# Retinoic Acid Decreases But Does Not Eliminate Murine Macrophage Phagocytic Activity and iNOS Expression

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## Introduction

Macrophages are important cells of the innate immune response that function as sentinels because they are one of the first cell types to recognize invading microbes and initiate an immune response[7, 12]. Macrophages express cell surface pattern recognition receptors (PRRs) that enable them to distinguish microbes from self by recognition of conserved microbial sequences known as pathogen-associated molecular patterns (PAMPs). PAMPs are typically conserved ultrastructural molecules unique to microbes. When PRRs bind to PAMPs, several events are triggered including increased phagocytosis and the stimulation of signal transduction pathways (STP). Phagocytosis stimulates intracellular killing and degradation of microbes followed by presentation of microbial fragments to adaptive immune cells. Activation of STPs may increase the production and secretion of cytokines and the particular pattern of cytokines secreted will guide the type of immune response that develops. Stimulating certain STPs may also increase the intrinsic killing ability of macrophages by increasing the expression of enzymes that help convert substances to toxic intermediates capable of killing and degrading ingested microbes. An important example of these enzymes is inducible nitric oxide synthase (iNOS), which is only expressed when macrophages are activated[12]. This enzyme results in production of nitric oxide, a molecule that vigorously enhances the ability of macrophages to kill ingested bacteria.

Macrophages are associated with inflammatory or Th1-like, and anti-inflammatory or Th2-like, immune responses[12]. Macrophages play a key role in triggering one response or the other through the production of particular cytokines. The inflammatory response is important because it facilitates the elimination of disease-causing microbes. However, if inflammation lasts too long or is too vigorous, the toxic products released may cause damage to host cell tissues leading to chronic inflammatory diseases, such as rheumatoid arthritis[5] and inflammatory bowel disease[14]. A better understanding of how the inflammatory response is triggered as well as dampened may aid in the development of treatments for some of these chronic disorders. Research focused on the mechanisms of inflammation and the molecules that may affect its development have indicated that vitamin A may influence inflammatory responses[6,10,13,18]. Vitamin A is found in colorful vegetables in the form of β carotene and in animal tissues in the form of retinyl esters[2]. Macrophages have the ability to convert the circulating form of vitamin A (or retinol) into retinoic acid (RA). Once formed, RA acts intracellularly by binding to nuclear receptors and influencing gene expression. Studies have shown that in macrophages, RA

increases the production of anti-inflammatory cytokines, which leads to the development of a more anti-inflammatory Th2-like immune response[6,10,13,18]. A lot of research has focused on examining the influence of RA on immune cells and their secreted products to elucidate how RA modulates immune responses.

The objective of my research was to analyze the direct influence of RA on phagocytic function and iNOS expression by macrophages. We tested the hypothesis that RA would increase phagocytic activity and decrease iNOS expression in stimulated macrophage cell lines. I used two different macrophage lines called RAW and J774. Both of these cell lines are mouse cell lines, but J774 macrophages are known to be more mature than RAW macrophages. The number of yeast ingested by unstimulated and lipopolysaccharide (LPS)-stimulated RAW and J774 macrophages with and without RA pre-treatment was microscopically quantified. Lysates were prepared from similarly treated macrophages and iNOS expression was detected using immunoblotting and ELISA. Our results indicate that RA decreased, but did not eliminate phagocytic activity in either RAW and J774 macrophages, and decreased iNOS expression in RAW macrophages.

## Materials and Methods

**Cell lines:** RAW 267.4 (RAW) and J774 macrophages (ATCC, Manassas, VA) were used for all experiments. RAW cells were grown and maintained in Roswell Park Memorial Institute RPMI medium (Thermo Fisher Scientific, Mississauga, ON, Canada) containing 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific), 2 mM L-alanine-L-glutamine, 55 μM 2-mercaptoethanol, and 50 μg/mL gentamicin (R10). Chemicals were obtained from Sigma-Aldrich Canada Co. (Oakville, ON). J774 cells were maintained in R10 medium supplemented with 1% (w/v) glucose. Both cell lines were grown in 75 cm$^3$ (T75) Falcon tissue culture-treated flasks or tissue culture-treated petri dishes (Thermo Fischer Scientific) incubated at 37°C in an atmosphere of 95% air and 5% $CO_2$. Cells were harvested from flasks or dishes by decanting the medium then washing once with phosphate buffered saline (PBS; 0.01 M phosphate buffer, 0.154 M sodium chloride, pH 7.2; Thermo Fisher Scientific). A sufficient volume of 20 mM EDTA (JT Baker Chemical Co., Phillipsburg, NJ) in PBS was added to cover the monolayer then incubated at 37°C for 5 minutes. Flasks were vigorously tapped to dislodge the cells, then the contents were pooled with the growth medium and cells were pelleted by centrifugation at 800g for 8 minutes. To enumerate viable cells, an aliquot of cells was stained with trypan blue then cells were counted using a hemocytometer.

**Phagocytosis Assay**: Phagocytosis experiments were conducted using Nunc sterile, 8 well tissue culture-treated slide chambers (Lab Tek; Thermo Fisher Scientific). Wells were seeded at 2.5 x $10^5$ macrophages/well. Retinoic acid was added at 50, 5 or 1 μM alone or in combination with 1 ng/well lipopolysaccharide (LPS, Sigma-Aldrich Canada Co.) and LPS was added either simultaneously or 24 h after RA. Twenty-four hours after LPS stimulation, congo red dyed yeast were added at a 5:1 yeast to cell ratio then incubated for one hour at 37°C. Yeast were prepared by combining 0.3 g of baker's yeast with 0.05 g of congo red dye (Sigma-Aldrich Canada Co.) in 10 mL of water then heating to a boil. Dyed yeast were enumerated with a hemocytometer. Just before combining with macrophages in the slide chambers, the yeast were pelleted by centrifugation at 2000g for 10 minutes, the supernatant was aspirated, and yeast were suspended

in PBS. After incubation with yeast, medium was aspirated and wells were gently washed with RPMI, then fixed with 1% (v/v) formaldehyde in PBS. The number of yeast ingested by 100 macrophages for each treatment was counted using phase contrast microscopy at 400X magnification.

**Electrophoresis and iNOS detection**: Cells were seeded at 8 x $10^6$ cells per flask in T75 flasks with 30 mL R10 for RAW cells or 30 mL glucose enriched R10 for J774 cells. Cells were incubated until they were almost confluent. Retinoic acid was added to cultures at 1 μM and cells were cultured for 24 h then were either left unstimulated or were stimulated with 1 ng/mL LPS and 1 ng/mL IFNγ (Peprotech Inc., Rocky Hill, NJ) and incubated for an additional 24 h. Cells were harvested as previously described, then cell pellets were washed 3 times by suspending the cell pellet in 4°C PBS followed by centrifugation at 1500g for 10 minutes. After the final wash, cells were lysed by suspending 1 - 2 x $10^7$ cells/mL in 4°C ice cold lysis buffer [PBS containing 1% (v/v) Triton X-100 (Thermo Fisher Scientific), 1 protease inhibitor tablet (2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1mM EDTA, 130 μM bestatin, 14 μM N-(trans-epocysuccinyl)-L-leucine 4-guanidinobtylamide, 1μM leupeptin, 0.2 μM aprotin (Sigma-Aldrich Canada Co.)] for 30 min at 4°C with occasional vortexing. Lysis buffer was stored at 4°C for no more than two weeks after it was prepared. After lysis, insoluble material was pelleted by centrifugation at 13,000g for 5 minutes at 4°C. Supernatant was divided into aliquots and frozen at -80°C until required.

Proteins in the lysates were separated using SDS PAGE. Lysates were combined with a Laemmli sample buffer containing freshly added 5% (v/v) 2-mercaptoethanol, boiled for 5 min then loaded onto a 4% polyacrylamide stacking gel and separated through a 8% resolving gel using the Laemmli buffering system and a minigel apparatus (Bio-Rad Laboratories, Mississauga, ON). Proteins were separated using 50 volts through the stacking gel and 100 volts through the resolving gel. Proteins were transferred to a nitrocellulose membrane (Bio-Rad Trans-Blot Turbo Transfer Pack) using a Bio-Rad blotting device on turbo blot setting. After transfer, the nitrocellulose membrane was allowed to air dry. For staining, the membrane was wetted in PBS-T (0.1% Tween-20 in PBS) and washed twice in PBS-T for 5 minutes each. It was then immersed in 3% blocking solution (Bio-Rad laboratories) overnight and washed twice in PBS-T again before staining. Membranes were stained for iNOS using rabbit anti-mouse iNOS IgG (Cell Signaling Technology, Danvers, MA) diluted in 1% bovine serum albumen (w/v) in PBS-T while rocking the membranes at room temperature for 1 hour. The membrane was twice washed with PBS-T then goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) was added followed by rocking for 1 hour then washed as already described. The amplification step and colorimetric detection was done using the Bio-Rad western blot amplification module and Opti-4CN Substrate Kit (Bio-Rad Laboratories).

**ELISA**: An ELISA kit was used to quantify iNOS in cell lysates (Antibodies-online, Inc., Atlanta, GA). Samples and standards were tested in duplicate and all steps were done as described in the iNOS ELISA detection kit. Briefly, ELISA wells were pre-coated with anti-iNOS antibodies, and biotin-conjugated antibodies specific for iNOS were used for detection of captured iNOS followed by avidin conjugated to horseradish peroxidase. Substrate was TMB (Thermo Fisher Scientific) and plates were read using an Elx800 microplate reader at 630 nm (BioTek, Winooski, VT).

## Results

**Phagocytic activity of J774 and RAW macrophages:** To assess phagocytic activity of the RAW and J774 cells, a slide chamber technique was devised to microscopically quantify the number of congo red-stained yeast ingested by macrophages following various treatments. Previous experiments had indicated both macrophage cell lines had basal levels of phagocytic activity regardless of stimulation. To assess whether treatments affected the basal activity, macrophages were divided into four categories based on the number of yeast a single cell ingested (0, 1-3, 4-6, or ≥7 yeast ingested). Preliminary experiments were done to titrate RA and, consistent with reports in the literature, a 1 µM concentration of RA influenced cellular activity, but was not toxic to cells (data not shown). Therefore, this concentration of RA was used in all subsequent experiments. The number of macrophages that did not ingest yeast ranged from 11% to 17% for J774 cells (Fig. 1A) and between 12 to 18% for RAW cells (Fig. 2A) regardless of treatment. Interestingly, the lowest percentage of macrophages that either did not ingest yeast or ingested only 1-3 yeast were lipopolysaccharide-stimulated (Fig. 1A & B and Fig. 2A & B). For all other treatments, 64-70% of macrophages ingested 1-3 yeast, so this category seemed to represent basal phagocytic activity for both macrophage cell lines.
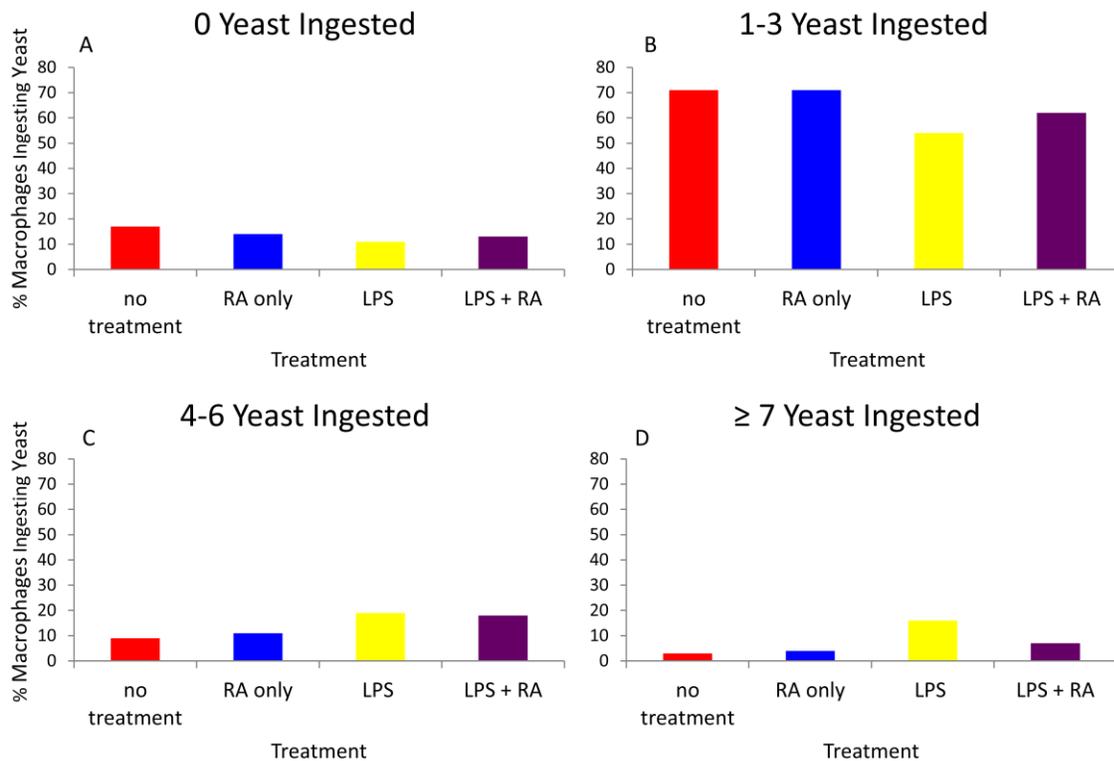


**Figure 1**: **RA decreases phagocytic activity of LPS-stimulated J774 macrophages**. The percentage of J774 macrophages that ingested no yeast (A), 1-3 yeast (B), 4-6 yeast (C) or ≥7 yeast (D) for each of the treatments is indicated on the x axis. No treatment = J774 cells cultured for 48 hours before yeast added. RA only = cells treated with 1 mM RA for 48 h before yeast added. LPS = cells cultured for 48 h with LPS stimulation 24 h before yeast added. LPS + RA = cells cultured with 1 mM RA for 24 h then LPS for an additional 24 h before yeast added.
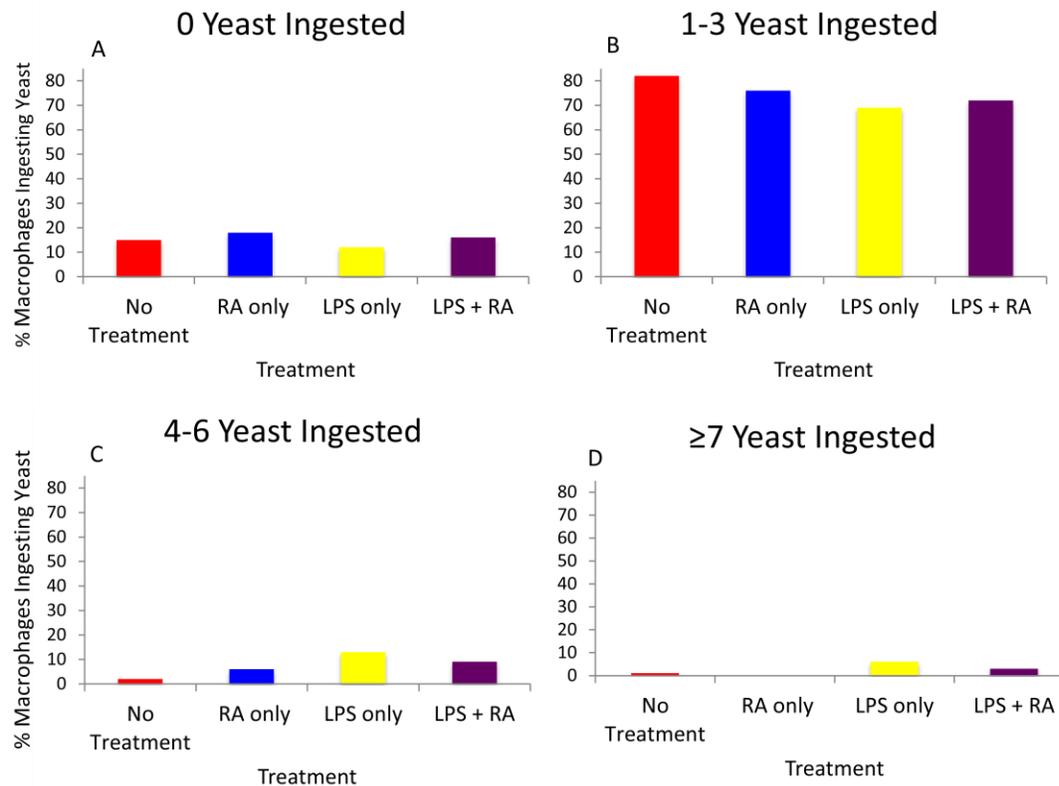
**Figure 2**: **RA decreases phagocytic activity of LPS-stimulated RAW macrophages.** The percentage of RAW macrophages that ingested no yeast (A), 1-3 yeast (B), 4-6 yeast (C) or $\geq$7 yeast (D) for each of the treatments indicated on the x axis. Treatments are as described in the legend for Fig. 1.

There was little variation in the percentage of macrophages that ingested 4-6 yeast whether macrophages were left unstimulated or were treated with only retinoic acid (Fig. 1C and Fig. 2C). The percentage of J774 macrophages that ingested 4-6 yeast increased from 9% for unstimulated cells to 19% when cells were lipopolysaccharide-stimulated. RA pre-treatment did not affect this increased phagocytic activity, since 18% of RA pre-treated cells still ingested 4-6 yeast. The phagocytic activity was similar for RAW macrophages in this category. LPS-stimulation increased the percentage of yeast ingested to 12%, and RA pre-treatment decreased this percentage slightly to 9% (Fig. 2C).

The highest degree of phagocytic activity was represented by macrophages that ingested 7 or more yeast. Very small percentages of J774 and RAW cells ingested this number of yeast if they were left unstimulated or were pre-treated with 1 μM RA (Fig. 1D and Fig. 2D). Only 3-4% of J774 cells and only 1% of RAW cells ingested $\geq$7 yeast in the absence of LPS stimulation. The percentage of J774 cells that ingested $\geq$7 yeast increased from 3% to 16% when treated with LPS and RA pre-treatment decreased this proportion by more than half to 7%. Similarly, the percentage of RAW cells that ingested $\geq$7 yeast increased to 6% when cells were LPS-treated and likewise this percentage decreased to 3% when macrophages were treated with RA prior to LPS stimulation (Fig. 2D).

**Inducible nitric oxide synthase (iNOS) expression in macrophages:** Since macrophage activation commonly causes increased expression of intracellular iNOS, we examined whether RA treatment affected iNOS levels in macrophages using electrophoresis and immunoblotting (Fig. 3). Lysates prepared from both J774 (data not shown) and RAW macrophages that had been treated with LPS + IFNγ expressed a detectable band between 150 and 100 kD, while macrophages without stimulation did not (Fig. 3, compare lanes 1 & 3 with lanes 2 & 4). The band had an apparent molecular weight of ~130 kD which is consistent with the known molecular weight of iNOS[17]. When RAW macrophages were pre-treated with RA prior to stimulation with LPS + IFNγ, the iNOS band was much fainter suggesting decreased expression of iNOS in these cells (Fig. 3, lane 5).
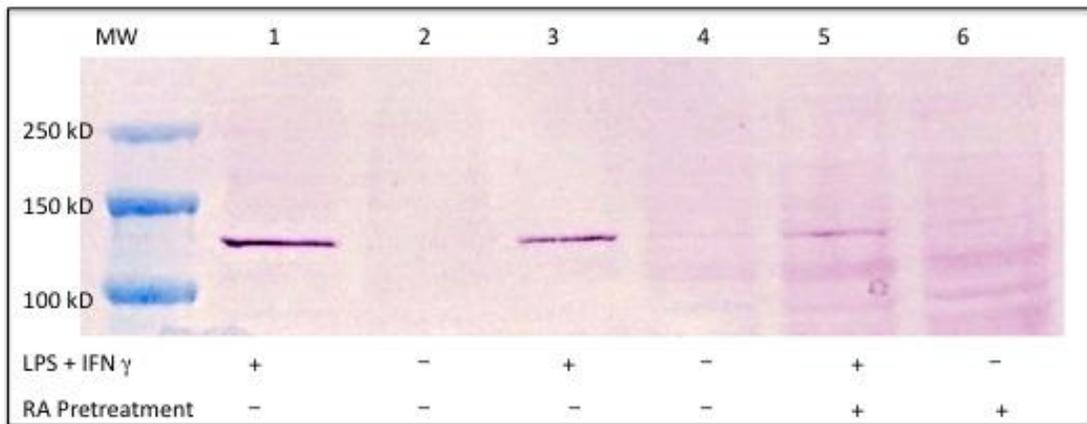


**Figure 3: Immunoblotting reveals iNOS expression in RAW cells is decreased by RA.** RAW cells were stimulated with LPS + IFNγ (lane 1 and 3), left unstimulated (lane 2 and 4), treated with 1 mM RA (lane 6) or pre-treated with 1 mM RA then stimulated with LPS + IFNγ (lane 5) prior to lysis. Lysates were separated on an 8% SDS PAGE gel, blotted and iNOS bands were detected with rabbit anti-iNOS IgG. Molecular weight standards and their sizes are indicated.

To verify and quantify the observations for iNOS expression, an ELISA was conducted on lysates from stimulated and unstimulated RAW macrophages that had been pre-treated and on those that were untreated. No iNOS was detected in lysates from macrophages without treatment or treated only with RA (Fig. 4). Macrophages treated with LPS + IFNγ expressed iNOS at a concentration of ~15000 IU/mL. However, macrophages pre-treated with RA decreased iNOS expression to ~8000 IU/mL.
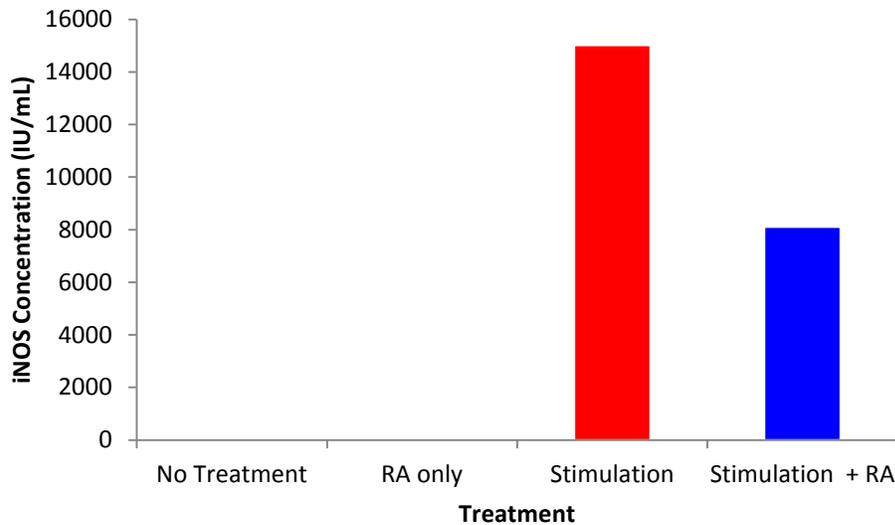
**Figure 4**: **RA pre-treatment decreases iNOS expression in RAW cells by 50%.** ELISA detection of iNOS in lysates prepared from RAW cells following the treatment indicated on the x axis. Treatments are as described in the legend for Fig. 3.

## Discussion

Previous studies have indicated that RA favours the development of a less anti-inflammatory or Th2-type immune response[7,12,13,22]. The development of a Th1-type response, which is characteristically inflammatory, or a Th2-type response, depends on the pattern of cytokines present that can interact with T cells[18]. Many of these cytokines are produced by macrophages; cells that act as immune sentinels and as the interpreters between microbes and adaptive immune cells. Certain cytokines produced by macrophages stimulate the development of Th1 cells, which produce cytokines, such as IFNγ, that favor an inflammatory response. Other cytokines produced by macrophages stimulate the development of Th2 cells, which secrete cytokines that favour a less inflammatory response. Retinoic acid has been shown to act on macrophages to favour the production of cytokines that stimulate the development of a Th2-type response and decrease the Th1-type response[6,10,11,18]. FewerTh1 cells result in less inflammatory cytokines being produced by T cells. Therefore, RA indirectly influences the kind of signals macrophages receive during an immune response and favours generation of a less inflammatory response. The present research aimed to determine if RA had direct influences on macrophage activity.

In this study, phagocytic activity was the first function of macrophages that was examined. In all phagocytosis assays, a higher percentage of RAW and J774 macrophages ingested higher numbers of yeast (4-6 and $\geq$7) when stimulated with lipopolysaccharide compared to unstimulated macrophages. This indicated that LPS stimulation increased this functional activity of macrophages that often initiates inflammatory immune activity. This is consistent with other reports that indicate that treatment of macrophages with stimulants causes an increase in various macrophage activities, including phagocytosis[7,21].

RA pre-treatment in the absence of stimulation did not affect basal phagocytic activity of either J774 or RAW cells. However, RA pre-treatment did decrease the highest phagocytic activity observed when macrophages were LPS-stimulated by approximately half. The decrease

in phagocytosis observed in our experiments contrasts with the results of some other studies. One study has found that RA increased phagocytosis in many different kinds of macrophages[8]. However, this was observed in macrophages that had not been stimulated, which suggests differing roles of RA depending on cellular conditions. Another study found that peritoneal macrophages isolated from rats fed a vitamin A deficient diet had decreased phagocytic and killing ability for the bacteria *Staphylococcus aureus* compared to controls[19]. Cells were isolated from the peritoneum and macrophages were phenotypically identified and counted using a hemocytometer. In contrast to these results, our results indicated a decrease in phagocytic activity when macrophages were first treated with RA. However, *S. aureus* are Gram-positive organisms whereas we used LPS, a component of Gram-negative bacteria, to stimulate phagocytosis by the macrophages. The type of stimulation may modulate the effect RA has on phagocytic activity of macrophages. It is possible that macrophage phagocytosis is influenced by multiple factors, such as stimulation, type of stimulant, and presence of other compounds such as RA. It is also possible that *in vivo* tests may differ from *in vitro* experiments. In summary, in our study pre-treatment of macrophages with RA decreased phagocytic activity of LPS-stimulated macrophages, suggesting that RA signaling supersedes PRR signaling for macrophages.

In the future, it may be beneficial to study macrophage phagocytic activity and how it is altered under different stimulation conditions. For example, using a stimulant other than LPS may assist in understanding how RA-treated macrophages respond to different types of microbes. A second change to stimulation would be to use LPS in addition to cytokines. Some reports suggest that both priming molecules, usually cytokines, as well as stimulants, such as bacterial products, are necessary for macrophage activation in inflammatory responses[21]. For example, some researchers found that IFNγ triggers cells to be responsive to doses of LPS that do not trigger a response when IFNγ is not present[9]. Testing the varying conditions could lead to more insight into the influence of RA on phagocytic activity. It may also be worthwhile to develop a method of assaying phagocytic activity that is more quantitative and less labour intensive. For example, a potential alternative is seeding macrophages into 96 well plates and incubating them with pre-labelled zymosan particles, which are prepared from yeast cell walls[4]. Non-ingested zymosan particles are blocked to prevent coloration, then the phagocytes are permeabilized and incubated with a detection agent and a substrate to produce color that can be quantified using a microtiter plate reader.

The second macrophage function we examined was the expression of inducible nitric oxide synthase in macrophages, assayed using immunoblotting and ELISA. Both immunoblotting and ELISA data indicated that pre-treatment with RA decreased the expression of iNOS in stimulated RAW macrophages. The detection of an iNOS-specific band with an apparent molecular weight of ~130 kD that was present only in cell lysates from stimulated cells, indicated that stimulation increased the expression of iNOS in RAW macrophages. Inducible nitric oxide synthase is expressed in response to inflammatory signals[3], and has been shown to be increased in macrophages treated with LPS and IFNγ compared to untreated macrophages[20]. Thus the detection of an iNOS specific band in macrophages treated with LPS and IFNγ was consistent with expectations. This increase was stronger in RAW cells than J774 cells (data not shown). This result was unexpected, since J774 cells are a more mature cell line and were expected to respond more strongly to an inflammatory stimulus.

Immunoblotting indicated that iNOS expression in lysates prepared from RAW cells treated with RA prior to stimulation with LPS + IFNγ was weak compared to RAW cells stimulated only with LPS + IFNγ. The ELISA data verified that the concentration of iNOS in

RA-treated and stimulated macrophages was 50% less than the iNOS concentration in stimulated macrophages without RA treatment. This indicates that RA decreased the expression of iNOS in stimulated macrophages.

Similar studies have produced varying results. For example, one study found that RA decreases iNOS expression in smooth muscle cells[16]. However, this study used a different cell type than the current study. A similar study examining the influence of RA on RAW macrophages was previously conducted[1]. In contrast to our study, they found that RA pre-treatment increased nitric oxide production, an indicator of iNOS activity, when RAW macrophages were stimulated with low concentrations of LPS and IFNγ. Their data indicated that the influence of RA decreased or increased when it was added at decreasing or increasing concentrations even though they used similar culture conditions. They suggest that the role of RA may depend on the state of RA in cells prior to RA treatment. The culture medium we used had 10% FBS, which may contain an adequate amount of RA for cell immune responses. However, their experiment used a 1% FBS solution and their cells were considered deficient in RA. It is possible that exogenous RA may have varying effects depending on how much RA was previously available to the cells. The influence of RA on iNOS expression may depend on multiple factors, such as cell type, maturity, previous RA state, concentration of stimulation, and length of time cells are stimulated. The opposing data suggests that instead of favoring only one type of immune response, RA may act as an immune modulating molecule capable of regulating the immune response by increasing or decreasing inflammation. Additional studies may provide a clearer understanding of the mechanisms and boundaries of these influences.

In conclusion, our study indicated that retinoic acid pre-treatment decreased, but did not eliminate the phagocytic activity of RAW and J774 cells. RAW inducible nitric oxide synthase expression was also reduced, but not completely eliminated, when macrophages were pre-treated with RA. Increased understanding about the role of RA on phagocytic activity and iNOS expression may be gained by examining *in vivo* responses to varying RA treatments. Macrophages from mice treated with varying RA diets could be isolated, purified, and then analyzed using the same phagocytic assay, immunoblotting, and ELISA techniques used in the current study. Our results, in combination with previous studies, suggest it is possible that in some conditions RA decreases the immune response and in others it increases it. A better understanding of the role of RA in increasing or decreasing particular immune functions and the specific cellular or systemic conditions prevailing during different responses to RA would increase understanding of immune system regulation. This could potentially lead to the development of treatments for inflammatory diseases such as inflammatory bowel disease and rheumatoid arthritis.

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