QUANTIFICATION OF CYTOKINE PRODUCTION FOLLOWING EXPOSURE TO 2,3,7,8-
TETRACHLORODIBENZO-P-DIOXIN (TCDD) IN A GRAFT VERSUS HOST (GvH) MODEL
Toxicology + Immunology = Immunotoxicology

Immune response to exposure of a toxin
OUTLINE

- Background information
- *In vitro and ex vivo* models
  - Purpose
  - Materials and Methods
  - Results
  - Discussion
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (aka “dioxin”)
- Present in Agent Orange, defoliant used during the Vietnam War
- Released by burning of industrial processes
- Stable environmental contaminant

Dr. Kerkvliet and lab studies effects of TCDD on the immune system and has shown that TCDD causes potent immuno-suppression in laboratory rodents.
AUTOIMMUNE DISEASES/DISORDERS

- Affect more than 23.5 million Americans
  - Leading cause of death and disability

- Over 80 types autoimmune diseases/disorders
  - Typically genetic
    - Women
      - Child bearing age
    - Races or ethnicities
WHAT IS THE RELEVANCE?

- Debilitating disease
- Difficult to diagnose
- Understanding mechanism of autoimmune disease may lead to therapeutic molecular targets

Immune suppression occurs when TCDD binds and activates a protein in cells called ‘Aryl Hydrocarbon Receptor’ (AhR) – transcription factor (Kerkvliet et al. 2002)

The “AhR pathway” of immune suppression is a novel target for drug development

- Immunosuppressive drugs can treat allergic and autoimmune diseases
- Current immunosuppressive treatments, such as corticosteroids, have many severe side effects
THE MECHANISM OF AHR
OBJECTIVE

- High level exposure to TCDD causes toxic and pathological effects

- One aspect of the Kerkvliet lab is to screen for other AhR ligands as AhR non-activation increases immune response
GVH STUDIES IMPROVE UNDERSTANDING OF AUTOIMMUNE RESPONSE

- Graft versus Host Response Model
  - GvH mechanism
IMMUNE RESPONSE

[Diagram showing the immune response process, including bone marrow, blood, lymph, and different cell types like B cells, T cells, NK cells, and various T cell subsets (CD4 T\(_{H1}\), CD4 T\(_{H2}\), CD4 T\(_{H17}\), and CD4 regulatory T cells).]
Donor CD4$^+$ and CD8$^+$ T-cells from a C57Bl/6 (H-2$^{b/b}$) mouse are transferred into a host B6D2F1 (H-2$^{b/d}$) mouse.
The “b” haplotype from the T-cell recognizes the “d” haplotype from the APC as foreign

\[ H-2^{b/b} \rightarrow H-2^{b/d} \]
CYTOTOXIC T-LYMPHOCYTE RESPONSE

Figure 8-36 Immunobiology, 7ed. (© Garland Science 2008)
TCDD IN THE MODEL

TCDD suppresses the CTL Response

Kerkvliet et al. 1996
Immune suppression is dependent upon the AhR expression in CD4\(^+\) T-cells, as well as in CD8\(^+\) T-cells\(^1\) when using a graft-versus-host (GvH) model.

Kerkvliet et al. 2002
**T_{regs}** are CD4 T-cells that suppress the immune response

- Suppresses similar to TCDD suppression
  - Express CD4, CD25 and Foxp3 proteins

- **T_{regs}** can release IL-10, which can affect the differentiation of dendritic cells (DCs)
How does TCDD work?

- Induce certain cytokines, so we will look at them in GvH model in day 2 or day 3
  - Inject day 0 and day 2 or day 3 look at response
Identify the production of cytokines, Interferon gamma (IFN-ϒ) and Interleukin (IL-10), as influenced by TCDD

- Phase 1
  - Method optimization for activation of CD4⁺ T-cells in vitro
    - Produce DCs → activate CD4⁺ T-cells → production of cytokines
- Phase 2
  - Use prior GvH supernatants from Kerkvliet lab
  - Test supernatants for IFN-ϒ and IL-10
WHY INTERLEUKIN-10 (IL-10)?

- IL-10 is anti-inflammatory

- Marshall et al, 2008 study found that TCDD-CD4+ cells secreted significant amounts of IL-10 when supernatants were harvested at 72 hours

TCDD-CD4+ cells produced more IL-10 than other treatment groups (t tests; *, p < 0.05)
IFN-γ can have both pro-inflammatory and immunosuppressive effects.

Vorderstrasse et al, 2001, observed an increase in IFN-γ production.
PHASE 1
DEVELOPMENT OF AN IN VITRO MODEL TO TEST EFFECT OF TCDD ON CYTOKINES: APC STUDIES
Mix MHC\textsuperscript{a} T cells and irradiated MHC\textsuperscript{b} non-T cells as antigen-presenting cells

Measure proliferation of T cells by incorporation of \textsuperscript{3}H-thymidine

Measure killing of \textsuperscript{51}Cr-labeled target cells to detect activated cytotoxic T cells

T-cell proliferation depends largely on differences in MHC class II alleles

Generation of cytotoxic T cells depends largely on differences in MHC class I alleles

Figure 14-46 Immunobiology, 7ed. (© Garland Science 2008)
Antigen Presenting Cells (APCs) present antigen to other immune cells

- Types: dendritic cells (DC), macrophages, B-cells
- DCs are the best type of APCs
To prepare antigen presenting cells (APC) that would activate CD4⁺ T-cells \textit{in vitro} model that is both cost effective and simple

- Donor CD4⁺ T-cells express AhR + TCDD Treatment = CTL response suppression
- AhR role unknown in CD4⁺ T cell, however, production of certain cytokines that regulate the CTL response may be involved

OBJECTIVE: IDENTIFY TCDD-EFFECTS MEDIATED ON CYTOKINE PRODUCTION
Specific Aim 1: Isolate APCs from mice spleen and evaluate different APC to CD4⁺ fractions

Specific Aim 2: Generate cultures and supernatants
Spleen dissociation via collagenase and processing media or Spleen Dissociation Medium (Stem Cell®) with the use of frosted slides

Pour suspension through conical tube, centrifuge at 300g, 4 °C, discard supernatant, add either Ficoll gradient or 35% BSA gradient, centrifuge at 9,500g, 4 °C, remove upper layer of suspension, and prepare cells to count on Coulter Counter
Stain cells with appropriate antibodies

Place titer tube in flow tube with cells to analyze on the FC 500

OBJECTIVE

- Compared Ficoll gradient to a 35% BSA gradient to identify maximum generation of APCs in spleen extraction
ISOLATION OF APC USING FICOLL

Band from Ficoll Gradient is mostly B-cells

![Diagram showing lineage marker expression]
Band from 35% BSA has B-cells and higher concentrations of DCs.
35% BSA gradient results as best technique to enrich for APCs

Both 35% BSA and Ficoll gradients had similar selections for B-cells
Isolate CD4^+CD62L^+ cells and stain with CFSE
- Isolate CD11c^+ cells

Co-culture at 1:10, 1:5, 1:1, 5:1, 10:1 of CD4^+:APC
- Measure culture proliferation (CFSE) and activation (CD25) on day 3 via flow cytometry
NEITHER APC FRACTION SUPPORTED T-CELL PROLIFERATION

(A) example of cell proliferation (B) 10:1 (C) 5:1 (D) 1:1 (E) 1:5 (F) 1:10].
RESULTS

- Ratios of different CD4\(^+\):APC co-cultures evaluated for activation and proliferation of CD4\(^+\) T-cells
  - No proliferation is evident amongst the various ratio
CONCLUSION

- 35% BSA gradient resulted in a better selection of dendritic cells (DCs) than the Ficoll gradient, as measured by percent positive CD11c\(^+\) and B220 cells
  - DCs are the best activators for CD4\(^+\) cells

- Both 35% BSA and Ficoll gradients had similar selections for B-cells
No proliferation or activation was observed in any of the tested ratio’s

- Semi-allogeneic splenic APCs cannot activate B6 CD4⁺ T-cells alone, even though ~60% of the APC population was B220⁺ cell (B-cells)

More DCs need to be present for T-cell activation to occur

- Duffner et al, 2004 stated that naïve, host B-cells were insufficient to activate donor CD4⁺ T-cells
SUMMARY OF PHASE 1

Based on the CD4\(^+\):APC co-cultures experiment conducted, this model presents a difficulty to induce activation and/or proliferation via ex \textit{vitro} cultures.
Study cytokines by collecting supernatants from prior GvH experiments

- IFN-g and IL-10
QUANTIFICATION OF CYTOKINES VIA ELISA IN DAY 2 AND DAY 3 GvHS
What do we expect to see?

- Samples obtained from animals dosed with TCDD should contain higher levels of IFN-\( \gamma \) and IL-10 than samples from vehicle-treated animals.
Following a day 2 or day 3 GvH, splenocytes (1x10^7) from host mice (B6D2F1) were cultured overnight.

- Cells were spun down and supernatant collected and flash frozen in liquid nitrogen.
- Stored at -80°C until needed.
ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) PROCESS

A Bind sample to support
B Add primary antibody; wash
C Add secondary antibody-enzyme conjugate; wash
D Add substrate
Day 1
- Plate coated with capture antibody in Capture Buffer and sealed overnight at 4°C

Day 2
- Aspirated and washed with Wash Buffer 5X
- Blocked with Assay Diluent, incubated at room temp. 1 hr
- Aspirated and washed with Wash Buffer 5X
- Dilute standards in Assay Diluent and samples added
- Plate sealed overnight at 4°C

Day 3
- Aspirated and washed with Wash Buffer 5X
- Detection enzyme, Avidin-HRP, added
- Aspirated and washed with Wash Buffer 7X
- Substrate solution, Tetramethylbenzidine (TMB), added, incubated at room temp. 15min
- Add stop solution / read plate at 450nm in spectrometer
Phase 2: Part 1

QUANTIFICATION OF IFN-γ VIA ELISA IN DAY 2 AND DAY 3 GvHS
ELISA IFN-y standard curve to quantify sample protein levels (pg/ml).
Standard Curve

Day 2 GvH

Day 3 GvH
Production of IFN-γ protein levels in both VEH and TCDD were measured on supernatants from a day 2 GvH
Supernatants from VEH and TCDD were quantified on a day 3 GvH for IFN-γ.

*Difference from VEH to TCDD-treated mice (p=0.011)
QUANTIFICATION OF IL-10 VIA ELISA IN DAY 2 AND DAY 3 GvHS
ELISA IL-10 standard curve to measure sample protein levels (pg/ml).
DAY 2 GvH

- Production of IL-10 in both VEH and TCDD were measured on supernatants from a day 2 GvH

- No difference between treatment groups
Supernatants from VEH and TCDD were quantified on a day 3 GvH for IL-10.

No significant difference between treatment groups.
CONCLUSION
Day 2 GvH production of IFN-γ
- IFN-γ protein levels similar between TCDD and VEH-treated mice

Day 3 GvH production of IFN-γ
- Increased IFN-γ protein levels in TCDD-treated compared to VEH
  - Statistically significant (p=0.011)
- TCDD up-regulated IFN-γ production

CONCLUSION
- IFN-\(\gamma\) consistent with prior studies
  - IFN-\(\gamma\) production increased in the presence of co-cultured T-cells and semi-allogeneic DCs in TCDD-treated mice from day 2 to day 3
  - Marshall et al, 2005
    - No significant difference day 2
- IL-10 production not influenced by treatment or time point of GvH
  - Difference in methods preparation of splenocyte cultures
    - *In vitro* method
- Inconsistent with Apetoh et al, 2010
  - AhR stimulates secretion of IL-10 during the differentiation of Type 1 regulatory T-cells
OVERALL CONCLUSION

- APC Initial Co-Culture
  - Incomplete
- Cytokines in GvH Supernatants
  - IL-10
    - No difference between VEH and TCDD-treatment
  - IFN-γ
    - No difference on Day 2
    - Significant difference on Day 3, TCDD-treatment induced
Look at IL-2 production on day 2 and day 3 GvH
- IL-2 inhibits IFN-γ production
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Credit: Nicole Nasholm
QUESTIONS?
WORKS CITED


Single cells analyzed one at a time as they flow past a laser beam
TCDD has been shown to inhibit T-cell responsiveness in experimental models such as

- Humoral responses to SRBC and OVA
  - Kerkvliet et al, 1990
- Cell-mediated CTL response to allogeneic tumor cells
  - Kerkvliet et al, 1991
- Adoptive transfer model
  - T-cells from one mouse strain and put them into a different strain
  - Shepherd et al, 2000
Cytokines

- Located in the supernatant
- Released by cells

Supernatant

BODY PARTS THAT CAN BE AFFECTED BY AUTOIMMUNE DISEASES

- Brain
- Eyes
- Mouth
- Spinal Cord
- Thyroid
- Lung
- Stomach
- Joints
- Pancreas
- Large Intestine
- Small Intestine
- Bladder
- Uterus
- Ovary
- Cervix
- Others: Glands, Muscles, Nerves, Trachea, Blood and Blood Vessels, Heart, Skin, Esophagus, Liver, Kidney
### Types of Autoimmune Diseases and Their Symptoms

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Symptoms</th>
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<tr>
<td><strong>Diabetes type 1</strong></td>
<td>• Being very thirsty&lt;br&gt;• Urinating often&lt;br&gt;• Feeling very hungry or tired&lt;br&gt;• Losing weight without trying&lt;br&gt;• Dry, itchy skin&lt;br&gt;• Losing the feeling in your feet or having tingling in your feet&lt;br&gt;• Having blurry eyesight</td>
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<td>A disease in which your immune system attacks the cells that make insulin, a hormone needed to control blood sugar levels. As a result, your body cannot make insulin. Without insulin, too much sugar stays in your blood. Too high blood sugar can hurt the eyes, kidneys, nerves, and gums and teeth. But the most serious problem caused by diabetes is heart disease.</td>
<td><a href="http://www.womenshealth.gov">www.womenshealth.gov</a></td>
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<td><strong>Addison’s Disease</strong></td>
<td>• Muscle weakness and fatigue&lt;br&gt;• Weight loss and decreased appetite&lt;br&gt;• Darkening of your skin (hyperpigmentation)&lt;br&gt;• Low blood pressure, even fainting&lt;br&gt;• Salt craving&lt;br&gt;• Low blood sugar (hypoglycemia)&lt;br&gt;• Nausea, diarrhea or vomiting&lt;br&gt;• Muscle or joint pains&lt;br&gt;• Irritability&lt;br&gt;• Depression</td>
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<td>A disorder that occurs when your body produces insufficient amounts of certain hormones produced by your adrenal glands... adrenal glands produce too little cortisol and often insufficient levels of aldosterone as well... occurs in all age groups and affects both sexes. Addison's disease can be life-threatening.</td>
<td><a href="http://www.mayoclinic.com">www.mayoclinic.com</a></td>
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