EPA Public Access

Author manuscript

Toxicol In Vitro. Author manuscript; available in PMC 2019 December 01.

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Published in final edited form as:

Toxicol In Vitro. 2018 December ; 53: 20–28. doi:10.1016/j.tiv.2018.06.025.

Development and Utilization of a Unique *In Vitro* Antigen Presentation Co-culture Model for Detection of Immunomodulating Substances.

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Abstract

Current regulatory immunotoxicity studies require the use of animal models. However, evolving regulatory requirements, the need to evaluate large numbers of chemicals efficiently and societal pressures are driving the development and utilization of alternative *in vitro* methods for identifying potential immunotoxicants. In line with these efforts, we developed a novel *in vitro* cell-based assay to evaluate effects on antigen presentation – a key step in successful immunization. In this assay, Ch27 B cells acquire and present hen egg lysozyme peptides to antigen-restricted 3A9 T cells, causing them to produce and secrete IL-2. IL-2 levels in the culture medium may be monitored to identify effects of immunotoxicant exposure on antigen uptake, processing or presentation by the Ch27 cells and on antigen recognition and IL-2 production and secretion by the 3A9 cells. IL-2 production was reduced in response to treatment with well-known immunotoxicants cyclosporin A (CYA), dexamethasone (DEX), azathioprine (AZPR), methotrexate (MOT) and benzo(a)pyrene (BAP) but was not affected by treatment with cyclophosphamide (CYPH). A negative control compound mannitol (MANN) altered neither cell viability nor IL-2 levels whereas the lysosomotropic compound ammonium chloride (AMCL) reduced IL-2 production. This novel *in vitro* assay of immune function may be suitable for integration into a tiered testing battery for screening and prioritization of potential immunosuppressants.

Keywords

Immune modulation; immunosuppression; *in vitro*; antigen presentation; immunotoxicology; alternative methods

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1. Introduction

A properly functioning immune system protects us from infections and, in some cases, the development of neoplasms, through a delicate interplay involving many cell types working in concert to properly regulate the immune response (Murphy, 2012). Modulation of the immune system in either direction can result in dysfunction. For example, immunosuppression may lead to decreased resistance to infection and the development of cancer while inappropriate activation of the immune system may lead to conditions such as allergic hypersensitivity or autoimmune disease. The incidence of conditions resulting from immune dysfunction is increasing; this increase is believed to be due, in part, to exposure to environmental chemicals (Hartung et al., 2013).

Given the potential health consequences of exposure to immunotoxic chemicals, it is critically important to be able to accurately identify them. Not surprisingly, the potential for chemicals to cause immunotoxicity is investigated as a standard part of risk assessment for medical devices, pharmaceuticals, and other chemicals, including pesticides (WHO, 2012). However, current, animal-based methods for evaluating immunotoxicity are (1) resource-intensive, (2) low-throughput, and (3) costly to perform. In addition, *in vitro* assays offer the ability to investigate multiple mechanisms of immunosuppression that can be extrapolated to potential effects in other species (Lankveld, 2009). For these reasons, animal-based methods are not practical for efficiently evaluating large numbers of chemicals, and only a small fraction (i.e. 14%) of HPV chemicals have been evaluated for immunotoxicity (Hartung et al., 2013). Consequently, there are large gaps in the basic data required to understand and characterize potential hazards, and there is a clear and pressing need for the development of alternative methods for screening and prioritizing potential immunotoxicants.

In response to the aforementioned limitations of animal models as well as evolving regulatory requirements and societal pressures, several *in vitro* models have been developed for assessing the potential of chemicals to cause immunotoxicity (Mishell et al., 1966, Gerberick et al., 2004, Gennari et al., 2005, Ringerike et al., 2005, Ulleras et al., 2005, Carfi et al., 2007, Corsini et al., 2009, Koeper et al., 2009, Collinge et al., 2010, Emter et al., 2010, Mitjans et al., 2010, Nukada et al., 2011). While some of these alternative methods hold promise, historical data indicate that, compared to assays such as those evaluating T and B lymphocyte responses to mitogens, assays of immune function are more sensitive and predictive of immunotoxicity (Luster et al., 1992, Koeper et al., 2009). This observation holds for *in vivo*, *in vitro*, and *ex vivo* experiments because functional assays preserve some of the complexity inherent to *in vivo* immune processes. However, it is widely accepted that no single assay will be able to reproduce the complexity of the immune system and responses, and an assay battery approach will likely be required (Galbiati et al., 2010).

The gold standard method for identifying immunosuppressive chemicals is the T-dependent antibody response (TDAR) assay in animals (Hartung et al., 2013, Boverhof et al., 2014). Antibody production involves a complex cascade of events requiring multiple cell types and signaling molecules (Koeper et al., 2009). Given its complexity, it is difficult to model this activity *in vitro*. One alternative, the Mishell-Dutton culture is an *in vitro* antibody response assay analogous to the TDAR assay (Mishell et al., 1966, Kawabata et al., 1987, Ban et al.,

1995, Koeper et al., 2009). In this method, mouse spleen cells are incubated in the presence of test compound and SRBCs for 5 days, after which the number of antibody-forming cells are determined (Koeper et al., 2009). Like other functional assays, the Mishell-Dutton culture identifies immunotoxic compounds more reliably than mitogen stimulation assays (Koeper et al., 2009). It also eliminates the need to directly expose mice to test article. However, donor mice are still required, and development of an *in vitro* model relying entirely on cell lines is the next logical step in the evolution of this assay system.

In this work, we focused on developing a novel *in vitro* cell-based assay for antigen presentation that does not require the use of donor rodents. Our method can be used to assess the impact of chemical exposure on a series of integrated events critical for immunization, including uptake, processing and presentation of antigen by antigen presenting cells, and antigen recognition and IL-2 production and secretion by T cells. We used a panel of known immunotoxicants and control compounds to investigate the utility of this *in vitro* assay of immune function for screening and prioritizing potential immunotoxicants.

2. Materials and methods

2.1. Chemicals

Test substances used for this study, including cyclosporin A (CYA; 95% purity CAS 59865-13-3), methotrexate (MOT; 98% purity; CAS 133073-73-1), urethane (URE; 99% purity, CAS 51-79-6), benzo(a)pyrene (BAP; CAS 50-32-8), cyclophosphamide monohydrate (CYPH; 97% purity, CAS 6055-19-2), D-mannitol (MANN; 98% purity, CAS 69-65-8), ammonium chloride (AMCL; 99.5% purity, CAS 12125-02-9) and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO). Dexamethasone (DEX; 98% purity, CAS 50-02-2) was purchased from Enzo Life Sciences (Farmingdale, NY). Azathioprine (AZPR; 98% purity, CAS 446-86-6) was purchased from Alfa Aesar (Ward Hill, MA). AZPR, BAP, CYA, DEX and MOT were prepared in DMSO for testing. CYPH, MANN, URE and AMCL were prepared in culture media for testing. The final concentration of DMSO was 0.1% in all assays. The top doses of these chemicals were selected to match those used in a Mishell-Dutton culture study published by Koeper and colleagues (Koeper et al., 2009). All test chemical dilutions were prepared fresh for each experiment.

2.2. Cell culture

The mouse 3A9 hen egg lysozyme (HEL)-specific I-A^K-restricted T cell hybridoma cell line and the mouse Ch27 B lymphoma cell line were generously provided by Dr. P. M. Allen (Washington University School of Medicine, St. Louis, MO). 3A9 and Ch27 cells were maintained in RPMI 1640 with L-glutamine containing 25 mM Hepes buffer (Cell Gro, Manassas, VA) supplemented with [1%] Penicillin/Streptomycin (Mediatech, Manassas, VA), [10%] fetal bovine serum (Hyclone, Logan UT) and [5 × 10⁻⁵ M] 2-Mercaptoethanol (Sigma Aldrich, St. Louis, MO) for microbiological use. Cells were maintained at a concentration of 0.1 – 1 × 10⁶ cells/mL in a humidified chamber at 37 °C and 5% CO₂.

2.3. Viability assay

All test chemicals were pre-screened to assess their impact on cell viability using Calcein AM staining (Invitrogen, Grand Island, NY). 3A9 and Ch27 cells were plated in opaque white 96-well tissue culture plates (BD Sciences, San Jose, CA) and incubated with test chemical (or vehicle) for 24 hours (37 °C and 5% CO₂). After incubation, the cells were adhered to the bottom of the tissue culture plate by centrifugation (IEC Centra 8GPR, 300 × g for 4 minutes), and the supernatant was aspirated and replaced with 50 µL Dulbecco's Phosphate-Buffered Saline (DPBS; Life Technologies, Grand Island, NY). Then, 50 µL of a 4 µM Calcein AM solution was added to each well, and the plates were gently swirled to distribute the stain. After a 20-minute incubation, the plates were centrifuged to concentrate the cells on the bottom of the tissue culture plate (IEC Centra 8GPR, 300 × g for 4 minutes) prior to signal quantification (Ex₄₈₅ nm, Em₅₂₀ nm) using a FLUOstar Optima plate reader (BMG LABTECH, Durham, NC.). Average blanks readings were subtracted from each well prior to calculating the percent control signal for each treatment.

2.4. Antigen presentation assay and test article treatment

The antigen presentation assay was performed using an add-together non-fixation system where the antigen presenting cell continuously processes the antigen. Here, the antigen (i.e. HEL) is taken up from the tissue culture media, processed by the antigen processing cell (i.e. Ch27 cell), and presented on its cell surface in complex with MHC II. 3A9 T cells are reactive against HEL peptide 46-61 and, upon recognition of the peptide-MHC complex via the T cell receptor, secrete IL-2 (Allen et al., 1984, Allen et al., 1985, Wahl et al., 2003). To optimize the assay for screening potential immune modulating compounds, the number of 3A9 cells, ratio of 3A9 cells to Ch27 cells, concentration of HEL, and the duration of the assay were extensively evaluated (refer to text and figure legends). Optimal assay conditions (20,000 3A9 cells, 2,000 Ch27 cells, 30 µg/mL HEL, 24-hour incubation) were used to investigate the impact of exposure to various immune modulating compounds. All conditions were plated in duplicate. Cells were pre-incubated with xenobiotic for 1 hour prior to the addition of HEL. After the 24-hour incubation with HEL, the cells were concentrated on the bottom of the tissue culture plate by centrifugation (IEC Centra 8GPR, 300 × g for 10 minutes). Cell-free supernatants were collected and stored at -80 °C.

2.5. IL-2 ELISA

IL-2 levels in tissue culture supernatants collected from antigen presentation assays were determined using the BD OptEIA IL-2 Mouse Cytoset ELISA kit according to the manufacturer's instructions (BD Biosciences). Briefly, 96-well flat-bottom Costar microtiter plates (Corning Life Sciences, Tewksbury, MA) were coated with 100 µL/well of anti-mouse IL-2 capture antibody in Coating Buffer (0.1 M BupH Carbonate-Bicarbonate Buffer, pH 9.4; Pierce Biotechnology, Grand Island, NY) and incubated overnight at 4 °C. After washing (with phosphate buffered saline (PBS) containing 0.5% Tween-20), the plates were incubated with 200 µL Assay Diluent (PBS + 10% heat-inactivated FBS (Hyclone)) at room temperature for 1 hour. Cell culture supernatants were diluted with Assay Diluent (10 µL into 300 µL) and mixed. Diluted samples (100 µL) were plated in duplicate and allowed to incubate at room temperature for 2 hours. After washing five times with PBS/0.05%

Tween-20, biotinylated anti-mouse IL-2 detection antibody and streptavidin-horseradish peroxidase conjugate were added to the wells and allowed to incubate at room temperature for 1 hour. After washing seven times with PBS/0.05% Tween-20, 100 μ L tetramethylbenzidine (TMB; Dako, Carpinteria, CA) substrate was added. After development, the reaction was terminated by the addition of Stop Solution (2N H₂SO₄). Optical density was determined at 450 nm (reference λ = 570 nm) using a SpectraMax 340pc plate reader (Molecular Devices, Sunnyvale, CA). IL-2 concentrations were determined within the linear range of the recombinant mouse IL-2 standard curve using Softmax Pro software (Molecular Devices). The concentration of test article required to reduce IL-2 release by twenty-five percent (i.e., IC₂₅) and fifty percent (i.e., IC₅₀) was determined by nonlinear regression analysis using GraphPad Prism v5 (La Jolla, CA).

2.6. Statistical analysis

Statistics were performed using GraphPad Prism® v5). Statistical significance was defined as $p < 0.05$ as evaluated by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test.

3. Results

3.1. Developing an *in vitro* antigen presentation assay for identifying potential immune modulating xenobiotics

Antigen presentation involves the display of antigen peptides complexed with MHC molecules on the surface of antigen presenting cells; the complex subsequently promotes activation of T cells bearing a cognate receptor (Figure 1A) (Murphy, 2012). *In vitro* antigen presentation assays have been used extensively to unravel the mechanisms involved in antigen uptake, processing, and presentation [reviewed in (Falta et al., 2010) and conditions from these assays served as a starting point to develop a protocol for this work. To develop an *in vitro* antigen presentation protocol suitable for identifying immune modulating xenobiotics, we carefully designed a series of experiments to optimize the 1) concentration of antigen (HEL), 2) number of 3A9 T cells, 3) ratio of 3A9 T cells to Ch27 B cells, and 4) assay duration (Figures 1-3).

In the current studies, under control conditions (i.e. no HEL), co-cultured Ch27 B cells and 3A9 T cells were only loosely associated and did not appear to form cell-cell clusters (Figure 1B). Addition of 30 μ g/mL HEL caused 3A9 T cells to form rosettes around antigen-bearing Ch27 B cells and to secrete IL-2 (Figure 1B and 2A). Increasing the concentration of HEL to 300 μ g/mL promoted the formation of additional rosettes and increased the amount of IL-2 secreted from 275.5 $\pm$ 14.4 pg/mL to 397.5 $\pm$ 2.3 pg/mL ($p < 0.05$; Figure 1B and 2A). While increasing the concentration of HEL to 3,000 μ g/mL increased the density of T cell rosettes (Figure 1B), the amount of IL-2 secreted into the culture medium did not increase further (Figure 2A). Although IL-2 secretion was maximized by using a HEL concentration of 300 μ g/mL, this concentration also resulted in loss of cell viability (Figure 2B). For further testing of this screening assay, we used HEL concentrations 30 μ g/mL to ensure that observed effects on IL-2 secretion would be associated with the effects of test article exposure and not with HEL-induced loss of cell viability.

As a next step, 20,000 3A9 T cells and varying numbers of Ch27 B cells were co-cultured at various ratios for 24 hours in the presence of 10, 20 or 30 µg/mL HEL. IL-2 secretion was measurable when 3A9 cells were stimulated by Ch27 cells in the presence of 10 µg/mL HEL, but only when 10 or fewer 3A9 T cells were present for each Ch27 B cell (Figure 3A). Increasing the concentration of HEL from 10 µg/mL to 20 µg/mL did not increase IL-2 secretion for any combination of 3A9 and Ch27 cells (Figure 3A). However, increasing the HEL concentration to 30 µg/mL increased IL-2 release for all conditions tested except for a ratio of 100:1 T cells to B cells (Figure 3A).

We also investigated the impact of the number of 3A9 T cells on production of IL-2 when co-cultured with different ratios of Ch27 B cells in the presence of 30 µg/mL HEL. A minimum of 10,000 3A9 T cells were required to achieve measurable levels of IL-2 release (Figure 3B). Depending on the ratio of 3A9 T cells to Ch27 B cells, IL-2 secretion peaked at either 30,000 or 100,000 3A9 cells (Figure 3B). The greatest IL-2 release was achieved when three T cells were present for every B cell (Figure 3B). IL-2 was not detectable in supernatants harvested from cultures with too many T cells for every B cell (i.e. 3A9:Ch27 = 100:1) (Figure 3B). A ratio of 10:1 3A9 to Ch27 cells yielded moderate, non-saturating levels of IL-2 secretion under a wide range of experimental conditions (Figure 3A, B, C).

To investigate the impact of assay duration on IL-2 production, 3A9 T cells (20,000) and Ch27 B cells (2,000) were incubated in the presence of increasing concentrations of HEL for 0, 6, 12 or 24 hours. IL-2 production was undetectable at 0, 6 and 12-hour time points for any concentration of HEL tested (Figure 3D and data not shown). At 24 hours, IL-2 release from co-cultures stimulated with 10 or 20 µg/mL HEL was too low relative to the sensitivity limits of the IL-2 ELISA to be useable. Increasing the concentration of HEL to 30 µg/mL resulted in ~200 pg/mL IL-2 released from stimulated 3A9 T cells (Figure 3D).

Based on the outcome of this series of assay optimization experiments, we selected the following conditions for our antigen presentation assay protocol: 1) 30 µg/mL HEL; 2) 20,000 3A9 T cells; 3) 10:1 ratio of T cells to B cells (i.e. 2,000 Ch27 B cells); and 4) an assay duration of 24 hours.

3.2. Pre-screening xenobiotics for effects on cell viability

In our assay, successful antigen processing and presentation is measured by quantifying IL-2 secreted by the 3A9 T cell – a process that requires the interaction of two different cell types. Since the assay uses a non-fixation, add-together protocol where the Ch27 B cell continuously processes antigen, xenobiotic-induced alterations in the viability of either the 3A9 or Ch27 cells could impact secretion of IL-2. To address this point, we pre-screened test substances for effects on cell viability. Since our co-cultures contain ten times as many 3A9 T cells as Ch27 B cells, we opted to evaluate the viability of each cell type individually rather than simultaneously in co-culture where it would be more difficult to detect decreases in Ch27 cell viability. Cells were singly plated (*i.e.*, 20,000 3A9 T cells/well or 2,000 Ch27 B cells/well) and incubated for 24 hours with test chemical prior to assessing viability using staining with Calcein AM and microscopic visualization. For the concentrations tested, results of the viability pre-screen can be organized into three categories: 1) non-cytotoxic (AZPR, BAP, CYPH, MANN and AMCL; Figure 4C, F, G, H); 2) cytotoxic to 3A9 cells

only (CYA, DEX and MOT; Figure 4A, B, D); and 3) cytotoxic to both 3A9 and Ch27 cells (URE; Figure 4E). CYA, for example, did not adversely impact the viability of Ch27 cells at the concentrations tested, but did cause a reduction in viability for the 3A9 cells, particularly at concentrations greater than 0.32 μ M (Figure 4A). URE was the only chemical to reduce the viability of both cell types by nearly 50%, but only did so at the maximum concentration tested (i.e. 100 mM; Figure 4E).

3.3. Immunosuppressive chemicals with different mechanisms of action alter IL-2 production

The effect of known immunosuppressive chemicals with different mechanisms of action on IL-2 production was investigated (Table 1). Treatment with CYA, a well-known immunosuppressive drug (Carfi et al., 2007, Markovic et al., 2015), completely eliminated detectable IL-2 release from 3A9 T cells co-cultured with antigen-bearing Ch27 B cells (Figure 5A) with an IC_{25} and IC_{50} for IL-2 production of 1.19 nM and 1.99 nM, respectively (Table 2). Treatment with other immunosuppressant compounds (i.e. DEX, AZPR, MOT, BAP and URE) also resulted in decreased IL-2 release from stimulated 3A9 T cells at non-cytotoxic concentrations (Figure 5B, C, D, E and F). URE, a weakly immunosuppressive chemical (Luster et al., 1982), was least potent in the assay, with an IC_{25} and IC_{50} for IL-2 secretion of 4.24 mM and 13.26 mM, respectively (Table 2). CYPH, a compound that acts as an immunosuppressant only after biotransformation to active metabolites (Connors et al., 1974), did not alter IL-2 release (Figure 5G). Also as expected, the negative control compound MANN did not alter IL-2 release whereas the positive control compound AMCL (Falo et al., 1987) reduced IL-2 production (Figure 5H and I).

4. Discussion

Currently, regulatory-driven studies for the detection of immune system effects require the use of animal models. However, public opinion, advances in scientific knowledge, recognition of practical limitations of animal models, and recent political pressure have provided the impetus to develop alternative, non-animal test methods to support hazard identification and risk assessment activities (Luebke, 2012). Several *in vitro* methods have been developed to assess immunotoxicity (Mishell et al., 1966, Gerberick et al., 2004, Gennari et al., 2005, Ringerike et al., 2005, Ulleras et al., 2005, Carfi et al., 2007, Corsini et al., 2009, Koeper et al., 2009, Collinge et al., 2010, Emter et al., 2010, Mitjans et al., 2010, Nukada et al., 2011). To expand upon these early efforts to develop *in vitro* methods for screening and prioritization of potential immunotoxic substances by assessing impacts on functional endpoints, we developed a unique, entirely cell-based co-culture assay of antigen presentation.

Proper function of the immune system requires antigen display on the surface of antigen presenting cells (e.g. Ch27 cells) in the form of peptide-MHC complexes. Extracellular antigen (e.g. HEL) is adsorbed onto the surface of the antigen presenting cell and taken into the intracellular space by endocytosis where it is degraded by acid proteases into peptide fragments that form complexes with MHC II molecules. Ultimately, these complexes are transported to the cell surface and “presented” to CD4+ T cells (e.g. 3A9 cells). Upon

recognition of the peptide-MHC II complex by the T cell receptor, the T cell is activated and, through a series of intracellular events, supports critical immune processes, including antibody production (Murphy, 2012). There are many points in the network of events leading to antigen presentation and T cell activation that can be disrupted. Thus, evaluating the effects of toxicant exposure on this process provides a relatively comprehensive and sensitive measure of potential impacts on immune function (Ladics, 2007). Like the *in vitro* antibody response test originally developed by Mishell and Dutton (Mishell et al., 1966), our *in vitro* assay preserves the functional integrity of cells by maintaining essential cell-cell interactions in co-culture. However, our assay differs from the Mishell-Dutton method because it relies on decreases in IL-2 rather than antibody production as an indicator of potential immunosuppression.

While *in vitro* antigen presentation models have been in use for a number of years, the conditions used for these assays were not optimized for our specific purpose. Consequently, development of our model required an extensive series of studies to identify optimal assay conditions. The concentration of antigen, T cell number, ratio of T to B cells, and assay duration were all meticulously evaluated for impacts on IL-2 secretion. And, although the intent of the assay is to detect xenobiotic disruption of cellular and molecular events involved in antigen presentation and recognition, IL-2 release may also be impacted non-specifically at xenobiotic concentrations that alter cellular viability. Therefore, we evaluated all test substances for cytotoxicity and used this information to guide our interpretation of assay results.

The end result of this effort is a high-performing assay that accurately identifies immunotoxic chemicals, as shown by the results achieved with a chemical training set composed of well-known immunotoxicants and control compounds. Chemicals selected for the training set represent different mechanisms of action targeting one or both cell types present in our assay. As presented herein, our assay format precludes the ability to deduce which cell type(s) is being targeted by the test article because either cell could be affected. However, the distinction is not vital since the purpose of the assay is to flag chemicals for additional screening. Exposure to several of the known immunotoxicants in our training set resulted in reduction in IL-2 secretion at non-cytotoxic doses. No less important, the negative control compound (i.e. MANN) had no effect on IL-2 secretion or cell viability. Immunosuppressive substances could be ranked by potency for disrupting *in vitro* antigen presentation using their IC values for reducing IL-2 secretion by 25% (CYA > DEX > BAP > AZPR > MOT > URE > AMCL; Table 2) or by 50% (CYA > DEX > MOT > AZPR > URE > AMCL; Table 2). These findings are consistent with those of Koeper and colleagues (2009), who confirmed the immunosuppressive potential of CYA, DEX, MOT, BAP, and URE *in vitro* using Mishell-Dutton cultures.

Some of the training set chemicals (i.e. CYPH, BAP, and AZPR) require biotransformation to active metabolites, providing an opportunity to gauge the metabolic capacity of the model used in our *in vitro* antigen presentation assay. In this assay, CYPH had no discernable impact on IL-2 secretion, and BAP-induced reductions of IL-2 secretion plateaued at about 40%. Meanwhile, AZPR was correctly identified as an immunosuppressive compound. It could be argued that using higher test concentrations of CYPH or BAP may have produced a

different result. However, we selected the same top dose of these chemicals used by Koeper et al. (2009) and their results were comparable (i.e., false negative result with CYPH). Chemical stability could impact assay results, but we only used freshly made test article solutions and the duration of chemical exposure is only 24 hours. A more likely explanation relates to the metabolic capacity of the 3A9 and Ch27 cells. Both CYPH and BAP are known to require biotransformation by cytochrome P450 isozymes (Connors et al., 1974, Dean et al., 1983, Carfi et al., 2007, Markovic et al., 2015). However, the mechanism of AZPR biotransformation involves conjugation with glutathione rather than cytochrome P450 activity (Maltzman et al., 2003). Together, these data suggest that the cytochrome P450 capacity of the co-cultures is limited, but that they do appear to have the capacity to perform phase II conjugation reactions. These findings are consistent with those reported by Koeper and colleagues using the *in vitro* Mishell-Dutton culture method (Koeper et al., 2009). In their work, CYPH was considered to be a false negative, but Takahashi and colleagues (2002) showed that disruption of *in vitro* antibody production by CYPH required the presence of S9. These results suggest that assay performance could be improved by inclusion of S9, and future assay development efforts should involve the addition of S9 fraction to co-cultures and an expanded set of test chemicals including more substances known to require metabolic activation.

To enable a higher understanding of mode of action, our antigen presentation assay could be complemented by a conjugation assay that utilizes fixed Ch27 B cells. Using this approach, antigen uptake, processing and presentation by the B cells could be separated from the T cell response. Depending upon the experimental question, investigators could treat either cell type with the test article in isolation from the other cell. In this way, investigators will be able to discern whether, or not the test article is acting on the B cell, the T cell or both cell types. Data like these would also be very useful for, as in the case of our data with cyclophosphamide, obtaining better understanding assay limitations and for informing future assay refinements.

Conclusions

Assays that directly assess changes in immune function are regarded as essential for screening and prioritizing potential immunotoxicants (Luster et al., 1992, Koeper et al., 2009). The production of antigen-specific antibodies is critical to support robust immune responses (Ladics, 2007). We show here that an *in vitro* antigen presentation model is capable of detecting several known immunosuppressive compounds that act by different mechanisms of action. This *in vitro* functional assay preserves the essential architecture of cell-cell interactions and is responsive to changes in antigen uptake, processing, and presentation as well as the ability of the T cell to recognize the antigen and to secrete IL-2. Compared to the Mishell-Dutton culture method, which requires 5 days of culture, this assay can be completed in 24 hours using an ELISA-based assay readout, which allows for more rapid determination of immunomodulating potential with greater throughput – all in the absence of animal use. Together, these attributes contribute to promising assay performance. Ultimately, this assay may be suitable for integration into a tiered testing battery for screening and prioritization of potential immunotoxicants.

Acknowledgements

The authors thank Dr. W. Mundy and D. Andrews for expert assistance establishing the viability assay and IL-2 ELISA assay protocols, respectively. We thank Dr. P. M. Allen for generously providing the 3A9 and Ch27 cells necessary for our work. We also thank Drs. R. Luebke, M. Ward, I. Gilmour and G. Lehmann for their thoughtful critical review of this manuscript.

Abbreviations:

ANOVA	analysis of variance
APC	antigen presenting cell
AZPR	azathioprine
BAP	benzo(a)pyrene
CYA	cyclosporin A
CYPH	cyclophosphamide monohydrate
DEX	dexamethasone
DPBS	Dulbecco's phosphate-buffered saline
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
HEL	hen egg lysozyme
IL-2	interleukin-2
MANN	D-mannitol
MHC	major histocompatibility complex
MOT	methotrexate
TMB	tetramethylbenzidine
URE	urethane
EPA	Environmental Protection Agency
SRBC	sheep red blood cell

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Figure 1. Antigen presentation.

(A) Ch27 B cells internalize the extracellular protein antigen hen egg lysozyme (HEL) by endocytosis. Once internalized, HEL is degraded by intravesicular acid proteases into peptide fragments that ultimately bind to MHC class II molecules. MHC II/peptide complexes are subsequently transported to the cell surface for presentation to antigen-specific CD4⁺ 3A9 T cells. Ligation of the T cell receptor promotes transcriptional activation leading to the production and secretion of IL-2 by the 3A9 cell. (B) HEL induces T cell rosette formation. 3A9 cells (20,000) and Ch27 cells (2,000) were co-cultured in the presence of 30, 300 or 3000 µg/mL HEL for 24 hours. Under control conditions, the cells are not closely associated. Upon addition of HEL, the 3A9 T cells aggregate around the antigen-bearing Ch27 B cells. Representative photos (40X power) were acquired with a Nikon Eclipse TE2000-U microscope and RT Ke Diagnostic Instruments Inc., image capturing system.

Figure 2. At high concentrations, HEL induces IL-2 secretion and decreases cell viability. 3A9 T cells (30,000) and Ch27 cells (3,000) were co-cultured for 24 hours in the presence of 30, 300 or 3,000 μg/mL HEL. **(A)** Cytokine release was measured in cell-free supernatants by ELISA. **(B)** Cell viability was determined by Calcein AM staining and reported as percent vehicle control. Representative data shown (+/- SD) from 2 independent studies. *p < 0.05 compared to 30 μg/mL HEL (ANOVA).

Figure 3. Identification of optimal *in vitro* antigen presentation conditions.

Optimal antigen presentation conditions were identified by quantifying IL-2 secretion in response to variations in the (A) concentration of HEL, (B) number of 3A9 T cells, (C) ratio of 3A9 T cells to Ch27 B cells, and (D) duration of incubation with HEL. For all panels, representative data shown (+/- SD) from 2 independent studies.

Figure 4. Cell viability in the presence of immune modulating chemicals. (A – I) 3A9 T cells and Ch27 B cells were plated in 96-well plates for 24 hours in the presence of immune modulating compounds or vehicle. Viability of each cell type was assessed using Calcein AM staining and reported as percent of vehicle control. Representative data shown (+/- SD) from 3 independent studies.

Figure 5. Immunosuppressant compounds modulate IL-2 production.

(A – I) 3A9 T cells and Ch27 B cells were co-cultured using the optimal conditions identified. IL-2 levels were quantified by ELISA and reported as percent vehicle control. Representative data shown (+/- SD) from 3 independent studies.

Table 1.

Characteristics and attributes of test chemicals.

Test substance	CAS No.	Classification	MOA	Metabolic activation	T cell targeting	B cell targeting	Reference
CYA	59865-13-3	Immunosuppressant	Calcineurin inhibitor	NA	✓		Markovic et al. 2015. Tox Lett. V233:8-15. Carfi et al. 2007. Tox. V229:11-22.
*AZPR	446-86-6	Immunosuppressant	DNA synthesis inhibition (purine analog)	Metabolized to 6-mercaptopurine by glutathione	✓		Maltzman and Koretzky. 2003. J Clin Invest. V111:1122-1124.
DEX	50-02-2	Immunosuppressant	Glucocorticoid agonist	NA	✓		Collinge et al. 2010. J Immunotox. V7:357-366.
MOT	133073-73-1	Immunosuppressant	Dihydrofolate reductase inhibitor	NA	✓	✓	Collinge et al. 2010. J Immunotox. V7:357-366.
URE	51-79-6	Immunosuppressant	Poorly described.	NA	Unknown	Unknown	Luster et al. 1982. Clin Exp Immunol. V50:223-230.
*BAP	50-32-8	Immunosuppressant	Indirect via immunotoxic metabolite formation. DNA synthesis inhibition	Metabolized by Cytochrome P450 to active metabolites.		✓	Markovic et al. 2015. Tox Lett. V233:8-15. Carfi et al. 2007. Tox. V229:11-22. Dean et al. 1983. Clin Exper Immun. V2:199-206.
*CYPH	6055-19-2	Immunosuppressant	Indirect via immunotoxic metabolite formation. DNA crosslinking	Metabolized by Cytochrome P450 to 4-hydroxy cyclophosphamide.	✓	✓	Conner et al. 1974. Biochem. Pharmacol. 23:115-129. Stockman et al. 2016. Cancer Treat Rev. 42:39. Cupps T. et al. 1982. J Immunol.
MANN	69-65-8	Immune inert	NA	NA	NA	NA	Koeper, LM and Vohr, HM. 2009. Food Chem Tox. V47:110-118.
AMCL	12125-02-9	Positive control	Lysosomotropic	NA	NA	✓	Falo et al. 1987. Journal of Immunology. V139:3918-3923.

* Requires metabolic activation.

MOA = mechanism of action; NA = non-applicable.

Table 2.

IC₂₅ and IC₅₀ values for IL-2 release.

Test substance	IC ₂₅ for IL-2 production	Percent viability at IL-2		IC ₅₀ for IL-2 production	Percent viability at IL-2		Correctly predicted by <i>in vitro</i> antigen presentation model?
		3A9	Ch27		3A9	Ch27	
CYA	1.21 (0.1) nM	99.8 (0.7)	98.9 (1.1)	2.04 (0.1) nM	99.7 (1.9)	99.7 (2.2)	Yes
DEX	2.10 (0.2) nM	97.3 (1.6)	102.1 (0.8)	4.76 (0.3) nM	99.9 (2.3)	100.9 (0.7)	Yes
AZPR	5.73 (1.6) μ M	98.6 (1.1)	99.7 (3.5)	32.75 (5.8) μ M	99.2 (1.0)	98.2 (0.9)	Yes
MOT	77.47 (7.1) μ M	102.0 (1.9)	99.6 (0.9)	273.83 (4.9) nM	94.2 (0.4)	98.7 (4.5)	Yes
URE	4.35 (0.8) mM	98.4 (2.6)	100.9 (1.7)	13.40 (2.4) mM	96.9 (4.7)	98.3 (4.3)	Yes
BAP	0.52 (0.2) μ M	98.6 (2.2)	99.1 (1.4)	> 10 μ M	NA	NA	Yes
CYPH	> 1 mM	NA	NA	> 1 mM	NA	NA	No
MANN	> 100 μ M	NA	NA	> 100 μ M	NA	NA	Yes
AMCL	9.35 (1.0) mM	100.2 (2.7)	100 (2.4)	26.09 (1.6) mM	101.5 (1.7)	101.1 (1.6)	Yes

Data shown from 3 independent experiments (+/–SD)