Published in final edited form as:

Immunol Cell Biol. 2009 ; 87(8): 630–633. doi:10.1038/icb.2009.48.

All-*trans* retinoic acid potentiates the antibody response in mice to a lipopeptide antigen adjuvanted with liposomal lipid A

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Abstract

Retinoic acid (RA), the bioactive metabolite of retinol, is essential for robust humoral immunity in animals and humans. Recent interest in RA as a vaccine adjuvant has been encouraged by reports demonstrating cooperative enhancement of antibody responses to tetanus toxin in rodents by all-*trans* RA (ATRA) and a Toll-like receptor 3 (TLR3) agonist. We hypothesized that retinoic acid would augment the antibody response to a co-delivered lipopeptide immunogen derived from the membrane proximal region (MPR) of HIV-1 gp41. The MPR is weakly immunogenic and could benefit from potent new humoral adjuvants. When co-formulated in liposomes and administered to BALB/C mice, ATRA alone did not elicit serum anti-peptide antibodies to an MPR-derived lipopeptide. However, addition of all-*trans*, but not 13-*cis*, RA to a liposomal formulation containing the TLR4 agonist monophosphoryl lipid A resulted in a four-fold enhancement of serum anti-peptide IgG titers as compared to a formulation containing lipid A alone ($p = 0.00039$). The difference did not arise from biophysical changes in the liposome formulation, including vesicle size, vesicle charge, and liposome association of antigen. Thus, ATRA warrants further study as a vaccine adjuvant.

Keywords

Retinoic acid; lipid A; liposomes; gp41; membrane proximal region

Introduction

Dietary retinol is essential for the development and maintenance of a healthy immune system¹. Retinol is also required to mount robust antibody responses to T cell-dependent and type 2 T cell-independent antigens in animals and humans^{2, 3}. Retinoic acids (RAs), the bioactive metabolites of retinol, exert a multitude of immunomodulatory effects *in vivo* through binding to retinoic acid receptors and retinoid x receptors^{1, 4} (Figure 1). Recently, Ross and colleagues demonstrated cooperative enhancement of antibody responses to tetanus toxin by all-*trans* RA (ATRA) and polyriboinosinic:polyribocytidylic acid, a Toll-like receptor 3 (TLR3) agonist, in mice and rats^{5, 6}. These reports have aroused interest in the utility of ATRA as a vaccine adjuvant. However, ATRA was not co-delivered with the antigen in these studies; ATRA was administered orally, whereas tetanus toxin was injected intraperitoneally.

We sought to determine if ATRA could promote antibody responses to a model antigen in mice when co-delivered in the same formulation with the antigen. A peptide derived from the

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membrane proximal region (MPR) of HIV-1 gp41 was selected for study because the MPR is a key target for development of a vaccine that elicits neutralizing antibodies⁷. This peptide (N-MPR) consisted of the epitope of the broadly neutralizing human monoclonal antibody 2F5 with flanking residues shown to enhance binding to 2F5 *in vitro*⁸. As this antigen is thought to be best presented in a membrane environment, the peptide was covalently attached to a lipid and formulated in lipid bilayer vesicles⁹. We immunized BALB/C mice with liposomes containing lipid-anchored N-MPR and either ATRA or monophosphoryl lipid A (MPL), a TLR4 agonist and potent liposomal vaccine adjuvant^{10, 11}. The results indicate that ATRA potentiates the adjuvant effect of MPL in BALB/C mice, supporting further investigation of ATRA as a humoral vaccine adjuvant.

Results and Discussion

This study sought to determine the ability of ATRA to promote the antibody response to a co-delivered lipopeptide antigen. A liposomal delivery system was desirable because liposomes efficiently co-deliver associated antigens and adjuvants to immune cells *in vivo*¹⁰. N-MPR was derivatized with diacylglycerol because covalent attachment of lipid anchors was previously found to substantially enhance the antibody response to MPR peptides formulated in liposomes¹². Incorporation of ATRA, 13-*cis* RA, or MPL into liposomes containing N-MPR-DSG (N-MPR-succinyl-distearoylglycerol) did not appreciably affect vesicle size or charge (Table 1). Moreover, liposome association of retinoic acid and N-MPR-DSG was not significantly altered by addition of MPL or RA. Regarding liposome association of MPL, our group has previously shown virtually complete association of lipopolysaccharides with liposomes when the endotoxin is taken to dryness with the constituent lipids prior to liposome formation, as was the case in this study¹³.

ATRA alone did not stimulate production of antibodies to a co-delivered MPR lipopeptide antigen, N-MPR-DSG. However, addition of ATRA, but not 13-*cis* RA, to a liposomal formulation containing MPL resulted in a four-fold enhancement of serum IgG titers to N-MPR in BALB/C mice (respective geometric mean titers of 6720 and 1600 for 'MPL + ATRA' and 'MPL', $p = 0.00039$; Figure 2a). The effect was reproduced with independent liposome preparations (Figure 2c) and persisted at least 15 weeks after the final immunization (respective GMT of 2460 and 340 for 'MPL + ATRA' and 'MPL', $p = 0.012$; Figure 2b). The magnitude of enhancement is comparable to the benefit observed in mice and rabbits when liposomes containing MPL and a recombinant malaria antigen were adsorbed onto aluminum hydroxide, the only adjuvant currently approved for use in the United States^{14, 15}.

Several previously reported immunomodulatory effects of ATRA were not observed in this study. Despite reports showing that ATRA can promote class switching and IgA production¹⁶, anti-N-MPR IgA antibodies were not detected in sera of mice from any group (data not shown). Additionally, the serum IgG1/IgG2a ratio was not significantly altered by incorporation of ATRA in the formulation ($p = 0.499$; Figure 2d), suggesting that the T helper profile of the response was unaffected. Although this finding conflicts with prior studies reporting that ATRA supplementation promotes a Th2 phenotype³, the discrepancy may be explained by the dominant effect of MPL. Additionally, it was hypothesized that attaching ATRA to a lipid anchor (Figure 1) would afford greater retention of ATRA in the formulation *in vivo*, assuring delivery of a higher fraction of the dose to immune cells. Lipid-anchored retinoic acid (RAL) was cleaved by phospholipase A₂ *in vitro* to release free ATRA (**SI Methods**). However, RAL failed to promote anti-N-MPR antibody responses in mice, raising the question of whether this prodrug approach can deliver retinoic acid to the correct compartment *in vivo* to enhance the immune response.

The enhancement of serum antibody titers mediated by ATRA does not appear to arise from biophysical changes in the liposome formulation, as all measured biophysical parameters were consistent among formulations (Table 1). Moreover, the enhancement effect was not caused by the addition of 13-*cis* RA, which differs from ATRA by only a single bond orientation (Figure 1). Thus, further study is needed to determine the mechanistic basis of the interaction between ATRA and MPL. One possibility is the activation of MAP kinases such as ERK, JNK and p38 by ATRA in antigen presenting cells⁴. These MAP kinases also play a role in MyD88-dependent immune activation upon MPL/TLR4 engagement¹⁷. Alternatively, ATRA is known to promote IL-2 signaling in CD4 T cells, which may increase T cell help to B cells¹⁸. Both of these effects are mediated by engagement of retinoic acid receptors (RARs). 13-*cis* RA binds RAR more weakly than ATRA and is believed to mediate immunomodulatory effects through isomerization to ATRA or 9-*cis* RA^{19, 20}. Thus, an RAR-dependent mechanism would be consistent with the lack of effect of 13-*cis* RA observed in this study. In summary, the data presented here indicate an interaction between ATRA and MPL in promoting the antibody response to a lipopeptide antigen, warranting further investigation of ATRA as a humoral vaccine adjuvant.

Materials and Methods

Synthesis of N-MPR-DSG, N-MPR-DPG, and RAL

Syntheses of N-MPR-DSG and N-MPR-biotin are described in detail elsewhere and in the supplementary information (**SI Methods**)¹². In brief, N-MPR peptide (NEQELLELDKWASLNGGK) was synthesized in an automated solid phase synthesizer with standard fluorenylmethyloxycarbonyl protocols. A carboxyl group was introduced to distearoylglycerol (DSG) via reaction of the available alcohol with succinic anhydride. Lipid conjugation was accomplished via amidation of the carboxylated lipid and the lysyl ϵ -amine at the peptide C terminus. Biotinylated N-MPR peptide was prepared for use in ELISA by an analogous method. Molecular weights determined by MALDI-MS were as follows: N-MPR-DSG, expected 2963.2 Da, observed 2963.2 Da; N-MPR-biotin, expected 2455.6 Da, observed 2455.1 Da.

All-*trans* retinoic acid phospholipid (RAL) was synthesized according to a general scheme published previously²¹. Detailed synthesis and characterization, including TLC, MALDI, and NMR, are reported in the supplementary information (**SI Methods**). First, 1-O-octadecyl-2-O-benzyl-sn-glycerol was converted to phosphocholine by phosphorylation with phosphorus oxychloride and coupling to the choline tetraphenyl borate salt. Then the benzyl group was removed by catalytic transfer hydrogenation. Finally, all-*trans* retinoic acid was attached to the 2-hydroxy group with the typical DCC/DMAP method.

Liposome preparation

Liposomes composed of 15:2:3 Dimyristoylphosphatidylcholine: Dimyristoylphosphatidylglycerol:Cholesterol with MPL, RA and lipopeptide as indicated were formed by hydration of dried lipid films in sterile PBS followed by extrusion through 400 nm polycarbonate membranes²². Vesicle size was characterized by dynamic light scattering and zeta potential was determined by electrophoretic mobility (Zetasizer 3000, Malvern, New Bedford, MA). Liposome association of N-MPR-DSG and retinoic acid was determined by absorbance following sedimentation by ultracentrifugation as described¹². Liposomes were prepared fresh prior to each injection. Detailed methods are described in the supplementary information (**SI Methods**).

Animal immunizations

All animal procedures were conducted in accordance with the policies and approval of the UCSF Institutional Animal Care and Use Committee. Eight week-old female BALB/C mice (Jackson Laboratories, Bar Harbor, ME) were housed in a UCSF specific pathogen-free barrier facility. Animals received subcutaneous immunizations in alternating hind hocks on Days 0 and 14. Each injection contained 50 µg lipopeptide, 25 µg MPL or 25 µg RA (for RAL, a molar equivalent to RA was used) and 1 µmol lipid vehicle in 50 µL sterile phosphate-buffered saline. On Days 28 and 119 blood was collected from the submandibular vein for characterization of antibody responses.

ELISA

Peptide ELISAs were conducted using MPR peptides biotinylated (**SI Methods**) and captured on 96 well streptavidin-coated plates (Pierce, Rockford, IL). Assays were performed according to the manufacturer's instructions. Titer was defined as the reciprocal dilution of immune sera yielding an optical density twice that of 1:200 pre-immune sera after subtraction of background wells lacking serum. IgG1/IgG2a ratios were calculated as an average of optical density quotients measured at 3 dilutions after subtraction of background values. All samples were assayed in duplicate.

Statistical analysis

Statistical significance was assessed by analysis of variance and two-tailed Student's t test using Microsoft Excel and SigmaPlot. Differences were considered significant if they exhibited p values < 0.05 in the Student's t test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Edward Dy, Nichole Macaraeg and Katherine Jerger for assistance with animal studies and Kevin Park for assistance with the digestion of RAL by phospholipase A₂. We thank Dr. Gary Fujii for suggestions regarding the liposome formulation. This work was supported by NIH R01 GM061851 and by a grant from the National Institutes of Health, University of California, San Francisco – Gladstone Institute of Virology & Immunology Center for AIDS Research, P30-AI027763. D. Watson was supported by a U.S. Department of Homeland Security Graduate Fellowship, administered by the Oak Ridge Institute for Science and Education under U.S. Department of Energy contract number DE-AC05-00OR22750. The opinions expressed herein do not necessarily reflect the policies and views of DHS, DOE, or ORISE.

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Figure 1.
Structures of all-*trans* retinoic acid, 13-*cis* retinoic acid, and lipid-anchored all-*trans* retinoic acid (RAL).

Figure 2. Effect of ATRA on total IgG anti-N-MPR antibodies to a lipopeptide antigen adjuvanted with lipid A

Panel A: ATRA, but not 13-cis RA, potentiates the MPL-mediated IgG response to N-MPR. Each group represents 5 animals except 'MPL + RAL', which consisted of 4 animals. Titers are expressed as geometric means and error bars represent standard errors of the mean. * $p = 0.00039$ vs 'MPL', 0.0017 vs 'MPL + 13-cis RA', 0.00095 vs 'MPL + RAL'. *Panel B:* ATRA-mediated enhancement of the anti-N-MPR serum IgG response persists at least 15 weeks following the final immunization. Each group consisted of 5 animals. Titers are expressed as geometric means and error bars represent standard errors of the mean. # $p = 0.012$ vs. 'MPL', 0.021 vs. 'MPL + 13cisRA'. *Panel C:* The ATRA enhancement was reproduced with independent liposome preparations. Experiments 1 and 2 consisted of 4 and 5 animals per group, respectively. Titers are expressed as geometric means and error bars represent standard errors of the mean. # $p = 0.026$ in Experiment 1; * $p = 0.00039$ in Experiment 2. *Panel D:* ATRA does not significantly alter the IgG1/IgG2a balance of anti-N-MPR antibody responses ($p = 0.499$). Each group represents 4 animals and error bars represent standard errors of the mean.

Table 1
Biophysical properties of liposomal lipopeptide formulations

Formulation parameters were not significantly altered by inclusion of MPL or RA. Measurements were collected as described in the **Methods**. Size and charge values represent means of 3 and 10 measurements, respectively. Liposome association values represent means of two independent experiments.

Formulation	Vesicle Diameter (nm)	Vesicle Charge (mV)	N-MPR-DSG Association (%)	Retinoic Acid Association (%)
None	259.6	-24.4	75.3	ND
MPL	244.3	-25.5	77.1	ND
ATRA	246.6	-26.8	79.7	98.0
MPL + ATRA	262.6	-26.8	74.1	99.8
MPL + 13-cis RA	335.1	-26.6	78.3	100.0
MPL + RAL	236.0	ND	ND	ND