Noncoding RNA danger motifs bridge innate and adaptive immunity and are potent adjuvants for vaccination

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The adaptive immune response is triggered by recognition of T and B cell epitopes and is influenced by “danger” motifs that act via innate immune receptors. This study shows that motifs associated with noncoding RNA are essential features in the immune response reminiscent of viral infection, mediating rapid induction of proinflammatory chemokine expression, recruitment and activation of antigen-presenting cells, modulation of regulatory cytokines, subsequent differentiation of Th1 cells, iso-type switching, and stimulation of cross-priming. The heterogeneity of RNA-associated motifs results in differential binding to cellular receptors, and specifically impacts the immune profile. Naturally occurring double-stranded RNA (dsRNA) triggered activation of dendritic cells and enhancement of specific immunity, similar to selected synthetic dsRNA motifs. Based on the ability of specific RNA motifs to block tolerance induction and effectively organize the immune defense during viral infection, we conclude that such RNA species are potent danger motifs. We also demonstrate the feasibility of using selected RNA motifs as adjuvants in the context of novel aerosol carriers for optimizing the immune response to subunit vaccines. In conclusion, RNA-associated motifs produced during viral infection bridge the early response with the late adaptive phase, regulating the activation and differentiation of antigen-specific B and T cells, in addition to a short-term impact on innate immunity.

facilitate the induction of class I–restricted immune responses during viral infections, thought until recently to occur primarily as a result of abortive or productive infection of APCs (12).

In the current study, we demonstrate that in addition to the single- versus double-stranded nature of RNA, oligonucleotide composition is a critical determinant for recognition of noncoding RNA motifs by innate immune receptors. In addition, heterogeneous synthetic RNA motifs have a potent and differential impact on adaptive immunity, mediating the major features of immunity against viruses. Similarly, naturally occurring dsRNA induced both activation of DCs and enhancement of specific responses. Finally, we show that defined synthetic RNA motifs can be effectively used in the context of vaccination to trigger enhanced antibody, Th1, and T cytotoxic 1 cells (Tc1) responses.

Methods

Antigens and immunomodulators. A panel of 18 single-stranded and double-stranded synthetic RNAs (Table 1) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and dissolved in sterile PBS. The RNAs were used as pools or individually. Low-endotoxin ovalbumin (OVA) was purchased from Sigma-Aldrich Cholera toxin subunit B (CTB) was purchased from Calbiochem-Novabiochem Corp. (San Diego, California, USA); CFA from Becton, Dickinson and Co. (Franklin Lakes, New Jersey, USA); and human IgG (hIgG) from Sigma-Aldrich. The identity of gp140 antigen was confirmed by Western blot analysis using HIV envelope–specific antibodies purchased from Cytiva Biotech, Piscataway, New Jersey, USA. The physicochemical properties of aerosol vectors, revealed by scanning electron micrographs, were defined prior to use. The majority of the particulate structures displayed a quasispherical shape, with diameters between 1 µm and 3 µm.

Immunization, challenge, and measurement of virus titers. Mice and rats were primed by intratracheal instillation or aerosolization using a PennCentury insufflator device (PennCentury Inc., Philadelphia, Pennsylvania, USA) according to the manufacturer’s instructions. Mice received two viral boosts intranasally at 2-week intervals. For induction of high-zone tolerance, the mice were primed by intravenous injection. Finally, for the induction of strong immune responses, mice were

<table>
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<th>Category of RNA</th>
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<td>p(C,I); p(A,U); p(A,G)</td>
<td>p(A,C,G); p(A,C,U); p(A,G,U)</td>
<td>p(C); p(G); p(A); p(U); p(C); p(G); p(A); p(U)</td>
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<td>Double-stranded</td>
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*Synthetic RNAs were dissolved in sterile PBS and used individually or mixed in equivalent amounts as shown above. The total number of 18 compounds was initially divided for screening purposes in four pools obtained by mixing ssRNAs, and in a fifth pool of dsRNAs. The total concentration of RNA in each pool was 2 mg/ml. *p, poly.

Table 1

Nucleotide composition of synthetic RNA compounds used for screening of immunomodulatory motifs.
immunized subcutaneously with antigen emulsified in CFA. The amounts of antigens used for priming, boosts, or induction of tolerance were: OVA, 100 µg; HIV gp140, 10 µg; hIgG, 200 µg; and sucrose-purified UV-inactivated WSN virus (UV-WSN), 20 µg. The amount of synthetic RNA used was 40–50 µg/dose with or without antigen, incorporated or not into SC-V. The amount of CTB per dose was 10 µg. Nonformulated antigens were delivered in saline. Formulated antigens were delivered in perfluorocarbon, an inert vehicle that is compatible with the SC-V matrix (total volume of instillation or aerosolization, 40–45 µl).

For virus challenge, C57BL/6 and TLR4–/– C3H/HeJ mice under Metofane anesthesia were infected with 10^4 TCID_{50} of live WSN virus via the nasal route. On day 5 after infection, the mice were sacrificed and lungs were harvested, homogenized, and stored at –70°C. Virus titers were measured by 48-hour incubation of serial dilutions of samples with permissive MDCK cells, followed by standard hemagglutination with chicken red blood cells (Animal Technologies Inc., Tyler, Texas, USA). The endpoint titers were estimated in triplicate measurements by interpolation (15) and expressed as total TCID_{50}/organ.

**Measurement of antibody and T cell response.** Antibody responses were measured by ELISA. In brief, wells were coated with antigen (2 µg/ml gp140, 8 µg/ml sucrose-purified virus, 10 µg/ml hIgG, or 10 µg/ml OVA) and blocked with Sea Block (Pierce Biotechnology Inc.). Serial dilutions of samples and bronchoalveolar lavage fluid were incubated in the wells for at least 2 hours at room temperature. After washing the wells, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma-Aldrich) followed by addition of substrate (pNPP; Sigma-Aldrich) and measurement with an automatic microtiter plate reader (THERMOMax; Molecular Devices Corp., Sunnyvale, California, USA) equipped with SoftMax software.

For measurement of cellular responses, splenic cell suspensions were obtained by passing spleenic tissue through 70-µm nylon Falcon strainers (Becton, Dickinson and Co.) followed by lysis of red blood cells with red blood cell lysis buffer (Sigma-Aldrich). The lymphocytes from the pulmonary lymphoid tissue were isolated by collagenase (Sigma-Aldrich) digestion of lung tissue followed by Ficoll-Paque (Amersham Pharmacia Biotech) gradient centrifugation. The T cell response was measured by ELISpot analysis as follows: 96-well 45-µm nylon Falcon strainers (Becton, Dickinson and Co.) equipped with SoftMax software. A mixed mouse NIH/NIAID C57BL/6 background of APCs such as CD11c+ DCs were separated from spleens by magnetic cell sorting (MACS; Miltenyi Biotec, Auburn, California, USA). For stimulation, we used graded amounts of antigen (OVA, gp140, hIgG, or sucrose-purified WSN virus) or peptides: class II–restricted HA SFERFIEFPKE, or class I–restricted SIINFEKL or HIV V3–derived R10K peptide (16). Both the H-2K^d–restricted OVA-derived SIINFEKL peptide and the H-2K^d–restricted V3-derived peptide are well-known epitopes recognized by MHC class I–restricted CD8^+ T cells (16, 17). At 72 hours after stimulation, the assay was developed with biotinylated rat anti–mouse cytokine antibodies (Pharmingen) followed by streptavidin-conjugated horseradish peroxidase (BioSource International, Camarillo, California, USA) and insoluble AEC substrate. The results were measured using an automatic imaging system (Navitar Micromate; Navitar Inc., Rochester, New York, USA) equipped with multiparametric analysis software (Image-Pro; Media Cybernetics Inc., Silver Spring, Maryland, USA).

**Measurement of chemokine gene expression.** The level of chemokine expression in the lungs of mice treated 1 day previously with synthetic RNA or control was measured by DNA array technology as follows: total RNA was isolated from lungs using an RNaseasy kit (QIAGEN Inc., Valencia, California, USA). RNAs were further purified by treatment with RNase-free DNase I (Stratagene, San Diego, California, USA). DNA array was performed using the Nonrad GEAArray kit from SuperArray Bioscience Corp. (Bethesda, Maryland, USA). Briefly, cDNA probes were synthesized using Moloney murine leukemia virus reverse transcriptase with dNTP mix containing biotin-16-DUTP. The GEAArray membranes were prehybridized at 68°C for 1–2 hours. Hybridization was carried out by incubation of the membranes with biotin-labeled cDNA. The hybridized membranes were washed twice in 2x SSC with 1% SDS and twice in 0.1x SSC with 0.5% SDS. The membranes were further incubated with alkaline phosphatase–conjugated streptavidin (BioSource International) and finally developed with CDP-Star chemiluminescent substrate (New England Biolabs Inc., Beverly, Massachusetts, USA). Signal intensity was measured with an Image-Pro analysis system equipped with Gel-Pro software (Media Cybernetics).

**Flow cytometry.** Cell suspensions from lungs of mice treated 1 day previously with 50 µg of synthetic RNA or saline were prepared by collagenase digestion and Ficoll gradient centrifugation as above. The cells were resuspended in PBS containing 1% (vol/vol) mouse serum (Sigma-Aldrich) and 1% (wt/vol) BSA, fraction V (Sigma-Aldrich), and stained with phycoerythrin-labeled (PE-labeled) rat anti–mouse CD11b, PE-labeled hamster anti–mouse CD11c, or the appropriate PE-labeled isotype control (all from Pharmingen) at a concentration of 1 µg antibody/10^6 cells for 40 minutes on ice. The analysis was carried out on a FACSCalibur instrument (Becton, Dickinson and Co.). Nonviable cells were gated out using propidium iodide.

**Magnetic separation and adoptive transfer.** Professional APCs such as CD11c^+ DCs were separated from spleens of BALB/c mice using magnetic beads coupled to rat anti–mouse CD11c antibodies (Miltenyi Biotec). In brief,
The impact of various RNA pools (see Table 1) on adaptive immunity was measured in C57BL/6 mice coimmunized with OVA via the respiratory tract. Mice were immunized intratracheally, followed by two boosts 2 weeks apart by intranasal instillation. The antibody response was expressed as mean ± SEM of IgG endpoint titers (n = 4/group). As controls, we used dose-matched OVA in sterile PBS, OVA with CTB, and PBS alone, respectively. (B) Effect of various dsRNA motifs on the induction of antibody response to OVA; results are expressed as in a. The data are representative of two independent experiments. Inset: the ratio of mean IgG2a and IgG1 titers to OVA for each group. The order from left to right is similar to that in the larger graph: PBS OVA, CTB OVA, pC:pG OVA, pI:pC OVA, and pA:pU OVA. (C) Magnitude and profile of T cell response induced by OVA together with various dsRNA motifs in female C57BL/6 mice. The results obtained by ELISPOT analysis were expressed as mean ± SEM of the number of IFN-γ and IL-4 spot-forming colonies (SFCs) per spleen (n = 4/group). The results are representative of two independent experiments.

Isolation of naturally occurring dsRNA. Permissive MDCK cells were infected with WSN influenza virus (10⁶ TCID₅₀/1 × 10⁷ cells), and after 24 hours, the cells were harvested and washed, and total RNA was extracted using an RNA separation kit (QIAGEN Inc.). The RNA was further purified by treatment with RNase-free DNase I (Stratagene). The single-stranded RNA (ssRNA) in the samples was then removed by 30 minutes of incubation at 37°C with 5 U of S1 nuclease (Ambion Inc., Austin, Texas, USA) per µg RNA. The RNA was analyzed as a mixture with recombinant gp140 antigen (25 µg/ml sterile PBS). The concentration of nucleic acid was measured by spectrophotometry at an absorbance of 260 nm. The absence of endotoxin was confirmed by measuring the average number of bacteria per molecule of fluorescent dye (approximately 240). For in vitro staining of splenic APCs, the cells were isolated by MACS as described above. Cells were resuspended in FACS buffer and stained at a concentration of 10⁴ cells/50 µl with 10 µg of tagged dsRNA (pApU-F) after a dose-effect optimization study. Following 30 minutes of incubation at 4°C, the cells were washed and analyzed by flow cytometry (FACSCalibur; Becton, Dickinson and Co.). For competitive inhibition, the cells were preincubated for 10 minutes at 4°C with different amounts of nontagged pApU, pIpC, or pA followed by the addition of pApU-F as described above.

Statistical analysis. Magnitudes of immune responses were compared using the Student t test, assuming a normal distribution of the values and equal variances.

Results
Systematic definition of RNA motifs that modulate immune responses. During viral infection, transient and unusual RNA species are created and may act as “danger” signals. We postulated that various RNA motifs are being recognized by the innate immune cells and profoundly
regulate the adaptive immune response. To address this hypothesis on a systematic basis, we screened a library of synthetic ssRNA and dsRNA motifs for the ability to modulate the specific IgG response to a protein antigen (OVA) administered via the respiratory tract. To simplify the process, we organized the screening into two rounds, with round one involving pools of RNA species (Table 1) and the second dissecting components within the pools having maximal impact on the immune response. As shown in Figure 1a, the pool of dsRNA (pool 5) displayed maximal impact on the antibody response, with substantial enhancement of specific immunity. In addition, mixtures of single-stranded species that complement each other (pool 1) partially reproduced this enhancement. This suggests that both the residues in and the secondary structure of an RNA determines its ability to act as a danger motif. In the second round of screening, we show that two categories of motifs organized as dsRNA, namely pA:pU and pI:pC, have a significant impact on the development of IgG response to a specific antigen in C57BL/6 mice (Figure 1b). Furthermore, both pA:pU and pI:pC had an amplifying effect on the specific T cell response (Figure 1c). Surprisingly, the profile of the T cell response was dependent on the nature of the RNA residues, suggesting that the immune system is able to discriminate RNA-associated danger motifs. RNA motifs containing A and U residues rather than I and C residues were able to direct the differentiation of IFN-γ-producing Th1 cells (Figure 1c) in C57BL/6 mice. This result was reflected in differential induction of IgG isotypes, consistent with the Th profile (Figure 1b, inset). Thus, pA:pU displayed both T1-promoting and antibody-enhancing effects. The stimulatory properties of selected dsRNA motifs on adaptive immunity were preserved in C3H/HeJ mice, which have defective TLR-4 and thus are unable to mount a normal response to LPS (Figure 2a).

The impact of dsRNA on antibody response was independently confirmed with additional foreign antigens, namely HIV envelope protein (recombinant gp140) and whole inactivated influenza virus, administered to BALB/c mice (Figure 2, b and c). In fact, pA:pU rather than pI:pC restored the titer of specific antibodies to influenza virus in BALB/c mice to a level similar to that triggered by infection (Figure 2c). In addition, in the context of immunization with inactivated virus, pA:pU restored the T cell response to a similar magnitude to that conferred by natural infection (Figure 2d). Thus, different RNA motifs exert a previously unknown and broad range of effects on T and B cell responses.

Motifs associated with dsRNA regulate the recruitment and activation of professional APCs. We hypothesized that dsRNA-associated danger motifs such as pA:pU and pI:pC influence the T cell response indirectly via components of innate immunity. To test this hypothesis, we first defined the expression of chemokine genes in the pulmonary lymphoid tissue after administration of RNAs. DNA array technique showed that IP-10, MIG, MIP-1α, MIP-1β, and MCP-1 were strongly induced by both pA:pU and pI:pC (Figure 3a). However, only pI:pC triggered expression of RANTES and MCP-3, CC chemokines that have the ability to engage receptors selectively expressed by Th2 cells. LPS induced a different chemokine expression: upregulation of CXC chemokines MIG and MIP-1α and the CC chemokine TCA-3 (Figure 3a). FACS analysis of pulmonary interstitial cells subsequent to mucosal administration of pA:pU and pI:pC showed prompt recruitment of CD11b+ monocytes and CD11c+ DCs (Figure 3b). There was no substantial

Figure 2
Increase of immune response to viral antigens by a specific dsRNA motif. (a) The induction of antigen-specific IgG response in C3H/HeJ mice deficient in TLR-4 was measured by ELISA after immunization with OVA together with pI:pC (filled diamonds) or pA:pU (filled squares), or with OVA alone (open triangles). Results are expressed as mean ± SEM of OD (measured at 405 nm) corresponding to different serum dilutions (n = 3 mice/group) and are representative of two independent measurements. (b) The effect on the antibody response to the HIV gp140 fragment was measured in female BALB/c mice immunized via the respiratory tract (intratracheal administration followed by two boosts 2 weeks apart, carried out by intranasal instillation) with gp140 alone or gp140 together with pA:pU. The results were expressed as mean ± SEM of IgG endpoint titers (n = 3 mice/group). (c) Similarly, influenza virus–specific IgG antibodies were measured after mucosal immunization of female BALB/c mice with UV-inactivated WSN virus (UV-WSN) alone or together with dsRNA motifs. As control we measured the antibody response after infection with the same strain of influenza virus (n = 4/group). (d) The T cell response to whole influenza virus was studied by ELISPOT analysis of splenocytes and was expressed as IFN-γ (gray bars), IL-4 (white bars), and IL-2 (black bars) SFCs/spleen (mean ± SEM, n = 4/group) after subtraction of background. The T cell response to antigen together with pA:pU was compared with the response to immunization with antigen alone or to influenza virus infection. The experiment was carried out in female BALB/c mice.
recruitment of leukocytes at the doses studied that may have interfered with the functional capacity of the lungs (25% increase in the case of pl:pc, 20% for pA:pU, and 15% for CTB, compared with naive mice). In addition, in vitro incubation of CD11c+ DCs from naive mice with antigen together with pA:pU (and to a lesser extent pl:pc) resulted in their activation, since subsequent adoptive transfer of antigen-pulsed cells into BALB/c recipients triggered enhanced class II–restricted T cell immunity (Figure 3c). Similar enhancement has been measured by APC incubation with anti-CD40 antibody or IL-12. Finally, pA:pU had a strong MHC class I–restricted T cell–promoting effect in the context of mucosal vaccination with recombinant HIV envelope protein gp140 or OVA (Figure 3, d and e), as revealed by ELISpot analysis with characterized peptides (16, 17) from the HIV envelope protein and OVA, respectively. In parallel, we confirmed that negatively separated CD8+ cells, rather than CD8– T cells, were responsive to the MHC class I–restricted peptide SIINFEKL (data not shown). Thus, dsRNA has the ability to trigger differentiation of professional APCs to a stage compatible with cross-priming of MHC class I–restricted T cells.

Since this process may involve binding to receptors specific for RNA motifs, we tested the binding profile of fluorescently tagged pA:pU to APCs in vitro and in vivo. Both CD11b+ and CD11c+ APCs isolated from secondary lymphoid organs or pulmonary tissue displayed subsets that were dimly or intensely stained with pA:pU-F. For example, as shown in Figure 4a, approximately 10% of splenic CD11b+ APCs stained intensely when pulsed ex vivo at 4°C. This staining was effectively inhibited by excess nontagged pA:pU and to a lesser extent by pl:pc. In contrast, even a large excess of nontagged pl:pc failed to affect the percentage of APCs highly stained with pA:pU-F. The dimly stained subsets were relatively unaffected in all circumstances, suggesting the coexistence of poorly saturable or nonspecific receptors. When administered via the respiratory tract by intratracheal inoculation in saline, pA:pU-F stained 3–4% of the local APCs (CD11b+ and CD11c+) (Figure 4b). CD11b– and CD11c– cells were not significantly stained.

To test whether naturally occurring dsRNA promotes the activation of APCs and enhances adaptive immunity, we infected permissive MDCK cells with influenza virus and isolated total RNA after 24 hours. The ssRNA and DNA were removed by treatment with nucleases (see Methods), and the absence of infectious material was confirmed using standard titration on MDCK cells. Incubation of CD11c+ DCs with naturally occurring dsRNA or synthetic pA:pU promotes increased production of IL-12 and TNF-α (Figure 4c). No increased production of IL-1β was measured (not shown). In addition, CD11c+ APCs did not display significant cytokine production. Finally, incubating APCs with control RNA from noninfected MDCK cells did not result in increased cytokine production (Figure 4c). Coadministration of naturally occurring dsRNA with limiting amounts of prototype

**Figure 3**

The impact of defined dsRNA motifs on innate immunity and APCs. (a) The chemokine expression triggered by 50 µg pA:pU (black bars) or pl:pc (white bars) dsRNA was compared with that induced by 1 µg of LPS (gray bars) 24 hours after mucosal administration. The chemokines that bind to receptors on Th1 and Th2 cells are indicated with solid or dashed lines. (b) Recruitment of professional APCs in the lungs of mice treated with dsRNA motifs was assessed by FACS. Results are expressed as number of CD11c+ (black bars) and CD11b+ (white bars) cells separated from the pulmonary interstitial tissue (n = 4/group). (c) Activation of professional APCs by the dsRNA motifs was ascertained by ex vivo overnight pulsing of CD11c+ cells with antigen (100 µg/ml of IgHA) together with 50 µg/ml of dsRNA, followed by adoptive transfer of APCs into naive mice. As controls, we used antigen-pulsed APCs or costimulated with either recombinant IL-12 or anti-CD40 mAb. Results are expressed as number of IL-2+ SFCs measured in the spleen. (d) Cross-priming was studied in BALB/c mice immunized with gp140 antigen together or without pA:pU. The response was measured by ELISpot analysis using the peptide R10K. Results are expressed as mean ± SEM of the number of IFN-γ+ (black bars) and IL-4 (white bars) SFCs/spleen (n = 4/group). (e) Cross-priming was studied in C57BL/6 mice treated with 100 µg of whole OVA together with or without pA:pU, by ELISpot analysis using in vitro stimulation with the peptide SIINFEKL. Results are expressed in d.
antigen (gp140) to BALB/c mice by the mucosal respiratory route resulted in significant enhancement of the specific IgG response (Figure 4d). Synthetic pA:pU displayed an immune-enhancing effect as well, in contrast to control material from noninfected MDCK cells that failed to promote an enhanced antibody response (Figure 4d and e). The effect of naturally occurring dsRNA and of synthetic pA:pU was to promote both T1-controlled IgG2a antibodies and IgG1 antibodies (Figure 4e).

Together, these data show a profound impact of synthetic and naturally occurring RNA motifs on elements of innate immunity that in turn has the ability to regulate the adaptive immune response.

dsRNA motifs block the induction of high-zone tolerance and trigger protective antiviral immunity. Danger molecules contribute to discrimination between innocuous antigens and those associated with infectious processes. In high doses, noninfectious purified protein antigens induce unresponsiveness or immunological tolerance. In order to test the danger-signal competence of pA:pU and pI:pC motifs, we used the model of tolerance achieved by intravenous inoculation of hIgG. Coinoculation of either pA:pU or pI:pC together with hIgG in saline prevented the induction of B cell unresponsiveness, as shown by antibody titers after boosting with hIgG in CFA (Figure 5, a and b). The dsRNA-associated motifs could both induce a higher primary response and rescue the secondary response to this prototype antigen. Identical profiles were noted in wild-type, LPS-responsive C57BL/6 mice (Figure 5a), C3H/SnJ mice (not shown), and LPS-resistant, TLR-4–deficient C3H/HeJ mice (Figure 5b). Similarly, assessment of the T cell profile revealed that IL-4 and IL-2 production were partially restored by coadministration of dsRNA with hIgG (not shown).

We postulated that danger motifs have the capability to rapidly mobilize protective arms of immune defense. Thus, we tested whether pA:pU and pI:pC have any impact on the evolution of primary infection with influenza virus. Mice treated with RNA motifs via the respiratory tract were infected with sublethal doses of influenza virus. Five days after infection, the pulmonary virus titers were quantified. Concordant results were obtained in C57BL/6 and TLR4–/– C3H/HeJ mice (Figure 5, c and d). Notably, pA:pU was more effective than pI:pC in orchestrating an effective reduction of pulmonary virus titers in C57BL/6 mice, and performed similarly in C3H/HeJ mice. Thus, in the absence of immune memory, dsRNA motifs are able to mobilize an effective primary response against viral infection, in addition to acting as a “master switch” for adaptive immunity (Figure 5e).

Enhancement of immunity to protein antigens by coencapsulation with RNA danger motifs and mucosal vaccination. Since the immune response to nonformulated subunit vaccines and the general response to purified protein antigens is minimal, we tested whether in vivo coinoculation of APCs to antigen and dsRNA danger motifs results in a more favorable outcome. Effective antigen administration to the bronchial lymphoid tissue requires the use of specialized delivery systems. To this aim, we coformulated a prototype antigen (OVA) with pA:pU or pI:pC in vectors...
with previous results, induction of T1 immunity was measured only with pA:pU particles (Figure 6b) and not with pI:pC, which displayed only enhancement of T2 immunity (not shown). Furthermore, the T cell response to pA:pU coformulated antigen displayed an important local component (Figure 6b). Finally, using the well-defined class I-restricted SIINFEKL peptide, we confirmed that pA:pU retains the ability to promote cross-priming in conjunction with the vectors used (Figure 6b).

A similar enhancement of the antibody response has been measured in Sprague-Dawley rats aerosolized with vectors loaded with OVA together with pA:pU or pI:pC (Figure 6c). Lower titers were achieved with SC-V devoid of dsRNAs or OVA in saline. The analysis of mucosal antibody titers (Figure 6d) revealed a similar profile.

Thus, coformulation of RNA-associated danger motifs and protein antigen using this formulation technology results in a substantial increase in the specific immune response with potential clinical implications.

**Discussion**

The mechanisms of immune response during microbial infection became a major area of investigation subsequent to advances in understanding the potential role of innate immunity. It quickly became evident that the innate immune cells are endowed with multiple categories of receptors that discriminate between various microbe-associated motifs or exogenous danger signals. Crosstalk between the innate and adaptive immune systems subsequent to such pattern recognition events decisively influences the magnitude and profile of T and B cell responses. The innate immune system is rapid though less discriminative, but instructs the adaptive immunity that evolves more slowly and is composed of more efficient effectors with a vast repertoire acquired by somatic mutation. This multipatterned recognition strategy that jointly employs innate and adaptive immunity shifted the immune discrimination paradigm from self/nonself to dangerous/nondangerous cognition. The poor immunogenicity of purified proteins, induction of immune mediators by microbial motifs, and characterization of the activity of such mediators (cytokines, chemokines, and costimulatory molecules) on adaptive immunity all supported this concept (18).

In the present study, we undertook a rational approach in delineating the role of noncoding RNA motifs as danger signals, with direct implications for understanding their role in controlling adaptive immunity during viral infections. In addition, we explored their use as adjuvants in conjunction with mucosal vaccination.

We employed a library of synthetic RNAs and a two-tier strategy, using as read-out the effect on adaptive rather than innate immunity. By this method, we found, surprisingly, that the oligonucleotide composition in addition to the nature of RNA as double-stranded or single-stranded plays a role in this concern. We show that AU-based motifs have the ability to turn on innate immune tolerance develops if antigen is abundant. If the antigen is limiting (immune ignorance), or coexisting antigens. In the absence of danger signals, the magnitude of immunity is low if the antigen is limiting (immune ignorance), or immune tolerance develops if antigen is abundant.

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IC- or CG-based motifs do. IC motifs defined earlier (19) resulted in enhanced T2 and B cell immunity in a manner dependent on the antigen and the animal strain (Figure 1 and Figure 2). CG motifs associated with dsRNA, and miscellaneous ssRNA motifs, had little effect on adaptive immunity unless mixtures of ssRNAs composed of complementary bases were used. Since these findings have been reproduced in the absence of functional TLR-4 (Figure 2 and Figure 5), a pathway common to that of endotoxin recognition can be ruled out at this point. Recently, it has been shown that TLR-3 plays a role in recognition of pl:pC (7), but a common recognition pathway with pA:pU seems unlikely due to the profoundly different profile of the immune response triggered by these two motifs (Figure 1). In addition, there was a clear-cut difference in the ability of pA:pU and pl:pC to antagonize the binding of tagged pA:pu to APC receptors (Figure 4), strongly supporting a model based on multiple receptors for RNA motifs. Thus, different TLR isoforms and/or coreceptors may participate in the process of discriminating RNA motifs based on the identity of nucleotides, in a process reminiscent of a rudimentary immune repertoire. In support of this concept, pA showed binding with reduced affinity to the pA:pU receptor on APCs (Figure 4). Thus, nucleotide composition and the single- versus double-stranded nature of the RNA may affect its secondary and tertiary structure, resulting in differential binding affinity to the same receptor or various isoforms. A possibility may be that TLR-9, shown to recognize palindromic unmethylated CpG oligodeoxynucleotide motifs (20), or isoforms of TLR, may be involved in dsRNA motif discrimination. Recent evidence does not support the involvement of TLR-9, since dsRNA induces a different spectrum of transcription factors and costimulatory molecules than unmethylated CpG motifs induce (21). Since both pl:pC and pA:pU induce CXC chemokines (Figure 3), it is likely that alternative mediators such as CC chemokines with the ability to selectively bind Th2 cells (22) may be responsible for the different Th profiles elicited by these motifs. In addition, this may explain the distinct effect of pl:pC and pA:pU relative to the type of APCs recruited at the site of administration (Figure 3).

Based on the data, we conclude that the newly characterized pA:pU-associated motifs can induce a large number of features of the adaptive immune response that are usually noted only after viral infection. Induction of T1 responses (both Th1 and Th2) was documented with protein antigens (OVA and gp140) and inactivated influenza virus (Figures 1–3). Induction of an MHC class I–restricted response to protein antigens suggests that this RNA motif activated APCs to a level compatible with this mechanism of processing and presentation, concordant with observations implying cross-priming in viral infections (23). This suggests that RNA-associated danger motifs rather than direct infection of APCs may be responsible for induction of cytotoxic T lymphocytes during infection with RNA viruses such as influenza virus. The enhanced magnitude of the immune response can be explained by rapid recruitment and activation (Figure 3) of APCs. Despite the fact that at this point, the differential activity of pA:pU and pl:pC cannot be ascribed to any single cytokine or chemokine, a more detailed analysis of the profile of activated APCs is likely to
address this issue. The induction of T1 immunity promoted by pA:pU was accompanied by isotype switching, resulting in generation of IgG2a antibodies. However, dsRNA could not induce an isotype switch to the IgA class. This was associated with inhibition of TGF-β (not shown), suggesting that dsRNA danger motifs act by virtue of induction of proinflammatory mediators and downregulation of anti-inflammatory mediators. These results are consistent with a considerable, but not exclusive, role of dsRNA motifs in shaping adaptive immunity during infections such as influenza, as depicted in the Figure 5e.

Since potent danger motifs should influence the outcome in terms of immune responsiveness versus tolerance, we studied whether dsRNAs prevent high-
zone tolerance to hlgG, a well-characterized model of immune unresponsiveness (24). We show that both pA:pU and pI:pC are potent inhibitors of immune tolerance (Figure 5, a and b), not only modulators of the adaptive response. This observation enriches the panel of features borne by dsRNAs and opens up the possibility that adjuvant effects of danger motifs in general are caused at least in part by prevention of immune unresponsiveness. Our data are concordant with a recent report showing that pI:pC promoted loss of tolerance to a defined autointigen (25). Lastly, since bases A and U, but not I, are found in natural RNA species, our data point to the first dsRNA motif with potential relevance to the immune response during viral infections. This concept is being strongly supported by the finding that naturally occurring dsRNA from virus-infected cells stimulated DCs and promoted increased specific responses to coadministered protein antigen (Figure 4).

The potency of pA:pU as a danger stimulator has been illustrated by its ability to control primary infection with influenza virus (Figure 5, c and d). This feature, which can be explained by rapid mobilization of innate and adaptive responses, is highly reminiscent of the ability of unmethylated CpG oligoDNA motifs to improve on immune defense during primary infection (26). By extrapolation, we conclude that the innate immune system has an exquisite ability to recognize foreign or endogenously generated infection-associated polynucleotide motifs and to accordingly regulate adaptive immunity.

Based on the results of this study, dsRNA motifs appear to be logical candidates for adjuvants in conjunction with subunit, recombinant, or inactivated vaccines. In particular, pA:pU appears likely to provide some of the beneficial features of live vaccines in the absence of vector replication. This concept was supported by our results on biocompatible aerosols as delivery vectors designed for mucosal vaccination (Figure 6).

In conclusion, by using a rational approach of selecting RNA motifs that impact the adaptive immune responses, we defined an unexpected heterogeneity of RNA-associated danger motifs. A systematic study of the adaptive immune response demonstrated that selected RNA motifs orchestrate a broad range of features that are reminiscent of natural infection. Finally, this study defines a practical approach consisting of novel formulations containing such RNA motifs that are of potential use in mucosal vaccination.

Acknowledgments
This work was supported by Alliance Pharmaceutical Corp. and by NIH grant 1R21 AI-47014 (to A. Bot).

cytes: more than an attraction. Immunol. 9:1–11.