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Research paper

Comparison of RAW264.7, human whole blood and PBMC assays to screen for immunomodulators

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ABSTRACT

The RAW264.7 mouse macrophage cell line is used extensively to carry out *in vitro* screens for immunomodulators. Compounds that are effective at reducing the expression of pro-inflammatory cytokines or nitric oxide (NO) from lipopolysaccharide (LPS)-stimulated RAW264.7 cells are often considered candidate anti-inflammatory agents for humans. There is, however, very little data on the reliability of this screen to identify *bona fide* human immunomodulators. We compared the efficacy of 37 purported immunomodulators to modulate LPS or *E. coli*-induced inflammatory responses in RAW264.7 cell, whole human blood and human peripheral blood mononuclear cell (PBMC) assays. Interestingly, there was no significant correlation ($R = 0.315$) between the responses obtained with RAW264.7 cells and the whole blood assay (WBA), suggesting that compounds demonstrating efficacy in RAW264.7 cells may be ineffective in humans, and, more importantly, compounds that are effective in humans may be missed with a RAW264.7 screen. Interestingly, there was also no significant correlation between the WBA and human PBMCs when the latter were cultured with 10% FCS, but a moderate correlation was seen when the PBMCs were cultured with 25% autologous plasma. The presence of plasma thus contributes to the overall inflammatory response observed in the WBA. We then asked if RAW264.7 cells, given that they are mouse macrophage-like cells, respond in a manner similar to primary murine derived macrophages. Intriguingly, there was no significant correlation ($R = 0.012$) with the 37 putative immunomodulators, pointing to distinct inflammatory response mechanisms in the two model systems. We conclude that the use of a WBA to confirm potential immunomodulators obtained from high throughput screening with RAW264.7 cells is advisable and that future screens be carried out using a WBA.

1. Introduction

Chronic inflammation (CI) is linked to the development of many diseases, including type 2 diabetes, Alzheimer's disease, heart disease and cancer (Coussens and Werb, 2002; Libby et al., 2002; Donath and Shoelson, 2011). To prevent CI, diet modification that incorporates anti-inflammatory components is thought to facilitate the resolution of the inflammatory state (Steckhan et al., 2016; Casas et al., 2014; Ricker and Haas, 2017). Since consumption of fruits and vegetables are associated with a reduced risk of various chronic ailments (Aune et al., 2017; Steinmetz and Potter, 1996), there has been a major interest in the identification of specific dietary components that can modulate inflammatory responses and ultimately prevent the development of CI-related disorders. On the other hand, with the advent of immunotherapy for cancer treatment, there is a growing appreciation for compounds that enhance the responsiveness of immune cells during inflammatory events, especially those that enhance a T_H1 response (Ikeda et al., 2004; Shalapour and Karin, 2015; Bose et al., 2015), since this may lead to more effective cancer killing (Dranoff, 2004; Kaczanowska et al., 2013). Thus identification of agents that can either increase or decrease pro-inflammatory responses, is highly desirable. To identify these immunomodulators, *in vitro* high throughput screens are usually performed, where extracts from plants or other natural sources are initially evaluated for their efficacy in modulating inflammatory responses, followed by bio-assay guided fractionation to identify the specific active component(s) responsible for the immunomodulatory activity.

One of the most commonly used model systems for evaluating the safety and efficacy of purported immunomodulators is the RAW264.7 cell system, a murine-derived macrophage cell line that produces a robust inflammatory response when challenged by an inflammatory stimulant such as lipopolysaccharide (LPS, also known as endotoxin), a Toll-Like Receptor (TLR) 4-agonist (Berghaus et al., 2010). The wide spread use of this RAW264.7 model system can also be attributed to the

Abbreviations: WBA, Whole blood assay; CI, Chronic inflammation; BMMØ, Bone marrow derived macrophage; MΦs, Macrophages

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fact that this cell line is commercially available, easy to culture and can be rapidly expanded for large scale screening. The extracts or pure compounds that are effective at modulating the expression of the pro-inflammatory mediators produced by these cells, such as interleukin-6 (IL-6), tumor necrosis factor α (TNFα) or nitric oxide (NO), are predicted to be efficacious *in vivo* at modulating inflammatory responses. However, despite the prevalent use of RAW264.7 screens, very little is known regarding the relevance of findings obtained with RAW264.7 cells to that obtained with human immune cells.

We recently developed a human whole blood assay (WBA) that can be used for high throughput screening of immunomodulators. Whole blood was chosen instead of peripheral blood mononuclear cells (PBMCs), which are often used to evaluate potential anti-inflammatory compounds (Dranoff, 2004; Kaczanowska et al., 2013; Berghaus et al., 2010), because it has been shown to more closely mimic *in vivo* conditions (Coch et al., 2013). As well, whole blood preserves the *in vivo* blood cellular composition and the extracellular factors in plasma that may modulate inflammatory responses (Yaqoob et al., 1999; Damsgaard et al., 2009). We also chose to challenge these cells with intact bacteria (*i.e.*, *E. coli*) rather than purified LPS, which only stimulates TLR4-mediated inflammatory responses, in an effort to mimic a more physiological inflammatory response. Related to this, Gao et al. (1999) have shown that a combination of bacterial DNA and LPS acts synergistically to enhance NO production, suggesting a potentially stronger physiological response when intact bacteria are used (Gao et al., 1999).

We also designed the WBA to focus on the early phase of the innate immune response, by incubating for only 7 h with *E. coli,* since this avoids confounding issues like red blood cell and neutrophil death and reduces the potential of *in vitro* drift from *in vivo* conditions. As well, the half-life of many phytochemicals that are commonly tested for potential anti-inflammatory compounds is relatively short (Mehta et al., 2015; Williamson, 2003). Lastly, compounds exposed to blood in *in vitro* or *ex vivo* assays are not distributed and metabolized like they often are *in vivo* (Dorresteijn et al., 2010), thus potentially confounding the outcome if incubation times are prolonged. We also incubated our whole blood samples in 5% O_2 rather than standard 20% O_2 incubators to more closely mimic *in vivo* oxygen and subsequent reactive oxygen species (ROS) levels (Atkuri et al., 2005). We choose IL-6 as our endpoint measure of inflammatory response since it is an early cytokine that is robustly produced after LPS stimulation of whole blood and has been shown to increase markedly *in vivo* after LPS stimulation of human volunteers (Dorresteijn et al., 2010).

The objective of this study was to compare the efficacy of 37 extracts and putative anti-inflammatory compounds at modulating inflammatory responses in the widely used RAW264.7 model system, as commonly performed in the literature, and our human WBA that we optimized to mimic physiological conditions as closely as possible. Also, since PBMCs are commonly used in high throughput screens, we also wanted to compare results with human PBMCs as well. Lastly, we compared results obtained with the RAW264.7 cell line to those obtained with primary murine macrophages to see if either mimics results obtained with whole human blood.

2. Materials and methods

2.1. Reagents

The IL-6 ELISA kit was from BD Biosciences (Mississauga, ON), One Shot INV110 *Escherichia coli* (*E. coli*) was from Life Technologies (Burlington, ON). All purified compounds tested in the assays were obtained from Sigma-Aldrich (Oakville, ON), unless otherwise stated. LPS from *Escherichia coli* serotype O127:B8 was from Sigma-Aldrich (Oakville, ON). Dried soursop (*Annona muricata*) leaf, dried mangosteen (*Garcinia mangostana*) skin, dried red yeast rice (*Monascus purpureus*), dried *Phaleria macrocarpa*, fresh *Curcuma xanthorrhiza*, fresh galangal (*Alpinia galanga*) and fresh turmeric (*Curcuma longa*) were obtained from a supermarket in Jakarta, Indonesia. Dried Chamomile tea leaves and mint tea leaves were purchased from a local market. Slippery elm (*Ulmus rubra*) and marshmallow root (*Althaea officinalis*) dry powder were from Finlandia Pharmacy and Health Centre (Vancouver, BC) and red reishi (*Ganoderma lucidum*) powder was from Purica (Duncan, BC). Triptolide was from Shanghai Yuanye Bio-Technology Company Ltd. (Shanghai, China).

2.2. Plant extraction procedures

Plant materials were blended with distilled water at a 20:1 ratio (water:plant) for dried plants and a 10:1 ratio for fresh plants with a Magic Bullet® blender for a total of 4 × 30 s. The blended plants where subsequently subjected to the following extraction methods.

2.2.1. H_2O extraction

The slurry (40 mL) was boiled for 45 min in a boiling water bath and subsequently filtered through a Whatman #1 filter. The filtrate was freeze-dried in a Labconco freeze dryer (Kansas City, MO) and stored at − 20 °C. Prior to testing, samples were reconstituted with PBS.

2.2.2. Methanol extraction

Blended plant materials (10 mL) were freeze dried and extracted with 10 mL of 100% methanol. The methanol extract was filtered through a Whatman #1 filter and the methanol evaporated under vacuum at 35 °C. The dried material in the round bottom flasks was reconstituted with 1–2 mL of distilled water and lyophilized. The freeze dried extract was stored at − 20 °C. Prior to testing, samples were reconstituted with 100% ethanol followed by dilution with PBS. Final ethanol concentration was 0.14%.

2.3. Whole blood assay (WBA)

All experiments using blood collected from human subjects were reviewed and approved by the joint Clinical Research Ethics Board of the University of British Columbia and the British Columbia Cancer Agency (#H12–00727). Blood from healthy subjects was collected into sodium heparin Vacutainer tubes (cat. no. 366480, BD, Mississauga, ON). The tubes were pre-screened for endotoxin levels using the Pierce LAL Chromogenic Endotoxin Quantitation kit (cat. no. 88282, Thermo Fisher Scientific). 50 µL of blood was aliquoted into individual wells of a 96-well round bottom tissue culture plate followed by the addition of 10 µL of the putative immunomodulator. Control cells consisted of cells that were exposed only to the vehicles used to dissolve the immunomodulators. The plate was incubated for 15 min in a 5% O_2, humidified incubator at 37 °C. Following this, 10 µL of either PBS or *Escherichia coli* (*E. coli*, One Shot INV 110, Life Technologies, Burlington, ON) at a final concentration of 2×10^4 cells/mL, was added to each well. Plates were then incubated for an additional 7 h in a 5% O_2, humidified incubator at 37 °C. Following incubation, 100 µL of PBS was added to each well and mixed thoroughly with the blood. The plate was centrifuged in an Allegra X-12R centrifuge at 424 × *g* at 4 °C for 5 min and supernatants were collected and immediately frozen at − 20 °C for subsequent IL-6 analysis.

2.4. PBMC assay

Peripheral blood mononuclear cells (PMBCs) were obtained from whole blood by density gradient centrifugation using Lymphoprep (StemCell Technologies, Vancouver, BC) according to the manufacturer's instructions. The PBMCs were washed twice with PBS and reconstituted in RPMI containing 10% fetal calf serum (FCS) or 25% human autologous plasma at a final concentration of 10^6 cells/mL. This cell suspension (50 µL) was aliquoted into the wells of a 96-well round bottom tissue culture plate. 10 µL of putative immunomodulators was