands were previously identified as nucleotides.

P2Y nucleotide and many G-protein coupled 7-transmem-
brane receptors can be desensitized to subsequent signaling. This increase in EC_{50} required for subsequent signaling can be observed for the same or different ligands that share a receptor (33–35). DCs were sequentially stimulated with ATP, ADP, or UTP; poly(A); and RANTES (Fig. 6). UTP stimulation did not inhibit subsequent signaling by poly(A) or RANTES (Fig. 6). ATP, at low concentrations (1–5 μM), desensitized DC to poly(A), with little or no effect on subsequent RANTES signaling (Fig. 6). Higher concentrations of ATP were toxic as previously demonstrated (20) and observed in this assay by a loss of calcium fluxes, confirming that RNA did not signal through the P2Y11 nucleotide receptor. In addition, the observation that poly(A) induced IL-12 clearly distinguished it from ATP-induced DC maturation, which has been demonstrated to inhibit IL-12 secretion (19).

We next sought to determine which P2Y nucleotide receptor signaled in response to poly(A) by utilizing the differential sensitivity to specific inhibitors of the members of this family. Pertussis toxin inhibits G_{i} proteins and completely blocks beta-chemokine-mediated signaling through their respective receptors (38). P2Y2, -4, -12, and -13 but not P2Y1, -6, or -11 are sensitive to pertussis toxin-mediated inhibition when ligand concentration is low (39–41). Poly(A) and RANTES but not ATP or UTP signaling were blocked by pretreatment of DC with pertussis toxin (Fig. 5 and data not shown).

Suramin is a synthetic polysulfonated napthylurea that blocks P2Y2 but not P2Y4 receptor signaling (40). When poly(A) was added to DCs that were preincubated with suramin, calcium flux was completely blocked, suggesting that P2Y2 was not responsible for RNA signaling (Fig. 5). Confirmatory investigation utilized cell lines (HL60 and U937) that expressed P2Y1 (HL60 only), P2Y2, and P2Y6 receptors and 293T cells transiently transfected with a P2Y4 expression plasmid. Both cell lines and P2Y4-transfected 293T cells fluxed calcium upon stimulation with ATP or UTP, respectively, but none responded to poly(A), suggesting that poly(A) signaled through a nucleotide receptor that could be desensitized with ADP and ATP, and whose signaling was blocked by pertussis toxin and suramin. The distribution of the poly(A) signaling activity was limited because neither poly(A) nor mRNA sig-

RNA-mediated DC Signaling and Activation

Fig. 4. mRNA activation of DC results in CD4\(^+\) T cells that produce IFN-γ. DC activated with mRNA (4.4 μg/ml), LPS (1 μg/ml), or poly(I):poly(C) (20 μg/ml) were cocultured with autologous CD4\(^+\) T cells, TSST-1 superantigen (0.01 ng/ml), and brefeldin A. After 6 h, cells were permeabilized, stained for IFN-γ, IL-4, and CD69, and flow cytometrically analyzed. Data from one subject are representative of two donors.
Our in vitro synthesized mRNA was prepared from nucleotide triphosphates and purified by LiCl precipitation and EtOH washing to remove free nucleotides. The lack of signaling of these RNA preparations on cell lines expressing P2Y1,-2,-4,-6, and -11 suggested that the RNA preparations did not contain contaminating nucleotides that were responsible for Ca$^{2+}$ signaling. In addition, homopolymers other than poly(A) made by polynucleotide phosphorylase catalyzed polymerization of ADP, UDP, CDP, and/or GDP also did not signal DC, suggesting that the receptor likely recognized stretches of A in RNA and not contaminating ADP.

The data suggested that poly(A) represented a subset, moderate induction of markers of DC activation and IL-12 secretion but no TNF-α or IFN-α (Table I), of the DC maturation activity of mRNA. The ability of in vitro transcribed encoding mRNA to induce IFN-α and TNF-α suggested that part of the mRNA’s DC maturation ability might be mediated by the formation of regions of dsRNA. This dsRNA would activate DC in a similar manner as poly(I):poly(C), although it likely may not provide the same intensity of activation due to the lower levels of dsRNA content of the mRNA (12). It was also possible that the poly(A) signaling altered the dsRNA DC activation effect. This was supported by the differences in DC maturation markers induced by poly(I):poly(C) and mRNA (Fig. 1 and Table I) and directly tested by demonstrating that adding poly(A) to poly(I):poly(C) stimulation of DC altered the activation markers CD80 and CD86 such that it resembled mRNA activation (Fig. 8). The magnitude of change in the level of the activation markers, although relatively small, was reproduced in three separate experiments. The samples normalized each experiment to equal untreated mean fluorescence for each condition and averaged the results of three experiments.

FIG. 6. Poly(A)-induced Ca$^{2+}$ flux is blocked by a preliminary stimulation with ATP, 2MeSATP, or ADP but not UTP. Fura-2-loaded DC were sequentially stimulated with ATP (5 μM), UTP (20 μM), ADP (5 μM), and 2MeSATP (1 μM) treated with CPK and CP to convert contaminating 2MeSADP, poly(A) (67 μM AMP equivalents), and RANTES (33 ng/ml). The ratio of emissions at 510 nm versus time is presented. A low concentration of ATP was used to avoid toxic effects observed at higher ATP concentrations. Data are representative of three preparations of DC from three donors. Portions of data from two donors are shown.

FIG. 5. Poly(A) induces a Ca$^{2+}$ flux in DC, which can be inhibited by pertussis toxin (PTX) and suramin. DC were preincubated with medium or pertussis toxin (30 μg/ml) for 5 h, loaded with Fura-2, treated with EGTA (200 μM) or suramin (30 μM) for 10 min, where indicated, and then stimulated with Gag-encoding mRNA with or without (noted with an asterisk) a poly(A) tail (30 μg/ml), ATP (100 μM), poly(A) (67 μM AMP equivalents), poly(U) (67 μM AMP equivalents), or RANTES (33 ng/ml) and followed for Fura-2 spectral changes induced by Ca$^{2+}$ release. The ratio of emissions at 510 nm is given versus time. The same scale of ratio of light emission and time was used for each assay shown. Ca$^{2+}$ fluxes presented were obtained from multiple samples of DC from different donors. Three to ten repetitions for each condition were performed.

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**DISCUSSION**

In the current report, we characterize a new signaling activity by extracellular mRNA that activates DC and determine the resulting phenotype and functional ability of mRNA-matured DC to activate T cells and compared it to other DC maturation stimuli. mRNA-activated DC expressed MHC classes I and II and coactivation molecules CD80 and CD86 at similar or greater levels than the potent DC activators, LPS, dsRNA, and CD40L. mRNA also induced IL-12, IFN-α, IL-8, and TNF-α production by DC. In vitro transcribed RNA containing a cod-
CD80

CD86

Mean fluorescence

none
Gag mRNA
poly(A)
poly(A)-poly(C)
poly(A)-poly(C)

P=0.01
P=0.05
P=0.05
P=0.05

FIG. 8. The addition of poly(A) to poly(I)-poly(C) alters the expression of CD80 and CD86 to levels observed with mRNA activation. DCs were activated with the indicated agents for 24 h followed by staining with directly conjugated specific antibodies and flow cytometrically analyzed. The mean fluorescence for each marker was calculated for each activation agent. P values were calculated by normalizing mean fluorescence based on the untreated samples from the three replicates and applying a one-tailed t test. A single-tailed t test was used as the predicted results of change for each activation marker was to only increase after activation. Error bars represent plus or minus two standard deviations.

do not signal to mRNA, but as new members of this subclass of P2Y receptors are identified, they will need to be screened for DC expression and mRNA signaling.

Based on our data, we cannot determine whether mRNA uses a new nucleotide receptor present on DC but absent on other lymphoid cell types (T cells, B cells, monocyte/macrophages) or on cell lines constructed to express most known P2Y receptors or if mRNA is a ligand for a heterodimer of known GPCR. Heterodimers of GPCR have been observed in multiple systems, including chemokine (43), γ-aminobutyric acid (44), opioid receptors (45, 46), and nucleotide receptors (P2Y) (47). The functional γ-aminobutyric acid type B receptor is a heterodimer of two GPCR receptors with low homology to each other. The expression of both receptors is required for signal transduction following ligand binding (44). Thus, GPCR heterodimers can significantly change the binding affinity for ligands and form receptors capable of binding ligands to which neither receptor alone sends signal.

The ability of poly(A) to signal a GPCR on DC is highly suggestive that this signaling activity is responsible for the DC-activating properties we observed. We cannot exclude that poly(A) has another activity in addition to GPCR signaling. It is interesting that ATP and poly(A) both activate DC through GPCR but do so through different receptors and signaling mechanisms with different results. ATP signaling through P2Y11 increases cAMP concentrations in DC, a property shared with prostaglandin E2, another DC-activating agent that synergizes with TNF-α (48). This activation of cAMP leads to a reduced ability to secrete IL-12 (19, 23). Poly(A), whose signaling activity is likely due to an ADP family, P2Y type nucleotide receptor, does not increase cAMP levels and induces IL-12 secretion. A recent report highlighted the ability of ligands utilizing similar signal transduction pathways to differentially activate DC. In this report, signaling through TLR 2 and 4, which share signaling pathways, including NF-xB and mitogen-activated protein kinase family member activation, led to DC that differed in the cytokines produced and the resulting phenotype of T cells activated (13). A number of host cell-derived molecules cooperate with TNF-α, which by itself does not completely activate DC (49), to induce DC activation (18, 19, 23, 49–51). Some of these molecules (ATP, PGE2) alter the cytokine secretion patterns of the mature DC and the phenotype of the resulting T cells (18, 23, 51). We also observed that the addition of poly(A) to poly(I)-poly(C)-activated DC changed the resulting DC phenotype, such that it more closely resembled mRNA activation of DC compared with dsRNA. dsRNA has recently been demonstrated to signal TLR3 at concentrations at least 4-fold higher than that required for mRNA to induce DC activation (53). Further studies will be required to determine whether mRNA, containing a coding sequence, signals through TLR3. mRNA lacking a poly(A) tail induced markers of DC activation but at a level below that observed for mRNA with a poly(A) tail. The signaling by the poly(A) tail both increased the mRNA ability to mature DC and alter the phenotype of the activated DC.

These studies identify a new class of ligands for P2Y nucleotide receptors, mRNA. Originally described as a receptor that bound and induced a response to ATP, seven 7-transmembrane, G-protein linked nucleotide receptors have been identified in humans. Although nucleotide receptors are present on nearly every cell type, including immunologic, neurologic, cardiac, salivary, and bone, we have been unable to identify another cell type that fluxes Ca2+ in response to mRNA, suggesting the mRNA nucleotide receptor is restricted in its expression, as has been described for the members of a new family of ADP receptors, P2Y12 (36) and P2Y13 (29). In addition to nucleotide receptors, a class of
receptors responsive to adenine dinucleotides has also been described (54). The ligand binding characteristics and the lack of selectivity to inhibitors (pertussis toxin and suramin) of these receptors do not parallel those of poly(A)-mediated signaling. A number of orphan receptors related to nucleotide receptors have been identified. Studies are now in progress to identify whether an identified orphan or new nucleotide receptor, specific for poly(A) and mRNA containing a poly(A) tail, is present on DC if receptor heterodimerization between known GPCR results in RNA signaling.

DCs obtain antigen from microbial pathogens through germ-line-encoded pattern recognition receptors. DCs are also responsible for developing immune responses to altered self where antigen loading occurs through the endocytosis and processing of infected or transformed cells. The use of mRNA to load DC with antigen has been observed to be an efficient and potent method for loading antigen-processing pathways for CD4+ T cells, CD8+ T cells (24, 25, 55–64), and B cells2 that exceeds those observed for DNA, viral, and protein immunization methods (52, 63). This study tentatively activates DC, adds to this hypothesis. The ability of mice injected with DC, pulsed with naked extracellular mRNA (1000- to 10,000-fold better than other antigen presenting cell) (25), and the ability of mice injected with DC, pulsed with naked extracellular mRNA or immunized with RNA, to mount a primary immune response (25, 55). The finding of this report, that extracellular RNA has a specific signaling activity that potently activates DC, adds to this hypothesis.

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REFERENCES

1. Steinman, R. M., and Inaba, K. (1999) J. Leukoc. Biol. 66, 205–208
2. Steinman, R. M., and Zitgraft, G. (1999) J. Leukoc. Biol. 65, 271–296
3. Lanzavecchia, A. (1996) Curr. Opin. Immunol. 8, 348–354
4. Imler, J. L., and Hoffmann, J. A. (2000) Rev. Immunogenet. 2, 294–304
5. Palendran, B., Palucha, K., and Blanchereau, J. (2001) Science 293, 253–256
6. Akira, S., Takeda, K., and Kaisho, T. (2001) J. Biol. Chem. 276, 41479–41485
7. Lanzavecchia, A., and Sallusto, F. (2001) Curr. Opin. Immunol. 13, 291–298
8. Liu, Y. J., Kanzler, H., Soumelis, V., and Gillett, M. (2001) J. Natl. Immunol. 2, 585–590
9. Wan, Y., and Brimson, J. (2001) Curr. Pharm. Des. 7, 977–992
10. Sallusto, F., and Lanzavecchia, A. (2000) Immunol. Rev. 177, 134–140
11. Allavena, P., Sica, A., Vecchi, A., Locati, M., Sznaji, M., and Mantovani, A. (2000) Immunol. Rev. 177, 141–149
12. Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999) J. Exp. Med. 189, 821–829
13. Re, F., and Strominger, J. L. (2001) J. Biol. Chem. 276, 37692–37699
14. Urban, B. C., Ferguson, D. J., Pain, A., Willcox, N., Plebanski, M., Austyn, J. M., and Roberts, D. J. (1999) Nature 400, 73–77
15. Urban, B. C., Wilcox, N., and Roberts, D. J. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 8750–8755
16. Ito, T., Amakawa, R., Inaba, M., Ikeharha, S., Inaba, K., and Fukuhara, S. (2001) J. Immunol. 166, 2861–2869
17. Berchtold, S., Ogivie, A. L., Bogdan, C., Muhl-Zurbes, P., Ogivie, A., Schuler, G., and Steinka瑟ser, A. (1999) PERS LETT. 458, 424–428
18. Schnurr, M., Then, F., Galambos, P., Scholz, C., Sigmund, B., Endres, S., and Egler, A. (2000) J. Immunol. 165, 4704–4709
19. Wilkin, F., Duhant, B., Bruyns, C., Suarez-Huerta, N., Boeynaems, J. M., and Conley, P. B. (2001) Nature 409, 202–207
20. Ferrari, D., La Sala, A., Ferrari, D., Corinti, S., Cavani, A., Di Virgilio, F., and Girolomoni, G. (2001) J. Immunol. 166, 911–1117
21. Steinbrink, K., Paraguas, J. X., Toledo, T., Knop, J., and Enk, A. H. (2000) Arch. Dermatol. Res. 292, 447–455
22. Moore, M. A., Lyerly, H. K., Gilboa, E., Thomas, E., and Nair, S. K. (1998) Cancer Res. 58, 2965–2968
23. Alexopoulos, L., Holt, A. C., Medzhitov, R., and Flavell, R. A. (2001) Nature 413, 732–738
24. Pintor, J., and Miras-Portugal, M. T. (1995) Br. J. Pharmacol. 115, 895–902
25. Nair, S. K., Boczkowski, D., Morse, M., Cumming R. I., Lyerly, H. K., and Gilboa, E. (1998) Nature Biotechnol. 16, 364–369
26. Gilboa, E., Nair, S. K., and Lyerly, H. K. (1998) Cancer Immunol. Immunother. 46, 82–97
27. Ashley, D. M., Faute, B., Nair, S., Hale, L. B., Dinger, D. B., and Gilboa, E. (1997) J. Exp. Med. 186, 1177–1182
28. Boczkowski, D., Nair, S. K., Snyder, D., and Gilboa, E. (1996) J. Exp. Med. 184, 365–472
29. Murphy, G. P., Tjio, M., and Simmons, S. J., Rade, G., Rogers, M., Elgama, A., Kenny, G. M., Troychak, M. J., Salgair, M. L., and Boynton, A. L. (1999) Prostate 39, 54–59
30. Nair, S. K., Heis, A., Boczkowski, D., Majumdar, A., Nair, M., Lebkowski J. S., Vieweg, J., and Gilboa, E. (2000) Nat. Med. 6, 1011–1017
31. Ying, H., Zaks, T. Z., Wang, F., Irvine, K. R., Kummala, U. S., Marincola, F. M., and Rosenberg, S. A. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 49–56
32. Zhang, W., He, L., Yuan, Z., Xie, Z., Wang, J., Hamada, H., and Cao, X. (1999) Hum. Gene. Ther. 10, 1151–1161
33. Van Tendeloo, V. F., Ponsarts, P., Lardin, F., Nigs, J., Lenou, J., Van Broeckhoven, C., Van Boockstael, D. B., and Berneman, Z. N. (2001) Blood 98, 852–857
34. Saenz-Badillos, J., Amin, S. P., and Granstein, R. D. (2001) Exp. Dermatol. 10, 134–154
35. Andersson, C., Vasonnelos, N. M., Sievertman, M., Haddad, D., Liljeqvist, S., Berglund, P., Liljestrom, A., Ashleigh, N., Stahl, S., and Berzins, K. (2001) Scand J. Immunol. 54, 117–124

2 D. Weissman, G. Cannon, and K. Karikö, unpublished observations.