Activation and Suppression of Macrophages: Implications for Cancer Immunotherapy

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Activation and Suppression of Macrophages: Implications for Cancer Immunotherapy

by

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Prepared under the mentorship of Dr. Paul Sondel (Human Oncology)

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Abstract

It is widely known that macrophages can be activated to kill tumor cells. It is also known that tumor-infiltrating macrophages can be immunosuppressed. The mechanisms of both tumor killing by activated macrophages and tumor-induced macrophage suppression are not entirely clear. To better understand the mechanisms that macrophages use to kill tumor cells, a murine macrophage cell line, RAW264.7, was fixed with paraformaldehyde, subsequently stimulated with lipopolysaccharide (LPS) and co-cultured with tumor cells. Macrophage activity was assessed by nitric oxide (NO) production and tumor cell growth inhibition in the ^{3}H-thymidine incorporation assay. It was found that fixed macrophages were still able to suppress the proliferation of tumor cells while the production of NO was abrogated. Additionally, a model of tumor-induced suppression of macrophages was developed by co-culturing them with tumor cell conditioned media before adding LPS. Inhibition of macrophage activity by tumor cell products was demonstrated by decreased NO production. This model mimics the suppressed immune system of cancer patients. Next, we will use this model to test substances that aim to reverse immunosuppression of macrophages allowing them to fight tumors.

Chapter I

Introduction

Immunotherapy is a method of cancer treatment which focuses on using substances to stimulate or support the patient's own immune system to fight invading cancerous cells (1). In order to be able to provide such beneficial immunotherapeutic treatments to cancer patients, it is necessary to further understand how the immune system functions in the presence of these foreign or cancerous cells. One such component of the immune system that is of particular interest in this field is the macrophage. Macrophages play an important role in the body's defense system as part of the innate immune response (2). Among their roles within the immune system, macrophages can become both activated and suppressed, although the exact molecular mechanisms of how these antagonistic processes occur are not entirely clear (3). In order to provide beneficial immunotherapeutic treatments, it is necessary to decipher these unknown mechanisms.

To better understand the mechanisms that activated macrophages use to kill tumor cells, a murine macrophage cell line, RAW264.7 (4), was fixed with paraformaldehyde (PFA), stimulated with lipopolysaccharide (LPS), and co-cultured with tumor cells. Macrophage activity was assessed by testing for nitric oxide (NO) production and tumor cell growth inhibition in the ^{3}H-thymidine incorporation assay. Increased NO production was taken as evidence of macrophage activation as it is one of the main cytotoxic pathways exhibited by activated macrophages (5).

Additionally, having a model of tumor-induced suppressed macrophages to use for experimentation would be beneficial to forming a deeper understanding of the

potential effectiveness of immunotherapeutic treatments. Therefore, a model of tumor-induced suppressed macrophages was developed by co-culturing RAW264.7 cells or peritoneal macrophages with tumor cell conditioned media before adding LPS. Tumor cell conditioned media were used because it has been demonstrated that molecules released by tumors can have suppressive effects on macrophages (6). Inhibition of macrophage activity by tumor cell products was demonstrated by decreased NO production. This model mimics the suppressed immune systems of cancer patients (1), and the goal is to use this model to test potential immunotherapeutic substances that aim to reverse the immunosuppression of the macrophages thereby allowing them to fight tumors.

Chapter II

Materials and Methods

Cell Lines

The RAW264.7 murine macrophage (RAW) cell line was maintained in complete RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100µg/mL) at 37° C in a humidified incubator with 5% CO_2. The cells were split every 2-3 days. The cells grew adherently and were passaged by using a cell scraper to release them from the bottom of the flask.

The B16 cell line, a murine melanoma, was maintained in the same complete RPMI-1640 medium. The cells were split every 3-4 days. The cells grew adherently and were passaged by using trypsin to release them from the sides of the flask.

Tumor Cell Conditioned Media

Conditioned tumor cell media were collected after culturing the B16 or MethA tumor cells for 72-hours. The collection was done by spinning the tumor cells followed by collecting the supernatant and filtering it. The conditioned tumor cell media was stored at -20° C until use.

RAW Cell Fixation

Two fixation methods were tested in these experiments. Fixation method A (7) involved fixing the RAW cells after they were allowed to adhere to the wells of a 96-well flat-

bottom plate. The desired number of effector cells were plated and allowed to adhere for 30 minutes. Then, the supernatant was removed and replaced with 1% PFA for 20 minutes. Finally, the PFA was removed, the wells were washed three times with PBS and once with RPMI, and fresh RPMI was put back into the wells.

Fixation method B (8) involved fixing the effector cells in suspension. The effector cells were adjusted to their final desired concentration with 1% PFA and the suspension was allowed to sit for 20 minutes. The suspension was then spun for 5 minutes at 1000 RPM and the PFA was decanted. 5 mL of PBS were added to the suspension mixture and allowed to sit for 5 minutes. The suspension was then centrifuged again for 5 minutes at 1000 RPM , decanted, and resuspended in fresh RPMI. The cells were then recounted and readjusted to their final desired concentration and plated on a 96-well flat-bottom plate.

Murine PEC Preparation

C57BL/6 mice were first sacrificed by CO_2 asphyxiation according to University of Wisconsin Madison Research Animal Resource Center guidelines. Then, 5 mL of cold RPMI-1640 complete medium were injected in each mouse with a 26 gauge (GA) needle. After injection, the peritoneal cavity was massaged vigorously for one minute followed by a peritoneal washing with an 18 GA needle. The resulting PECs were plated at 2-2.5x10^5/well in a 96-well flat-bottom plate.

Quantification of Nitrite

The Griess reagent assay was used to measure the amount of NO production released by different cell types, particularly the RAW cells or peritoneal macrophages. In this assay, RAW or peritoneal macrophages were co-cultured with B16 tumor cells or tumor cell conditioned media with and without LPS +/- interferon gamma (IFN γ) for 48 hours. Supernatants were then collected and tested for nitrite production using Griess reagent. Equal volumes of supernatant and Griess reagent were allowed to mix for 10 minutes before the absorbance was read at 540 nm on a microplate reader. These results were compared to a standard curve with concentrations ranging from 0 to 120 μM.

^{3}H-Thymidine Incorporation Assay

A ^{3}H-thymidine incorporation assay was used to measure tumor cell proliferation in the macrophage and tumor cell co-culture setting. This in vitro assay measured tumor cell proliferation by quantifying the amount of radioactively labeled thymidine that was incorporated into the DNA of proliferating cells. The principle of this assay is that only living actively proliferating cells are able to incorporate ^{3}H-thymidine into new DNA. The ^{3}H-thymidine was added to the macrophage and tumor cell co-culture in the last 6 hours of the co-culture period (total of 48 hours) at 1 μCi/well. After 6 hours, the supernatants were removed from the wells, and 50 μL of trypsin was added to each well for 10 minutes to detach tumor cells from the plastic. After that the cells were harvested onto glass fiber filters using the Packard harvester. The filters were allowed to dry and counts were detected over 5 minutes by a liquid scintillation beta counter.

Effector Cell Activation

Both RAW cells and peritoneal macrophages were activated by the addition of gram negative bacterial endotoxin, LPS. The peritoneal macrophages needed an additional activation signal provided by murine recombinant IFN γ.

Statistical Analysis

A two-tailed Student's *t*-test was used to determine significance of differences between experimental and relevant control values.

Chapter III:

Results and Discussion

Toxicity of PFA Fixation Method

In order to address whether the RAW cell PFA fixation procedure was toxic to the target tumor cells, several experiments were performed using fixed B16 cells as the "effector" cells and viable B16 cells as the target cells. The principle of these experiments was that the viable B16 cells in the presence of the fixed B16 cells should proliferate as actively as the control B16 cells in the absence of fixed B16 cells did if the fixation method of the "effector" cells was not toxic to the growth of the target cells.

The results in Figure 1A indicate that PFA method A was not toxic to the target cells while PFA method B was. The results that PFA method A was not toxic to the target cells were confirmed in a similar experiment (Figure 1B). Therefore, the conclusion was reached that PFA method A of fixing the RAW cells after they have been allowed to adhere to the plate was the optimal fixation method, and this was the fixation method used throughout the rest of the experiments.

Tumoristatic Activity of Viable RAW Cells

Tumoristatic activity of viable RAW cells against B16 tumor cells correlated with NO production as shown in Figures 2A and 2B, first two columns. Additionally, it can be noted that viable RAW cells that are not stimulated with LPS do not produce detectable amounts of NO (2A, 3A).

Tumoristatic Activity of PFA-Fixed RAW Cells

In experiment 2A, the fixed RAW cells did not exhibit any significant NO production while the amount of B16 cell proliferation of the cells plated with the fixed RAW cells was less than the proliferation of the B16 alone control group (2B). The B16 alone control group exhibited high level of tumor cell proliferation. This experiment was repeated with the similar results (Figures 3A and 3B). Please note that the ^{3}H-thymidine incorporation of control B16 tumor cells varies per experiment.

The observation that fixed RAW cells still exhibited some degree of inhibition of B16 proliferation even when NO production was abrogated suggests that while NO production is a factor in the RAW cell line method of tumor cytotoxicity, it is not the only factor. This difference in the degree of cytotoxicity may be due to an inflammatory mediator other than NO or a contact-dependent killing mechanism that involves RAW cell surface receptors.

Concentration Dependence in Viable RAW Cells

In experiments 2 and 3, it was shown that viable RAW cells plated at 2x10^5/well produced detectable amounts of NO, but those plated at 1x10^5/well did not. However, the RAW cells plated at 1x10^5 RAW cells per well in both experiments showed significant amounts of killing despite this lack of NO production.

This observation suggests that an effector cell concentration-dependent mechanism is taking place. One possibility may be that if a certain number of RAW cells are not present or a certain amount of NO is not produced, then the macrophages kill

tumor cells by a mechanism not involving NO production. Alternatively, killing may be mediated by a mechanism other than NO, both at 1x10^5 and 2x10^5 RAW cells/well.

Concentration Dependence in PFA-Fixed RAW Cells

Fixed RAW cells also exhibited concentration dependence. In experiment 2, some interesting differences were noted between the concentration of NO produced and the tumoristatic activity in the fixed RAW cells. In experiment 2, the PFA-fixed RAW cells at concentrations of 2x10^5/well and 1x10^5/well both did not produce detectable amounts of NO (2A). They also showed different degrees of ^{3}H-thymidine incorporation in B16 target cells (2B).

The observation that the degree of B16 cell proliferation is different suggests that there may be an effector cell concentration dependent effect occurring in the fixed RAW cells as well.

Effect of Tumor Cell Conditioned Media on NO Production by RAW Cells

RAW cells that were plated at 2x10^5/well with RPMI show approximately a 40-50% inhibition in the amount of NO they produced when cultured with B16 conditioned tumor cell media, or MethA conditioned tumor cell media (Fig 4). The RAW cells were allowed to co-culture with the conditioned tumor cell media for 30 minutes before LPS was added at 1 ug/mL, 100 ng/mL, or 10 ng/mL. The wells in which the conditioned tumor cell media was present during the total 48 hour incubation period exhibited significantly less NO production than do the wells in which only RPMI 1640 media was

used. These results indicate that the conditioned tumor cell media exhibit an immunosuppressive effect on the RAW cells.

Kinetics of Tumor Cell Conditioned Media-Induced Suppression of NO Production of RAW Cells

To further optimize the model of tumor-induced immunosuppression of macrophages, it was beneficial to determine whether the tumor cell conditioned media needed to be continually present or if it exerted its immunosuppressive effects in the first 30, 60, or 120 minutes of contacting with RAW cells. To do that, two separate plates were set up in which the RAW cell and conditioned media co-culture incubated for 30, 60, or 120 minutes. After the specified time had passed, the wells on one plate were washed 3 times with RPMI-1640 and were refilled with fresh RPMI-1640 complete medium along with LPS. The wells on the other plate were not washed after the specified time had passed, only LPS was added. The results from these experiments show that the tumor cell conditioned media needed to be continually present, or present for more than 120 minutes, in order to exert their immunosuppressive effects (Fig 5A and 5B).

Furthermore, it was beneficial to determine how long the RAW cells needed to be co-cultured with the tumor cell conditioned media for it to exert its suppressive effect. In the same experiment as described above, in which the co-culture of RAW cells and tumor cell conditioned media was incubated for either 30, 60, or 120 minutes before adding LPS without washing the wells, this was able to be determined. The results of these experiments indicated that there was no substantial immunosuppressive differences

between allowing the RAW cell and tumor cell conditioned media co-culture to incubate for 30, 60, or 120 minutes before LPS addition(Fig 5B).

Effect of Tumor Cell Conditioned Media on Murine PECs

In order to move one step further from showing the immunosuppressive effects of the tumor cell conditioned media on the RAW cell line, its effect on naïve C57BL/6 PECs was determined. This was an important step because the RAW cell line is an in vitro propagated, transformed macrophage cell line that may not function like healthy macrophages. In this experiment, PECs were collected from 6-8 week old female C57BL/6 mice and plated at 2.5x10^5/well with either RPMI or B16 conditioned media on a 96-well flat-bottom plate. After allowing the co-culture to incubate for 90 minutes, LPS and IFN γ were added to the wells. After 48 hours, a NO assay was performed. The results from this assay indicate that the B16 tumor cell conditioned media had a small, but significant immunosuppressive effect on the C57BL/6 murine macrophages (Fig 6).

Chapter IV:

Summary

Due to the observation that NO production was abrogated in fixed RAW cells and yet they still exhibited tumor cell killing, albeit quite reduced, in the ^{3}H-thymidine incorporation assay, we conclude that RAW cells exert some of their tumoristatic effects through an additional mechanism to NO production. There are at least two possibilities to what this additional mechanism may be, such as a membrane-dependent mechanism or the release of a different inflammatory molecule. However, the second possibility seems unlikely, because PFA fixation should abrogate all metabolic activity of the cells as exemplified with the loss of NO production. Additionally, our results show that both fixed and viable RAW cells exhibited concentration-dependence in that they did not produce detectable amounts of NO production when plated at 1x10^5 cells/well although they still exhibited significant amounts of B16 tumor cell killing per the ^{3}H-thymidine incorporation assay.

As for the tumor-induced model of immunosuppression in macrophages, tumor cell conditioned media exert an immunosuppressive effect on both RAW cells and C57BL/6 murine macrophages. As shown for RAW cells, the tumor cell conditioned media need to be continually present for the 48 hour incubation period. Additionally, there was no difference in the immunosuppressive effects between co-culturing the tumor cell conditioned media with the RAW cells for 30, 60, or 120 minutes prior to LPS activation.

Chapter V:
Future Directions

The results of this study suggest that there is an additional mechanism of cytotoxicity in the RAW cell line besides that of NO production. Thus, the investigation of other mechanisms is warranted. Studies are needed to determine which cell surface molecules are present on the fixed RAW cells since there may be a contact-dependent mechanism coming into play.

Along with doing a number of in vitro studies, the antitumor effect of fixed RAW cells in vivo could also be examined. This might be done by fixing RAW cells with PFA in vitro and then injecting them intratumorally into B16 melanoma tumors grown subcutaneously in C57BL/6 mice. This group would be compared to a control group of mice receiving intratumoral PBS injections and another group receiving an intratumoral injection of viable RAW cells.

In order to further develop the tumor cell conditioned media induced model of immunosuppression, it would be helpful to test the suppressive effects of other tumor cell conditioned media than the B16 and MethA conditioned media. It would also be beneficial to assess the activity of the macrophages in a ^{3}H-thymidine incorporation assay. Additionally, testing the applicability of an in vivo model would be useful as well. A possibility for an in vivo model is to inject the tumor cell conditioned media into mice and subsequently harvest their PECs and test their activity levels.

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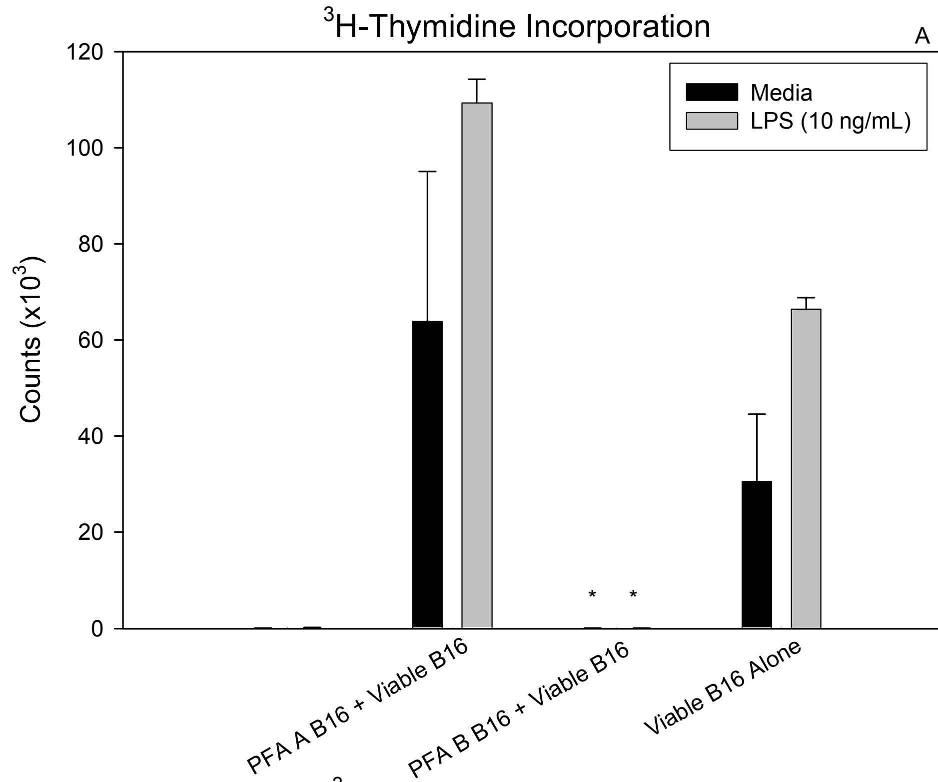

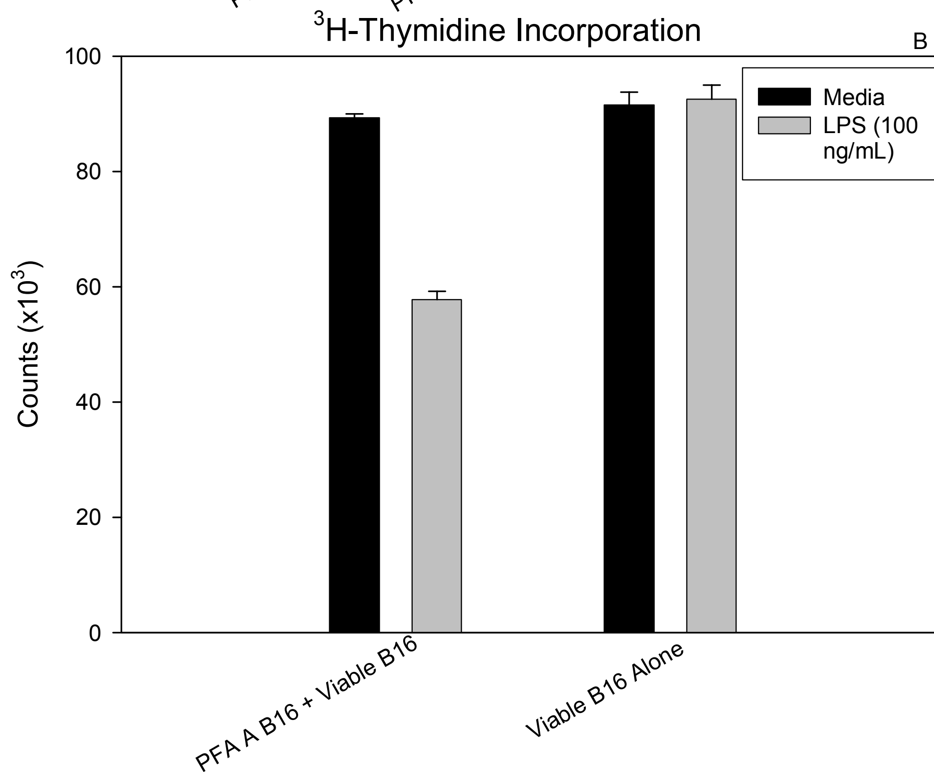

Figure 1. Evaluation of Toxic Effects of PFA Fixation on Cells. The fixed or viable B16 cells were plated at 1x10^4/well. In PFA fixation method A, the cells were allowed to adhere to the wells before fixation and the addition of the viable B16 cells. In PFA fixation method B, the cells were fixed in suspension and added to the wells where the viable B16 cells were already adhered. After a 48 hour incubation period, a ^{3}H-thymidine assay was done. The results of 2 similar experiments (A and B) are shown and indicate that PFA method A was not toxic to the target cells while PFA method B was.

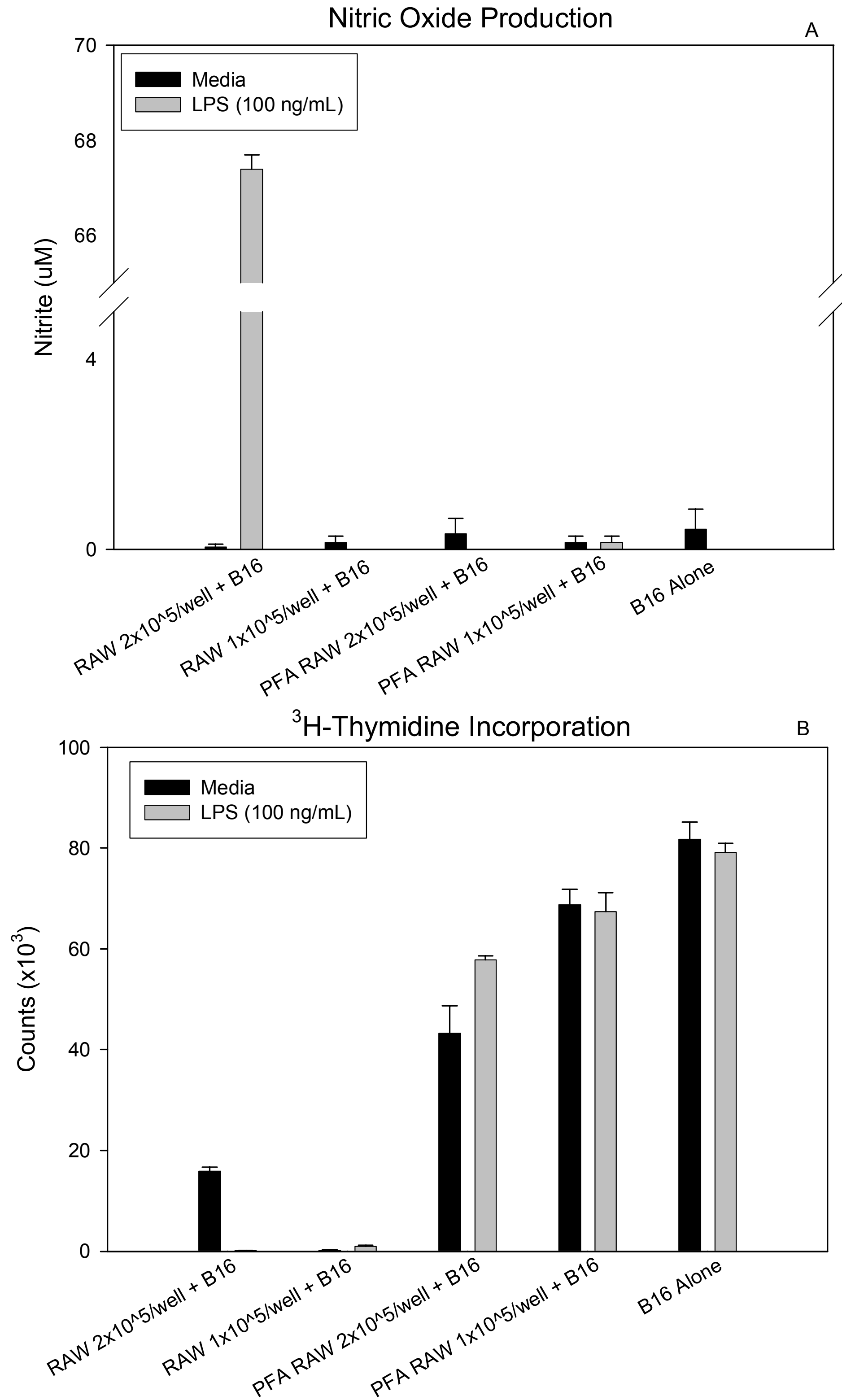

Figure 2. Effect of PFA Fixation on RAW Cell Functions. Fixed or Viable RAW cells were plated at either 2x10^5/well or 1x10^5/well with viable B16 cells (1x10^4/well). The RAW cells were stimulated with LPS for 48 hours. Then, a NO (A) and ^{3}H-thymidine incorporation assay (B) were done. The results indicate that RAW cells exert their cytotoxic effects through a mechanism other than just NO production. Additionally, concentration-dependent cytotoxicity was demonstrated in both the viable and fixed RAW cells.

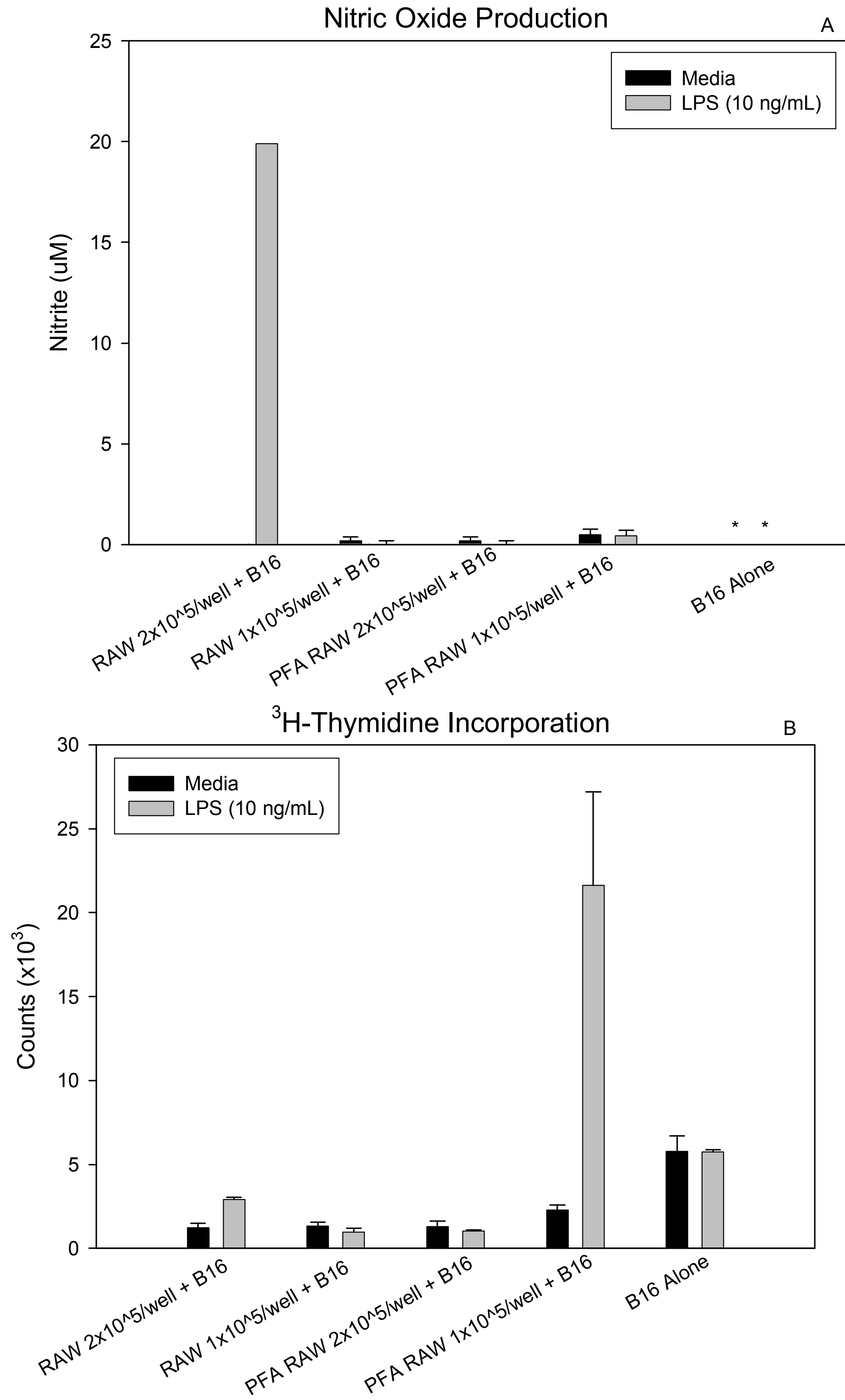

Figure 3: A repeat of the experiment exhibited in figure 2.

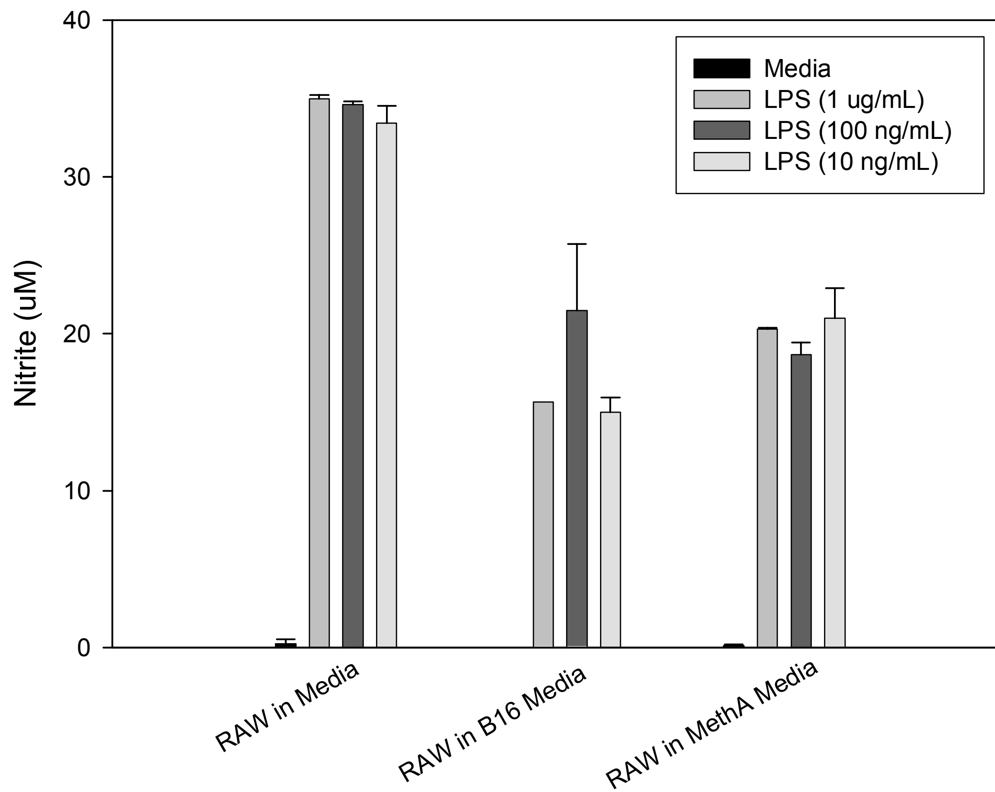

Figure 4. Effect of Tumor Factors on RAW Cell Activity. RAW cells were plated at 2x10^5/well with RPMI, B16 conditioned media, or MethA conditioned media for 30 minutes. Then, LPS was added at 1ug/mL, 100 ng/mL, or 10 ng/mL. A NO assay was performed after a 48 hour incubation period. The results indicate that the tumor cell factors exert a suppressive effect on the RAW cells.

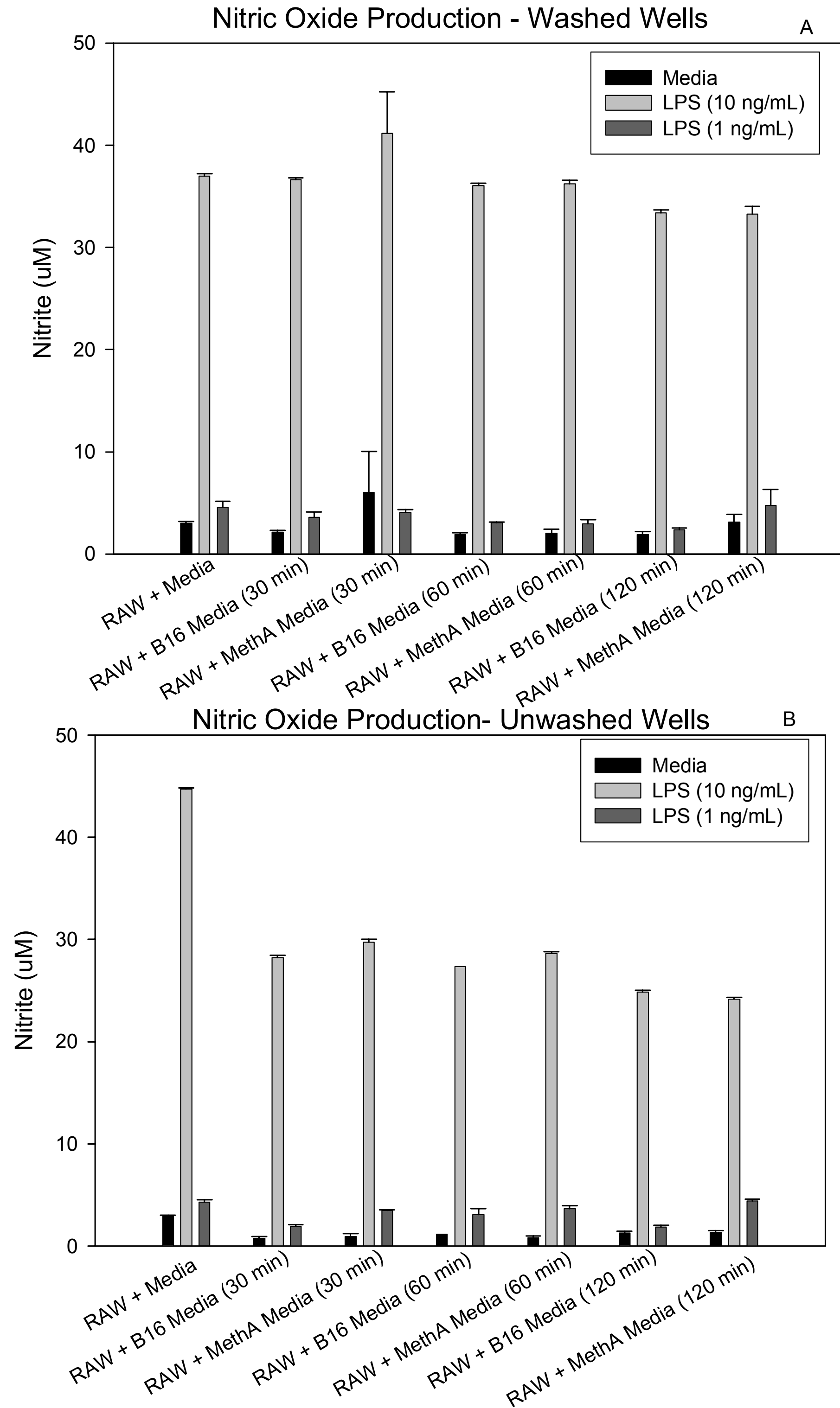

Figure 5. Kinetics of Tumor Cell Conditioned Media Induced Suppression of NO by RAW Cells: RAW cells (2x10^5/well) were plated with either RPMI, B16 conditioned media, or MethA conditioned media. The co-cultures incubated for 30, 60, or 120 minutes. At the specified time, the wells in one set were washed out and replaced with RPMI and LPS (A).
In the other set, no washing was done before LPS was added (B). The results show that the conditioned tumor cell media need to be continually present in order to exert an immunosuppressive effect on the RAW cells. Additionally, there was no difference between adding the LPS at 30, 60, or 120 minutes to the co-culture of RAW cells and tumor cell conditioned media.

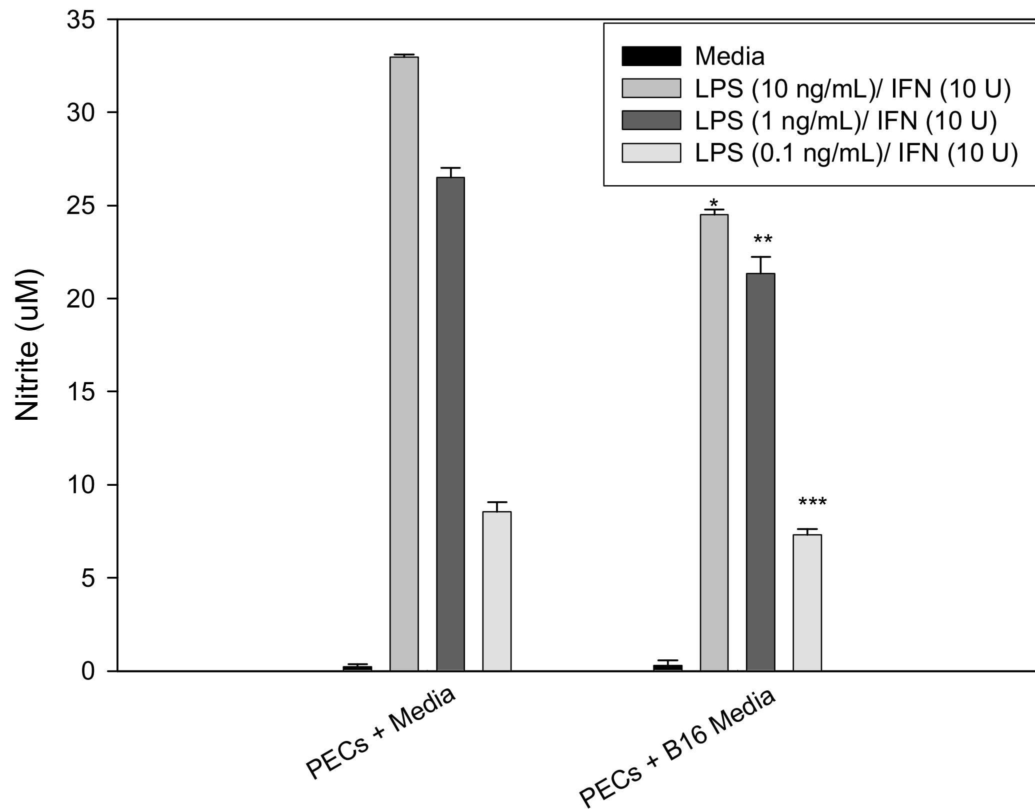

Figure 6. Effect of B16 Tumor Factors on PEC Function. PECs were collected from C57BL/6 mice and plated at 2.5x10^5/well with either RPMI or B16 conditioned media. After 90 minutes, LPS and interferon gamma were added to the wells. A NO assay was done 48 hours later. *P< 0.0001. **P< 0.0075. ***P not significant The results show that B16 conditioned media exert a significant immunosuppressive effect on C57BL/6 murine macrophages.