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

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IMMUNOTOXICOLOGY

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

TOXIN TO POTENTIAL HEALTH MODEL

- 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

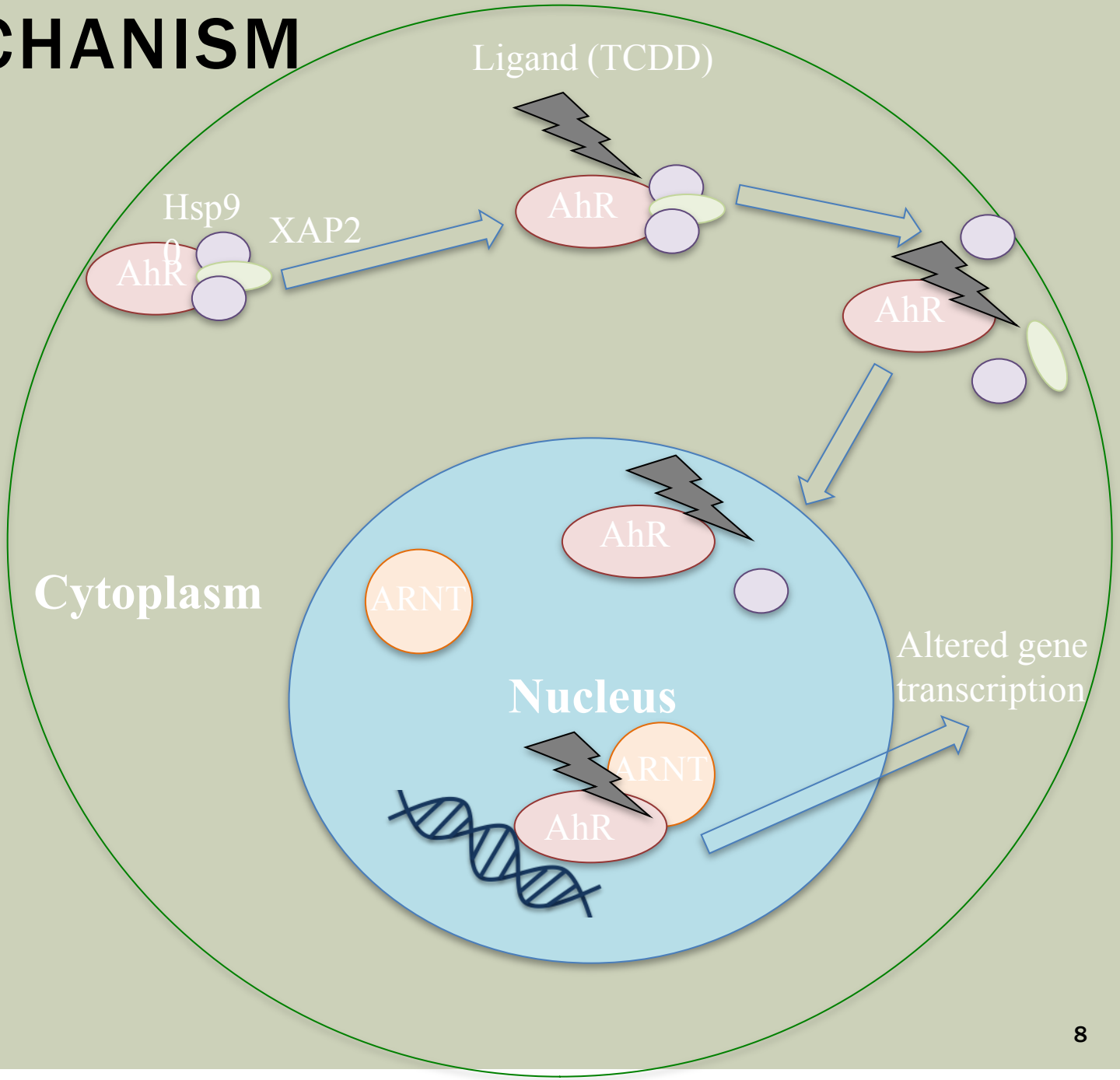
http://celebritiesfamous.com/venus-williams.html/venus-williams-18

MECHANISM OF IMMUNOSUPPRESSION

- 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

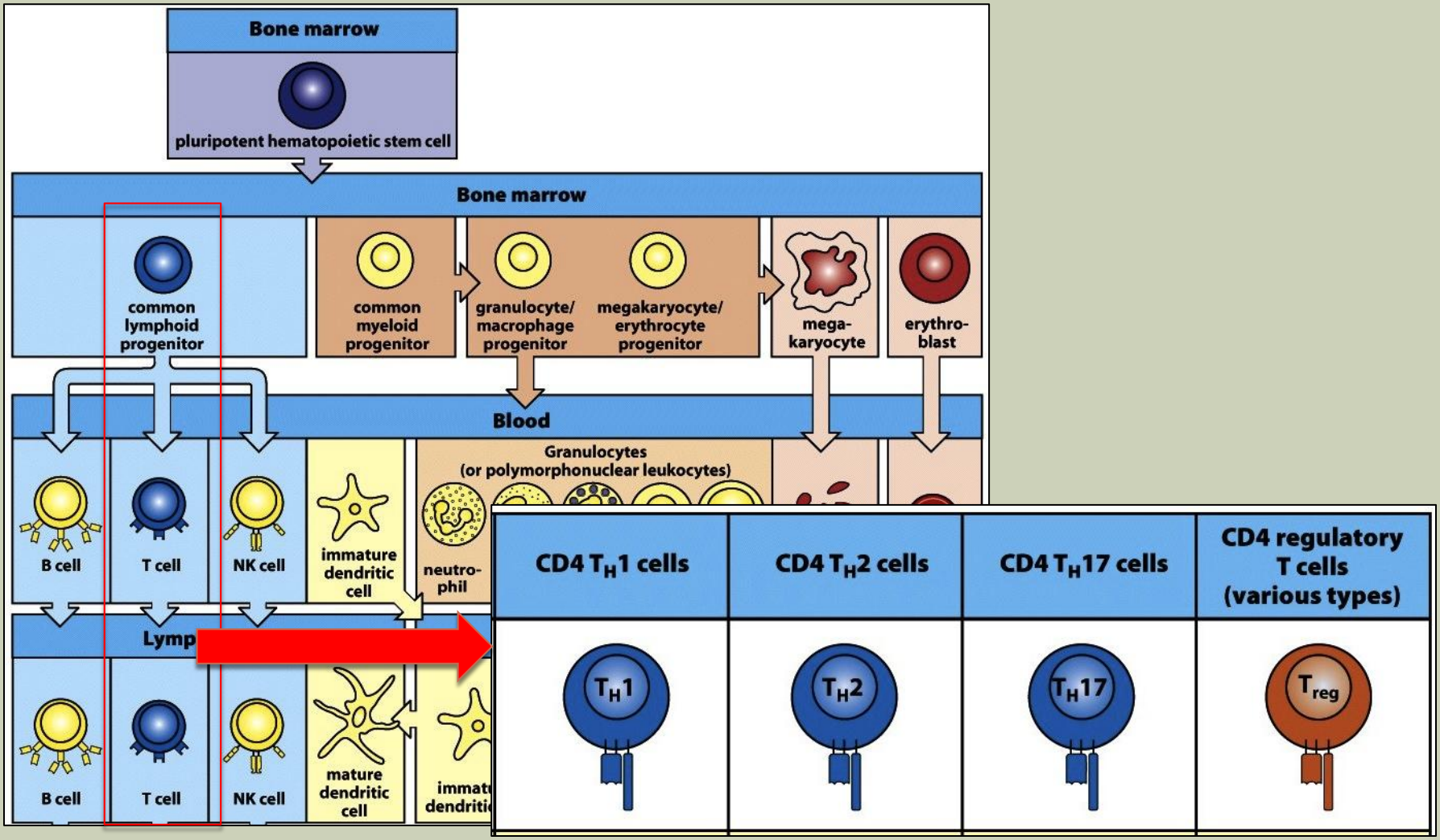
- High level exposure to TCDD causes toxic and pathological effects
- One aspect of the Kerkvliet lab is to screen for other AhR ligands as AhR non-activation increases immune response

Viktor Yushchenko travel.webshots.com

GVH STUDIES IMPROVE UNDERSTANDING OF AUTOIMMUNE RESPONSE

- Graft versus Host Response Model
  - GvH mechanism

IMMUNE RESPONSE



HOW THE GvH MODEL WORKS

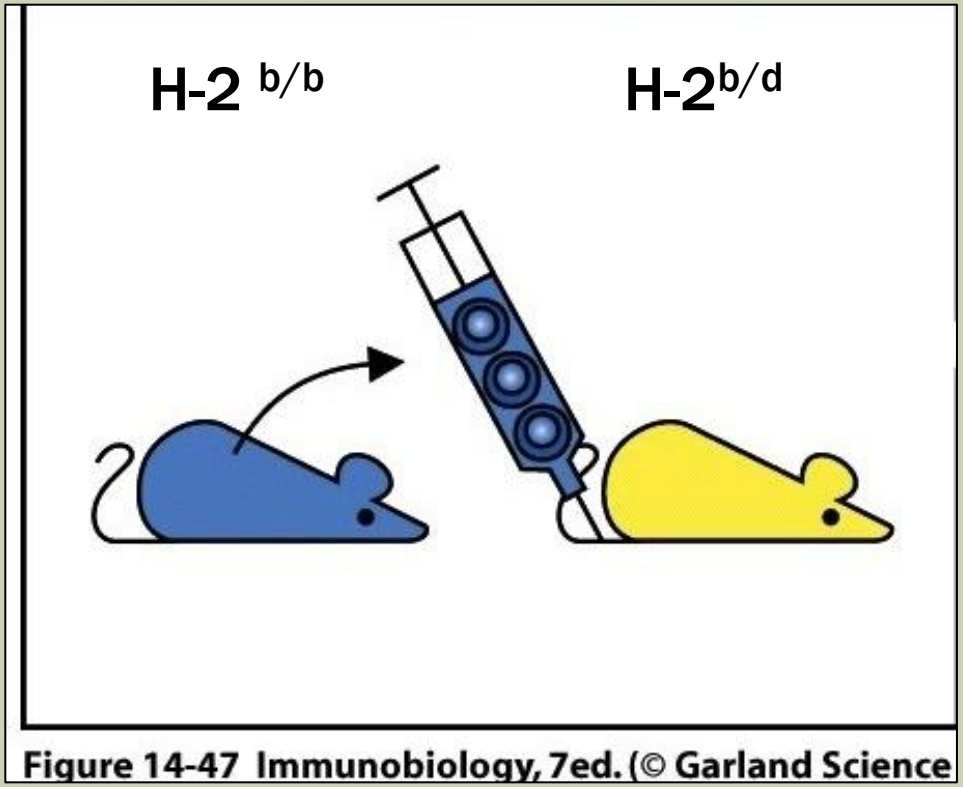
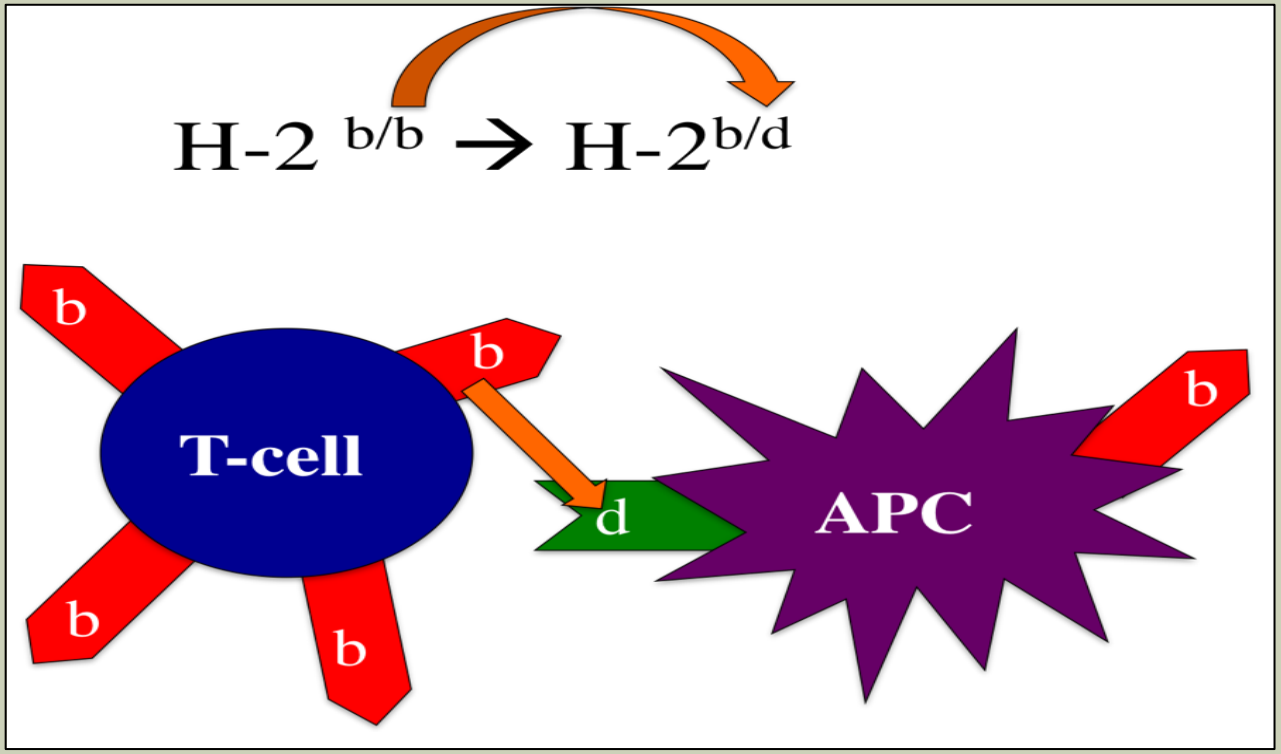


Figure 14-47 Immunobiology, 7ed. (© Garland Science

- Donor $CD4^+$ and $CD8^+$ T-cells from a C57Bl/6 ($H\text{-}2^{b/b}$) mouse are transferred into a host B6D2F1 ($H\text{-}2^{b/d}$) mouse

HOW THE GvH MODEL WORKS

- The “b” haplotype from the T-cell recognizes the “d” haplotype from the APC as foreign



CYTOTOXIC T-LYMPHOCYTE RESPONSE

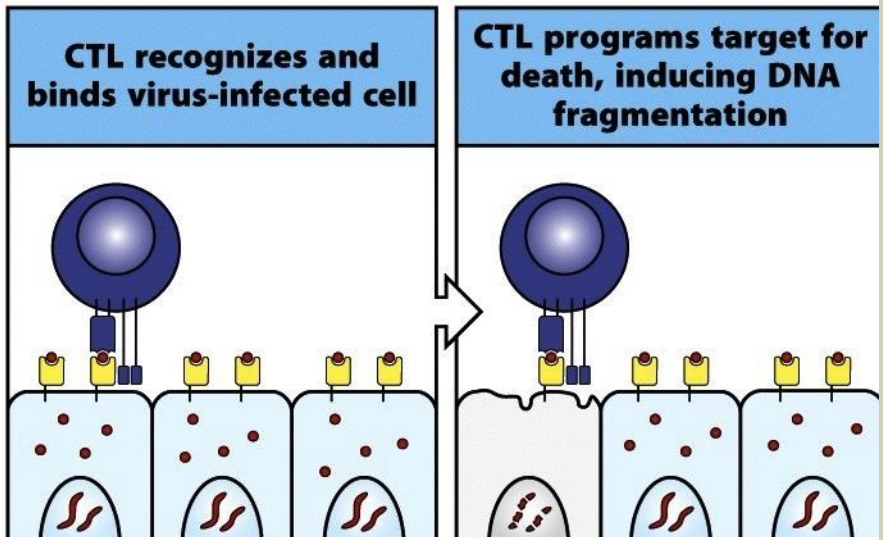
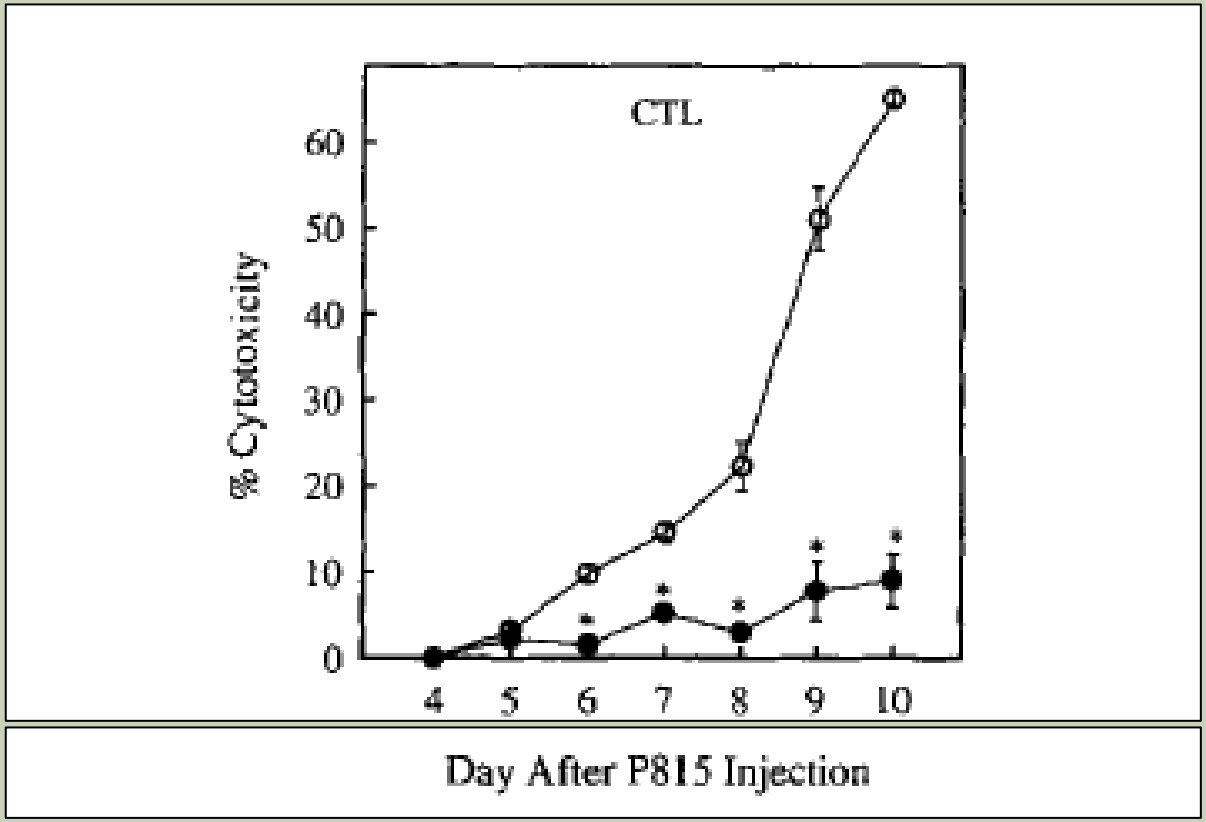


Figure 8-36 Immunobiology, 7ed. (© Garland Science 2008)

TCDD IN THE MODEL

TCDD suppresses the CTL Response



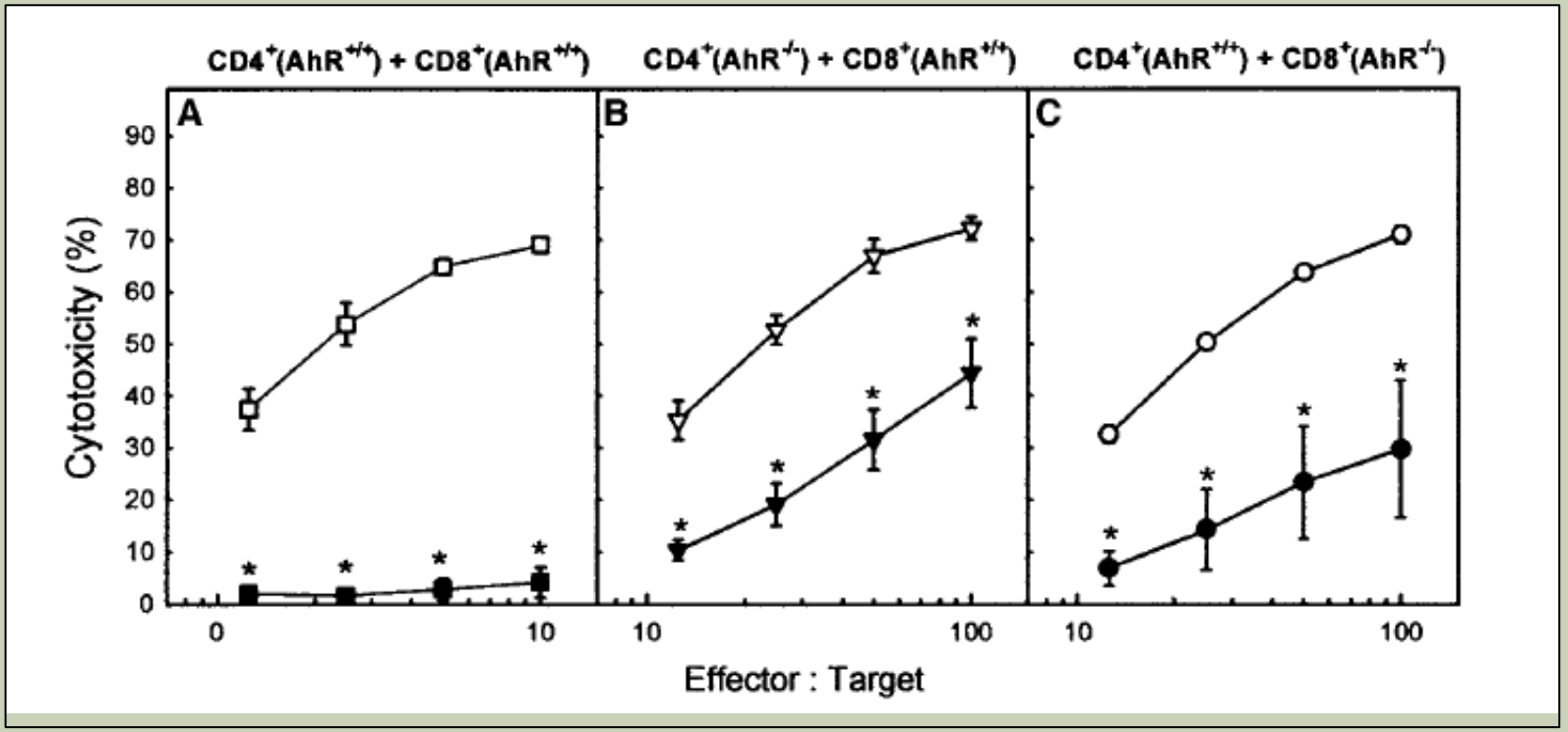
Key

TCDD black circles

VEH white circles

Kerkvliet et al. 1996

HOW THE AHR WORKS



Key

TCDD black shapes

VEH white shapes

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

REGULATOR T CELLS

- 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)

GENERAL PURPOSE OF STUDY

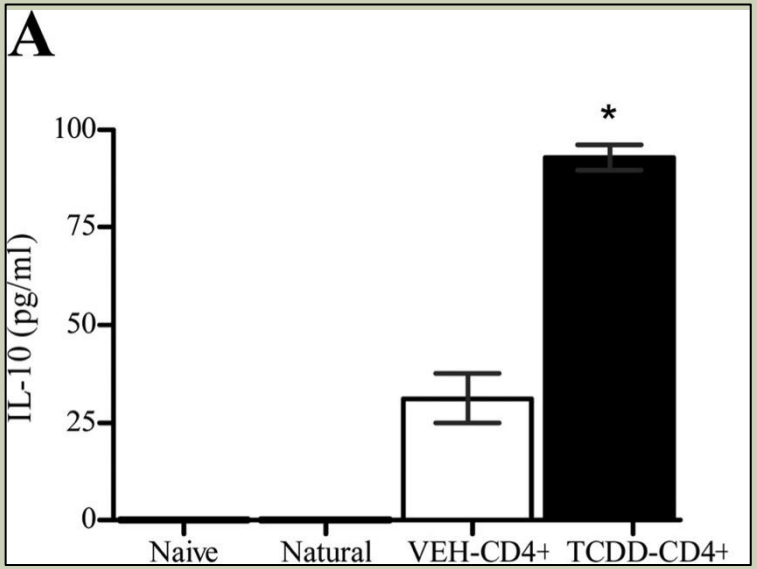
- How does TCDD work?
  - Induce certain cytokines, so we will look at them in GvH model in day 2 or day 3
    - Inject day 0 and day 2 or day 3 look at response

OBJECTIVE

- 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 INTERLUEKIN-10 (IL-10)?

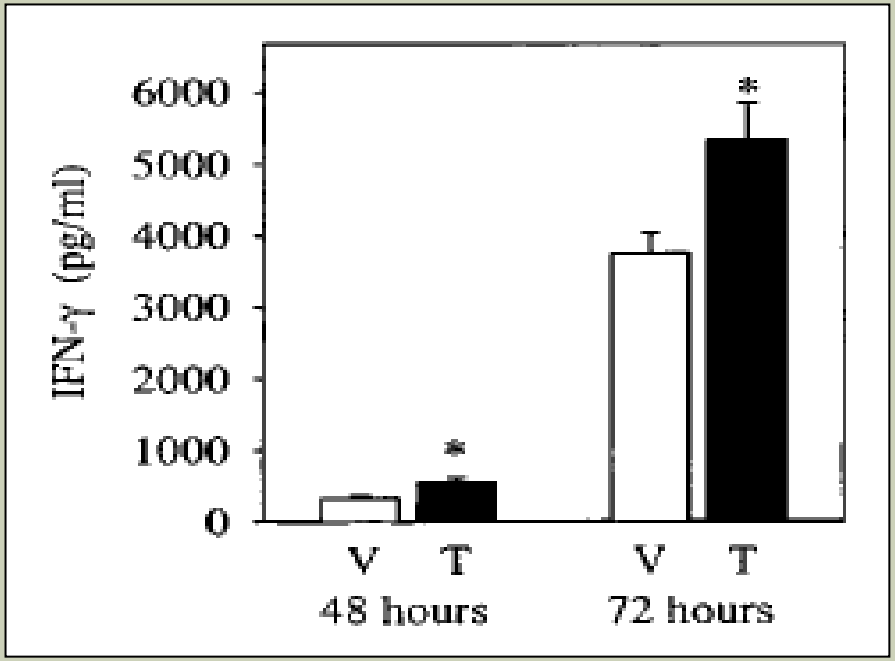
- IL-10 is anti-inflammatory
- Marshall et al, 2008 study found that TCDD-CD4+ cells secreted significant amounts of IL-10 when supernatants were harvested at 72 hours



TCDD-CD4+ cells produced more IL-10 than other treatment groups (*t* tests; *, $p < 0.05$)

WHY INTERFERON-GAMMA (IFN-ϒ)?

- IFN-g can have both pro-inflammatory and immunosuppressive effects
- Vorderstrasse et al, 2001, observed an increase in IFN-ϒ production



IFN-ϒ production in host DCs following treatment with TCDD (4).

PHASE 1

DEVELOPMENT OF AN *IN VITRO* MODEL TO TEST EFFECT OF TCDD ON CYTOKINES: APC STUDIES

Phase 1

MIXED LYMPHOCYTE REACTION (MLR)

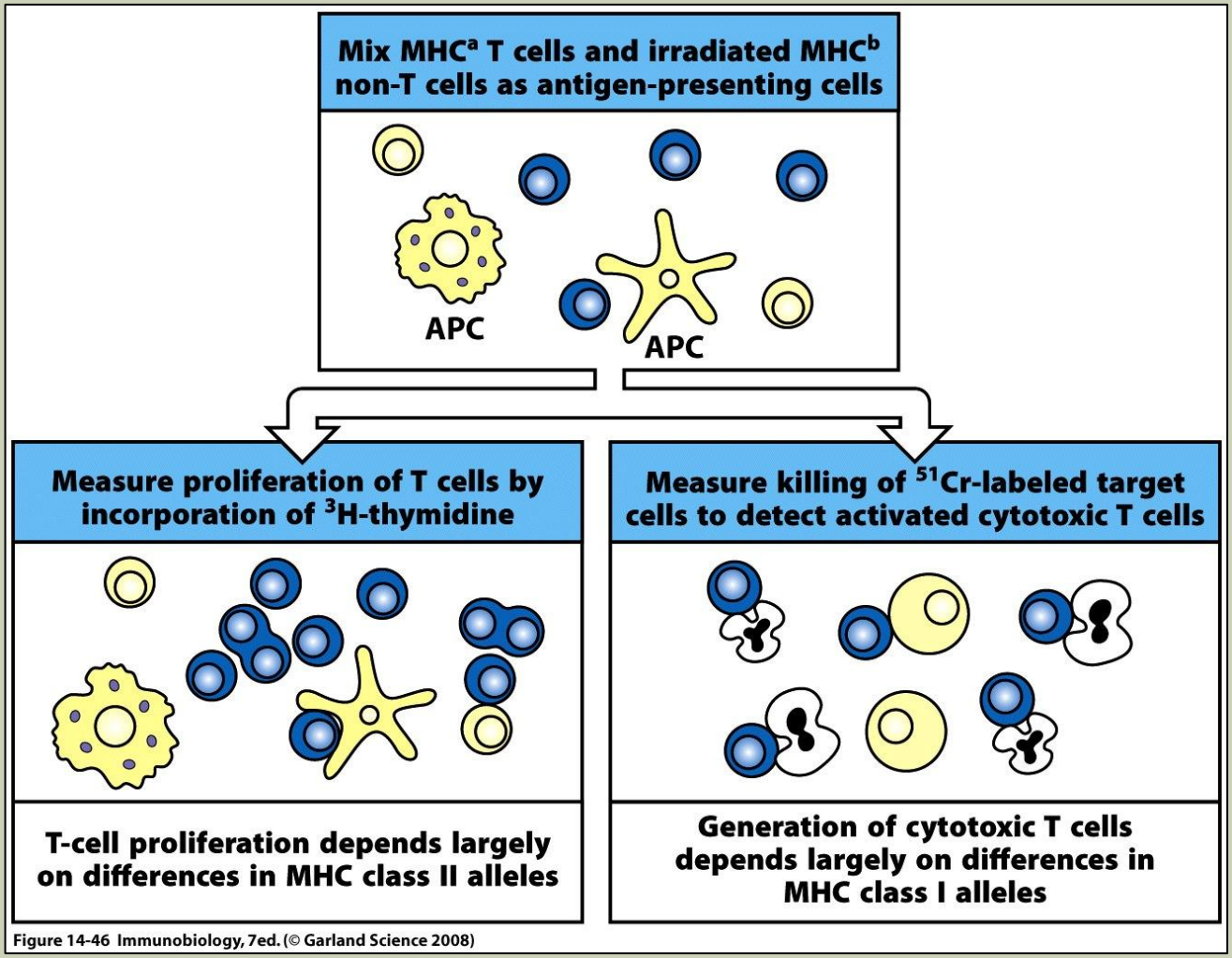
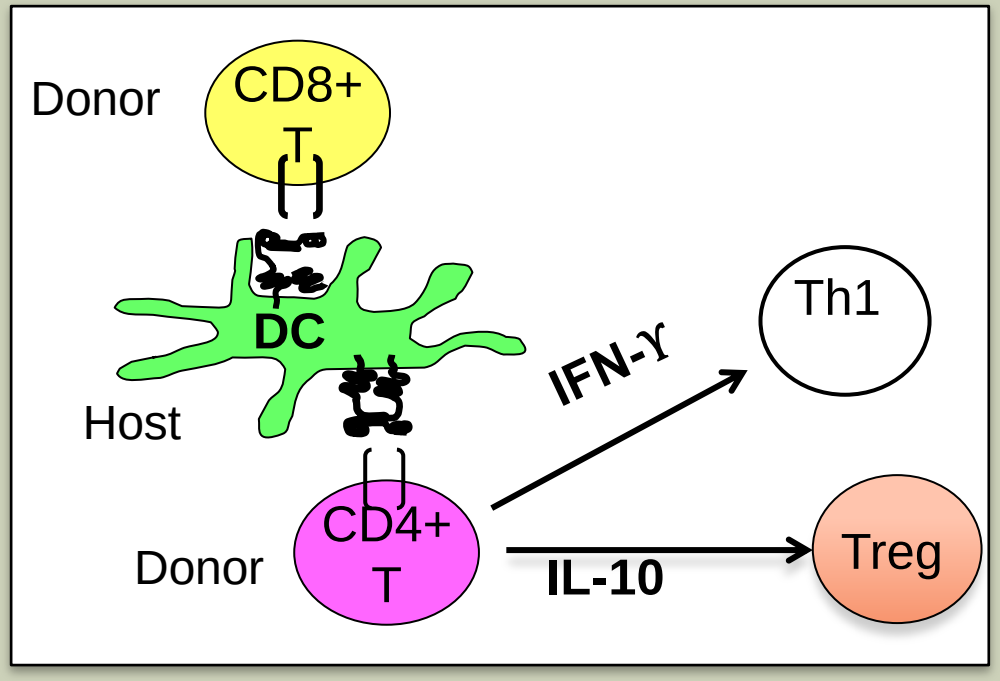


Figure 14-46 Immunobiology, 7ed. (© Garland Science 2008)

WHY ANTIGEN PRESENTING CELLS?

- Antigen Presenting Cells (APCs) present antigen to other immune cells
  - Types: dendritic cells (DC), macrophages, B-cells
  - DCs are the best type of APCs



OBJECTIVE: IDENTIFY TCDD-EFFECTS MEDIATED ON CYTOKINE PRODUCTION

- To prepare antigen presenting cells (APC) that would activate $CD4^+$ T-cells *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

AIMS

- *Specific Aim 1:* Isolate APCs from mice spleen and evaluate different APC to $CD4^+$ fractions
- *Specific Aim 2*: Generate cultures and supernatants

ANTIGEN-PRESENTING CELLS (APC) ISOLATION

- 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

FLOW CYTOMETRY

- Stain cells with appropriate antibodies
- Place titer tube in flow tube with cells to analyze on the FC 500

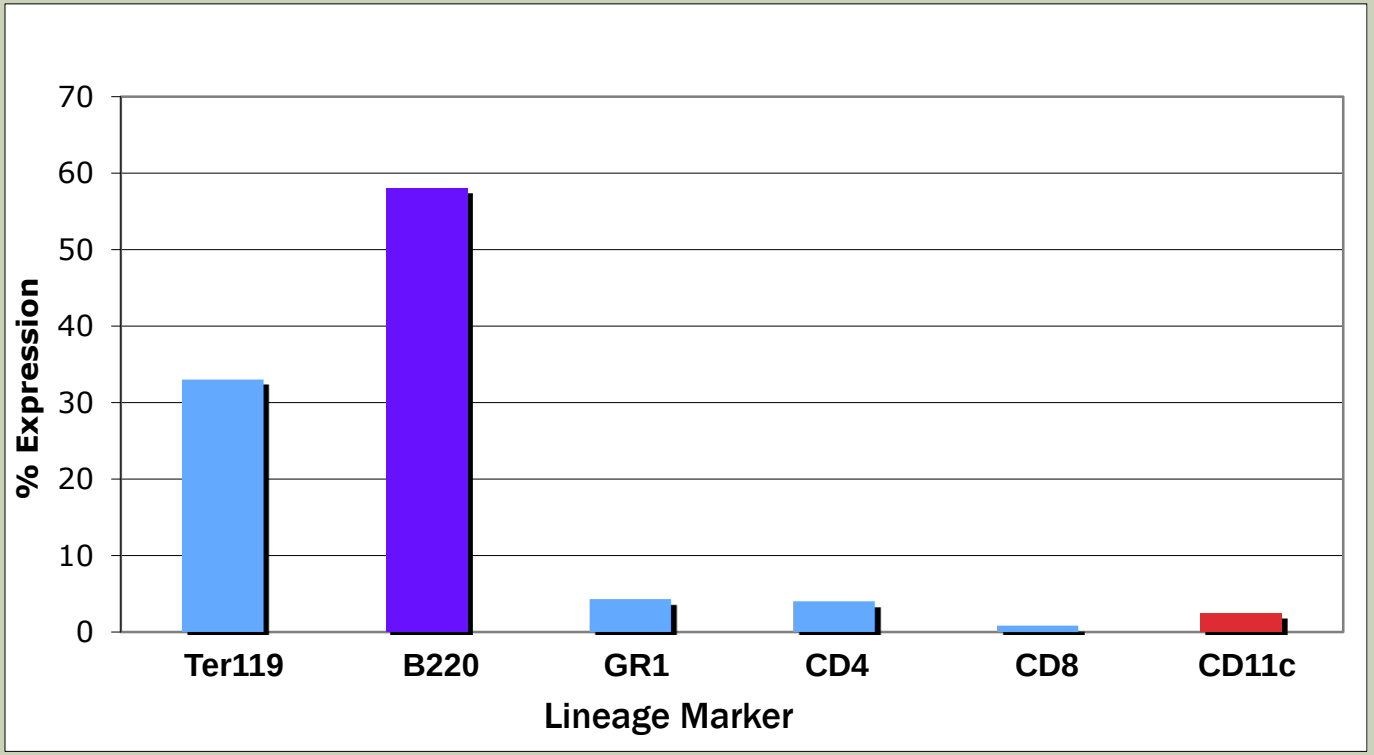
http://www.immunotox.com/Facilities/FlowCytometer.html

OBJECTIVE

- Compared Ficoll gradient to a 35% BSA gradient to identify maximum generation of APCs in spleen extraction

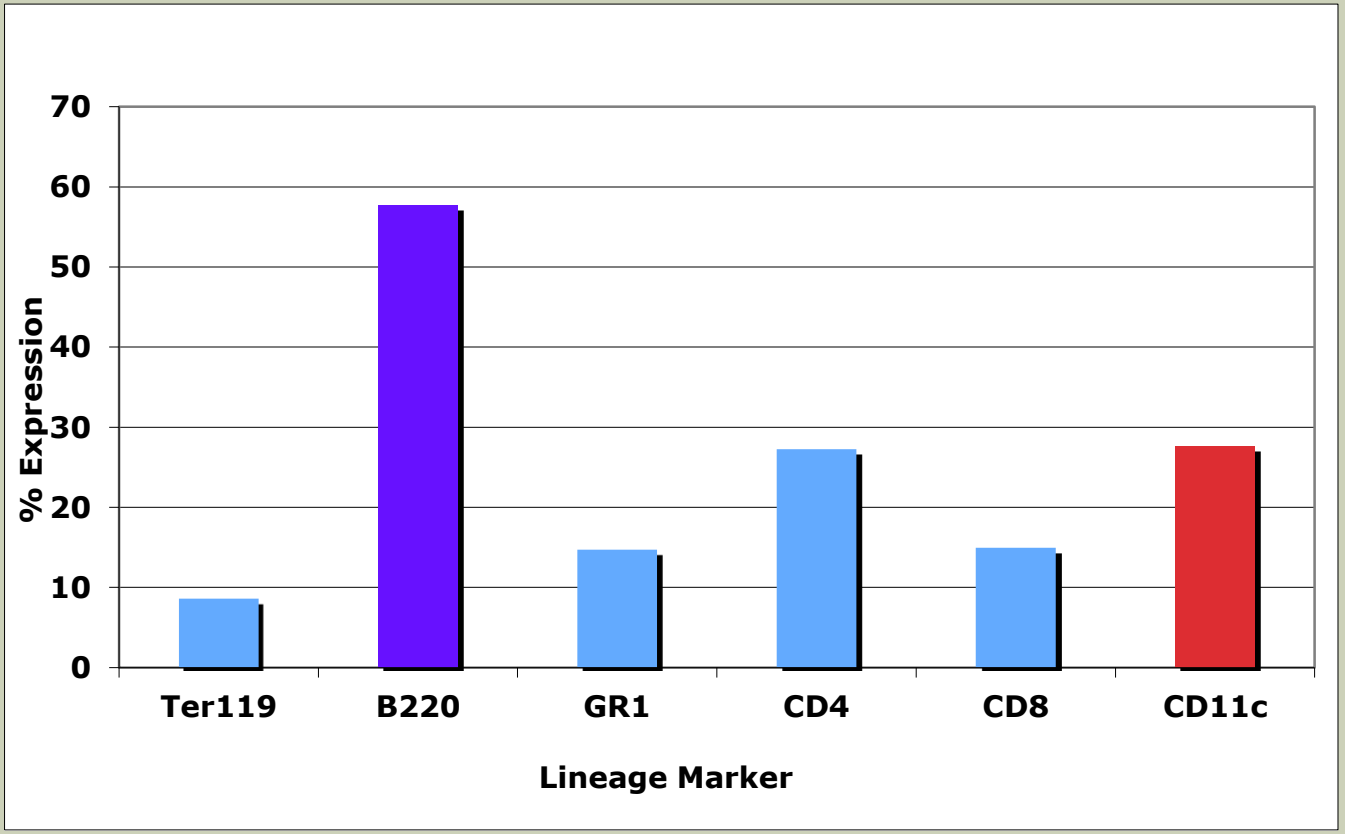
ISOLATION OF APC USING FICOLL

Band from Ficoll Gradient is mostly B-cells



ISOLATION OF APC USING 35% BSA

Band from 35% BSA has B-cells and higher concentrations of DCs



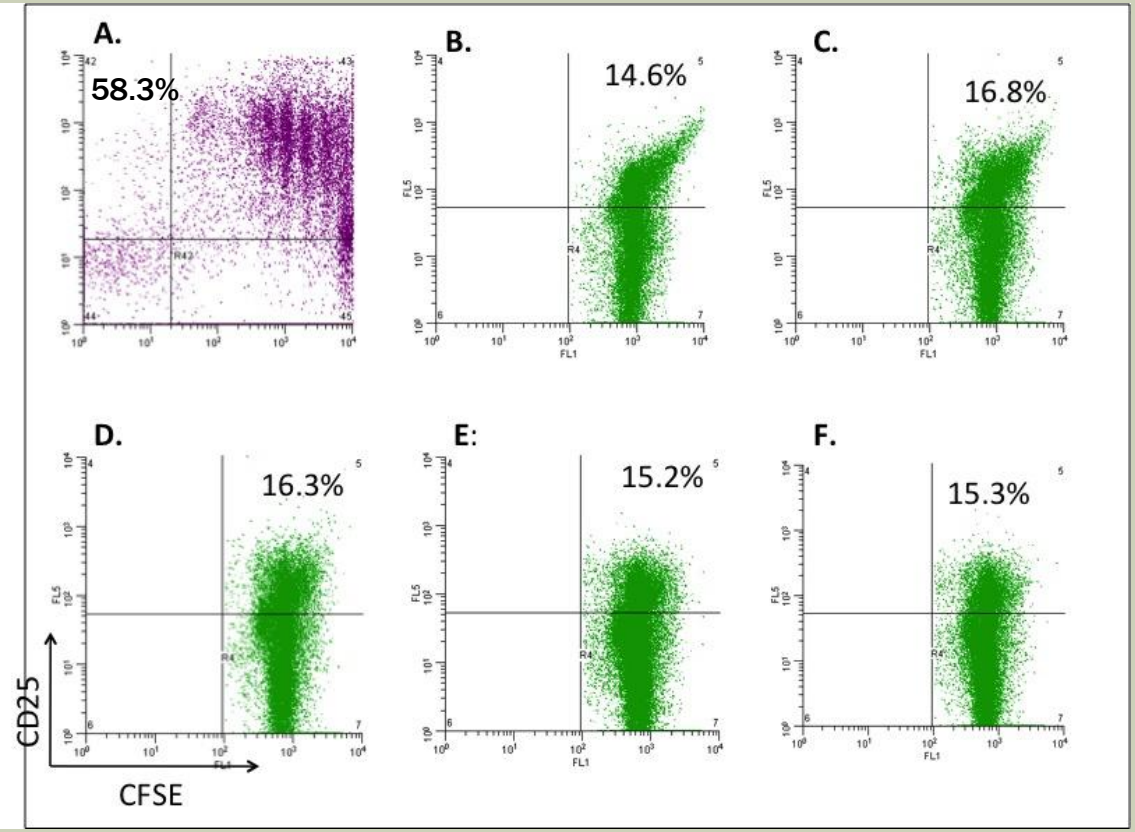
RESULTS

- 35% BSA gradient results as best technique to enrich for APCs
- Both 35% BSA and Ficoll gradients had similar selections for B-cells

APC INITIAL CO-CULTURES

- 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

CONCLUSION

- 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 vitro* cultures

NEXT STEP

- 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

Phase 2

PREDICTIONS

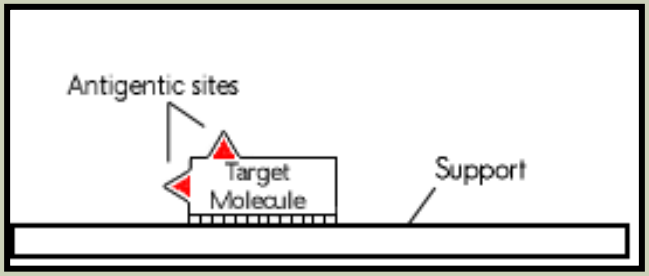
- What do we expect to see?
  - Samples obtained from animals dosed with TCDD should contain higher levels of IFN-ϒ and IL-10 than samples from vehicle-treated animals

PREPARATION OF SUPERNATANTS

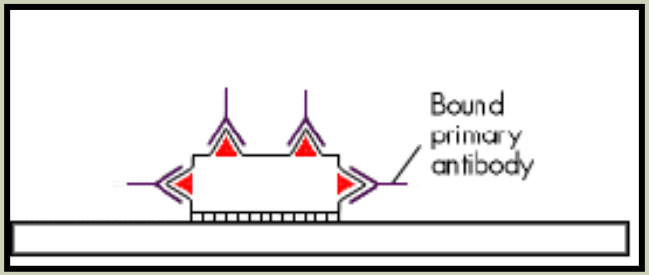
- Following a day 2 or day 3 GvH, splenocytes ($1x10^7$) from host mice (B6D2F1) were cultured overnight
  - Cells were spun down and supernatant collected and flash frozen in liquid nitrogen
- Stored at -80˚C until needed

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) PROCESS

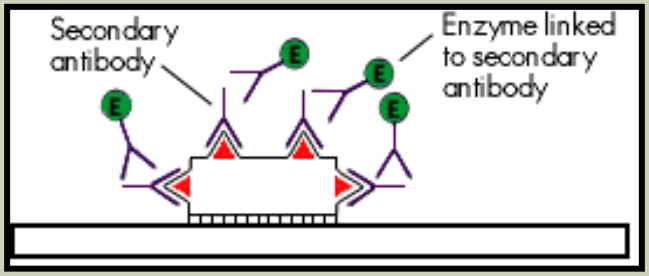
A



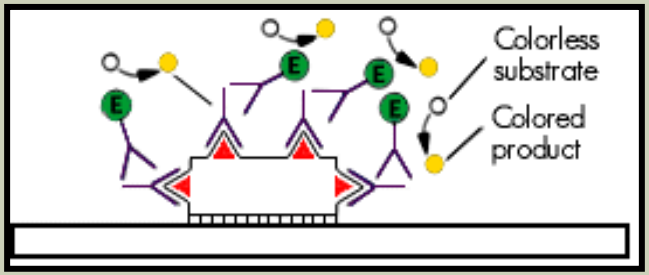
B



C



D



A Bind sample to support

B Add primary antibody; wash

C Add secondary antibody-enzyme conjugate; wash

D Add substrate

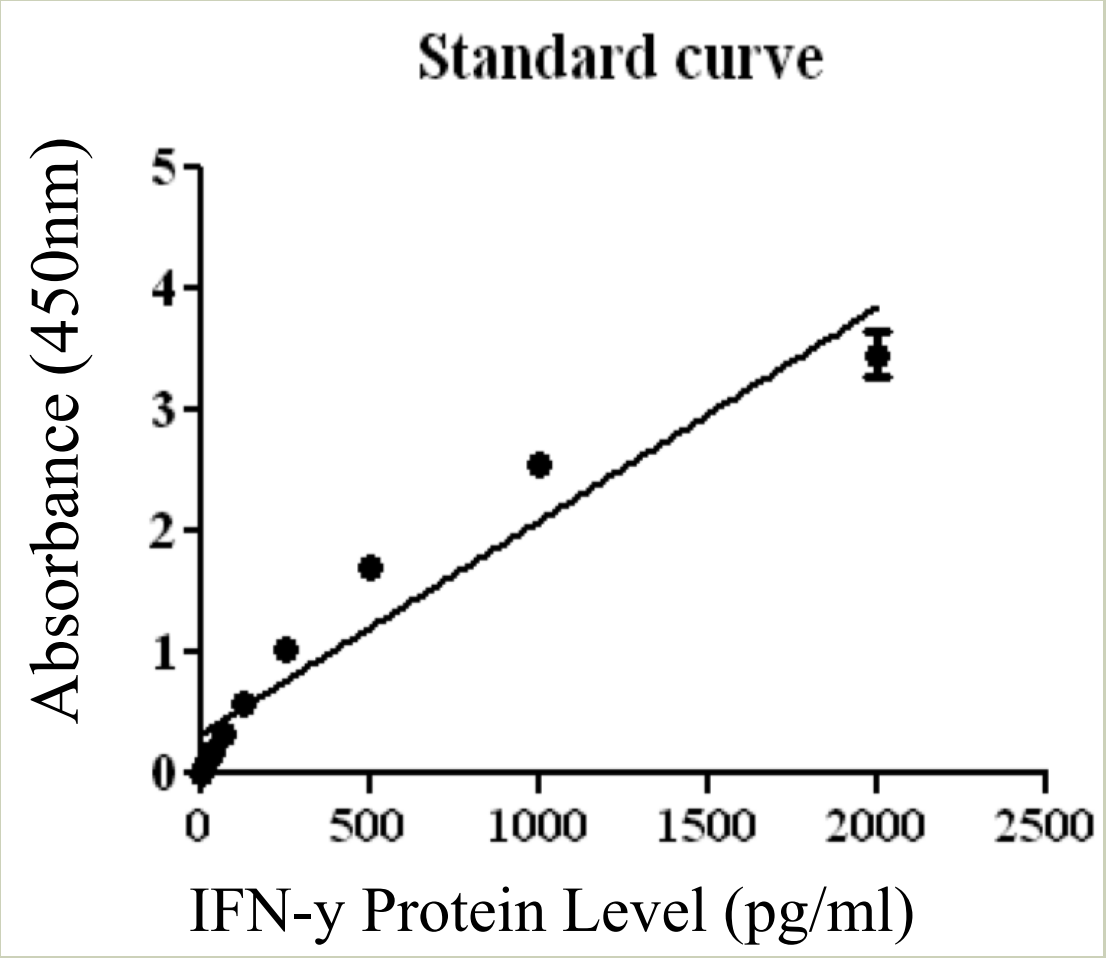
THE EBIOSCIENCE© ELISA READY-SET-GO™ KIT

- 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

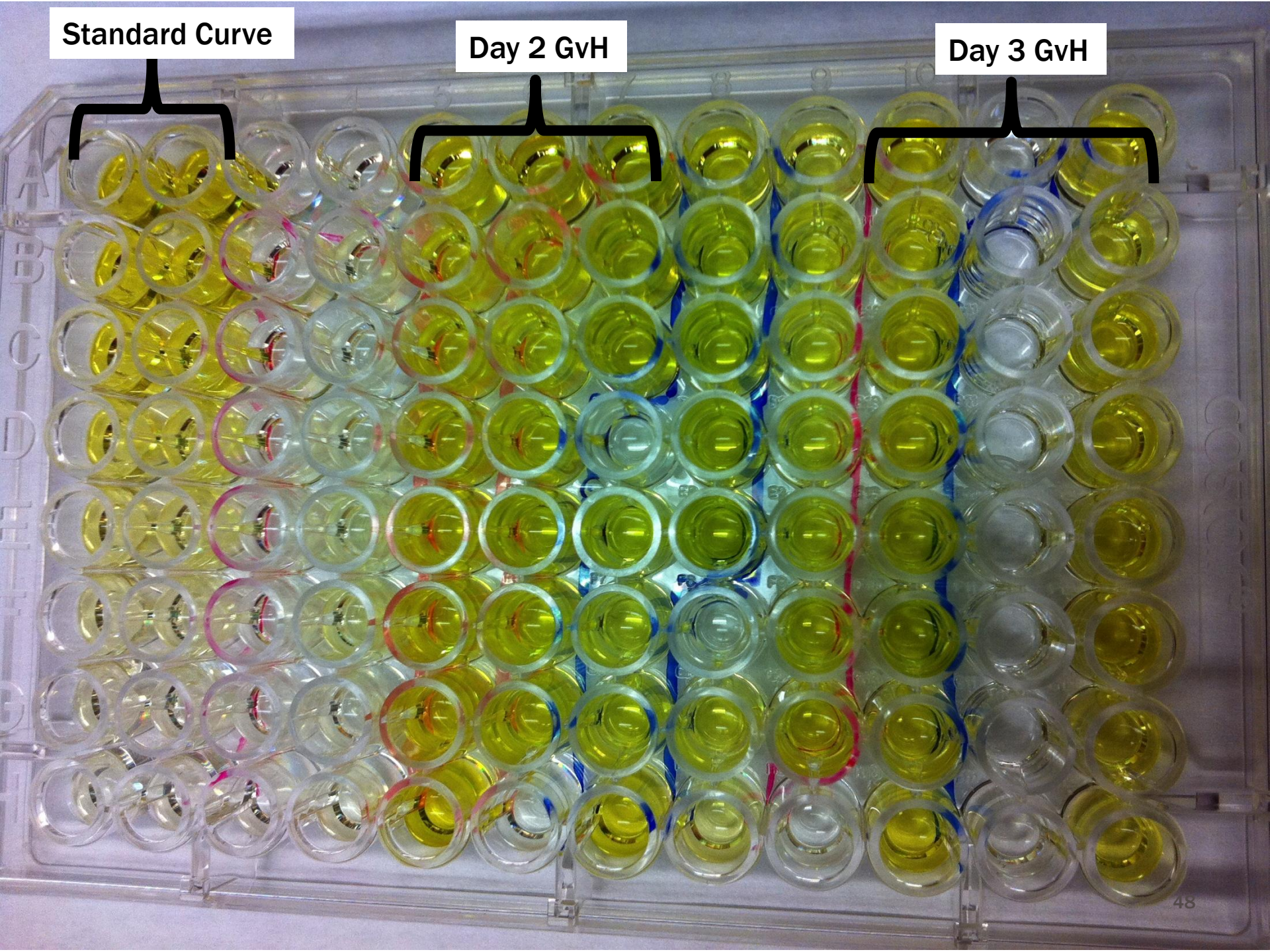
QUANTIFICATION OF IFN-ϒ VIA ELISA IN DAY 2 AND DAY 3 GvHS

Phase 2:
Part 1

CYTOKINE ANALYSIS

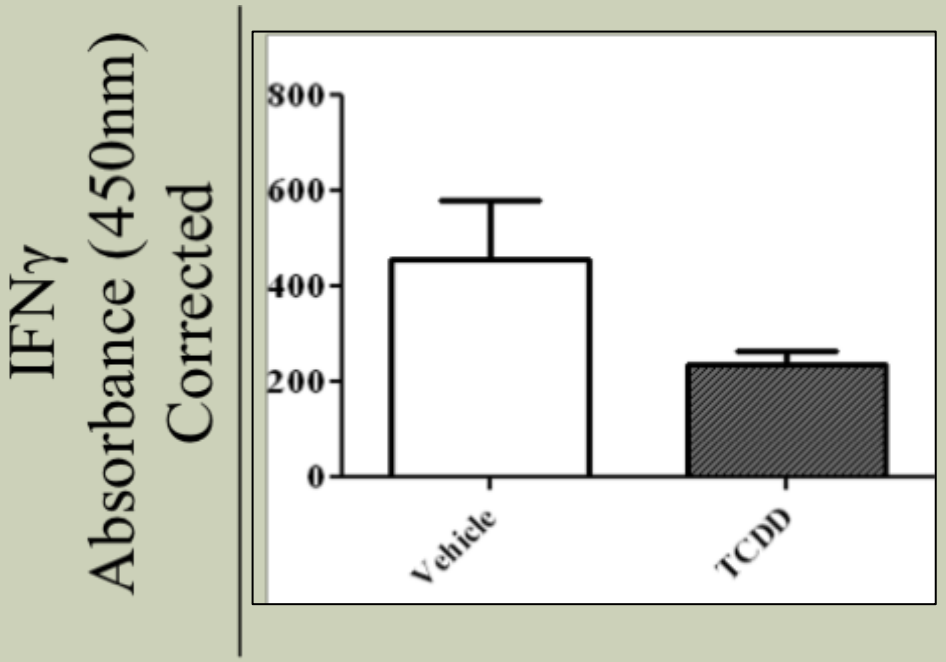


ELISA IFN-y standard curve to quantify sample protein levels (pg/ml).


Standard Curve
Day 2 GvH
Day 3 GvH

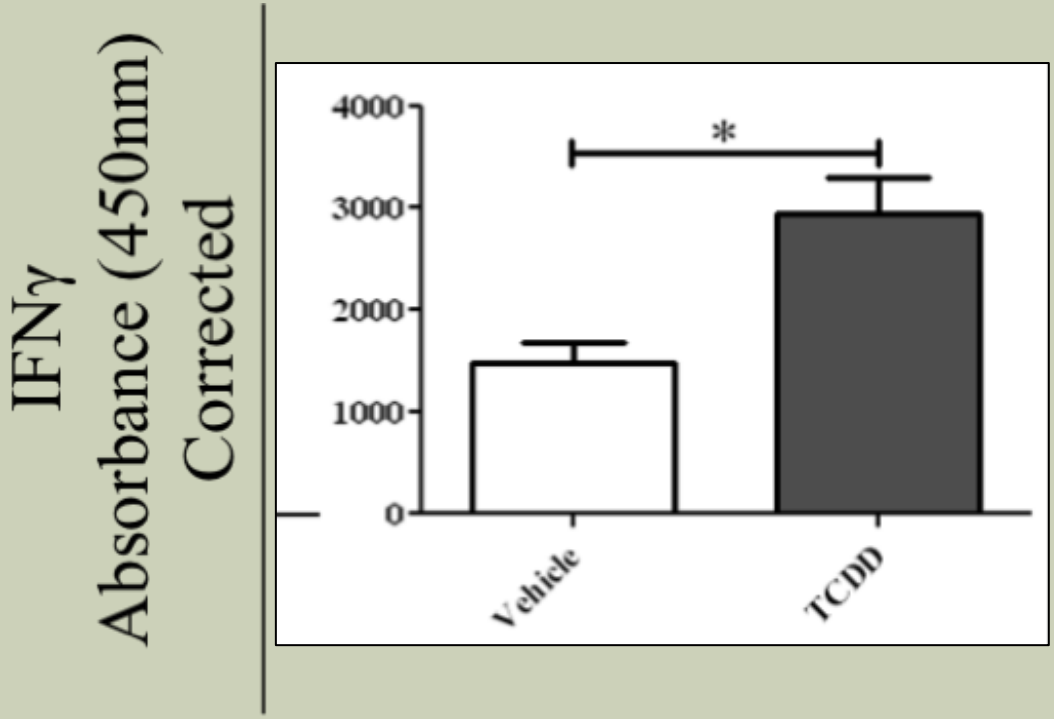
DAY 2 GvH

- Production of IFN-ϒ protein levels in both VEH and TCDD were measured on supernatants from a day 2 GvH



DAY 3 GvH

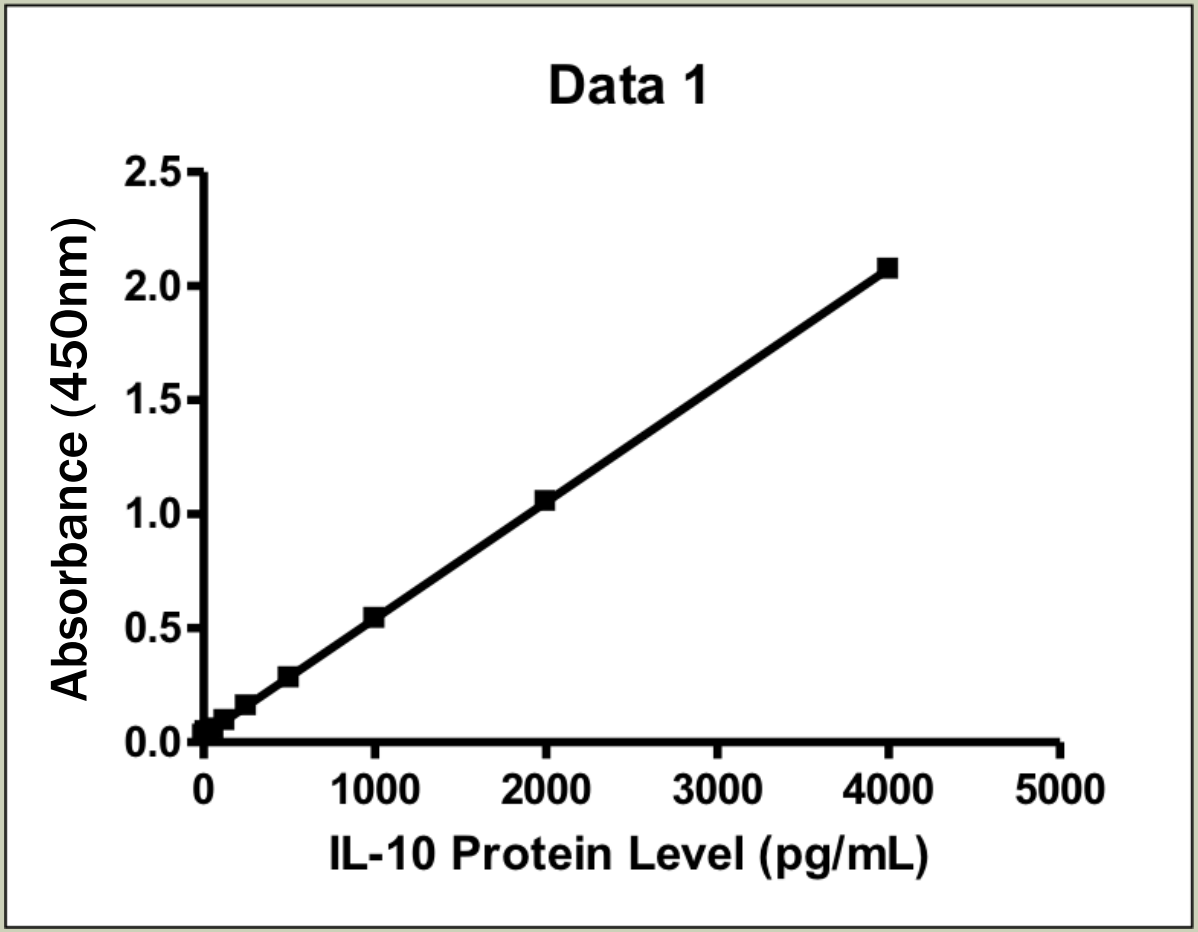
- Supernatants from VEH and TCDD were quantified on a day 3 GvH for IFN-ϒ
  - *Difference from VEH to TCDD-treated mice (p=0.011)



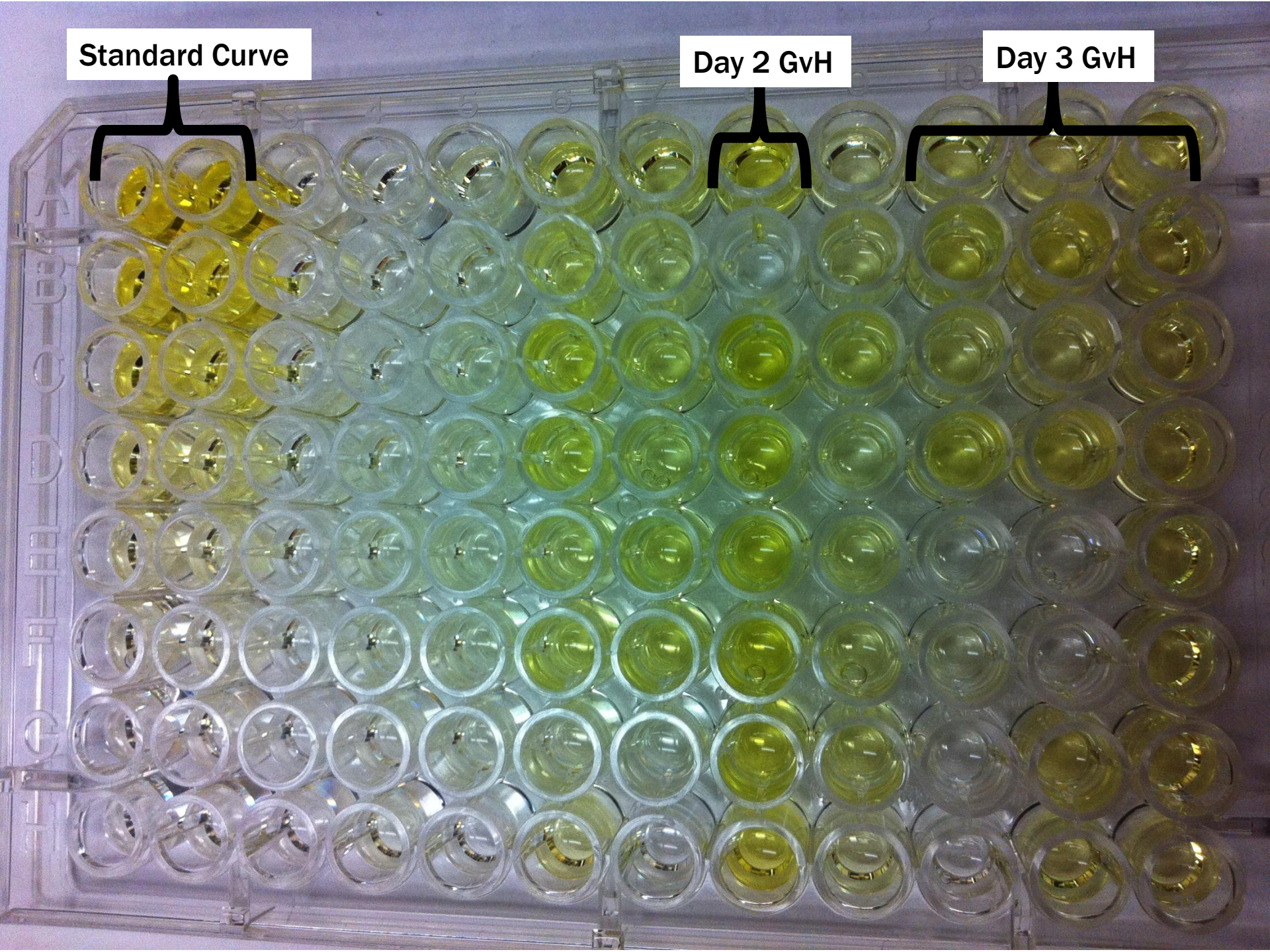
QUANTIFICATION OF IL-10 VIA ELISA IN DAY 2 AND DAY 3 GvHS

Phase 2: Part II

CYTOKINE ANALYSIS

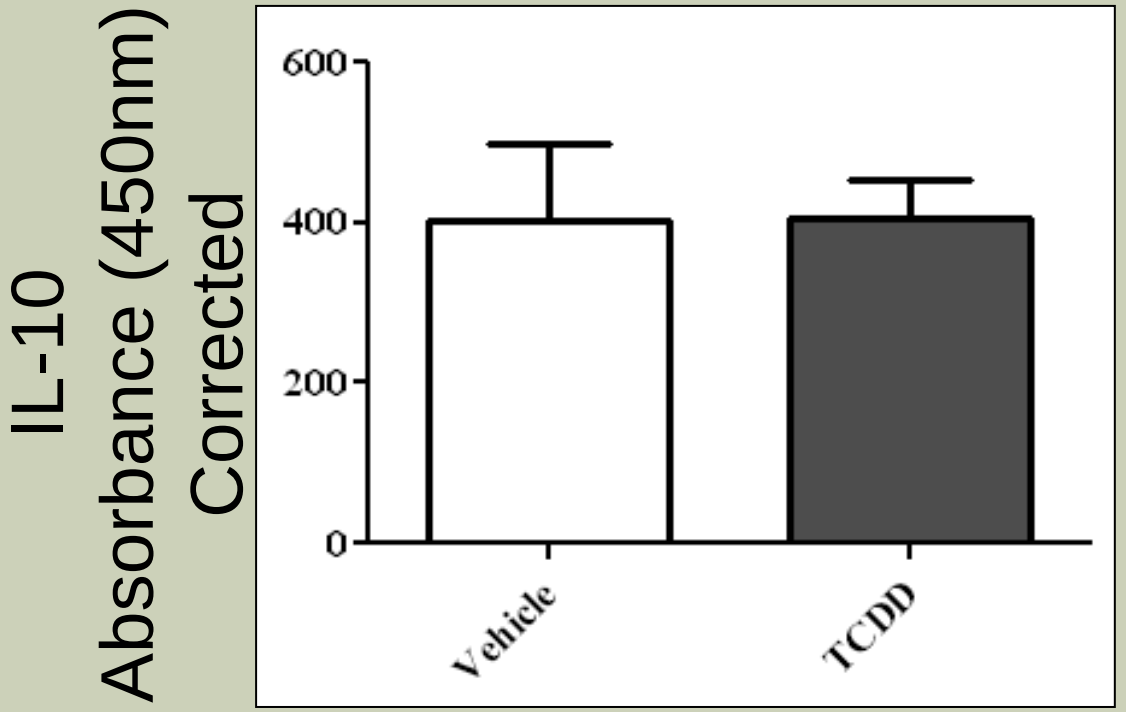


ELISA IL-10 standard curve to measure sample protein levels (pg/ml).


Standard Curve
Day 2 GvH
Day 3 GvH

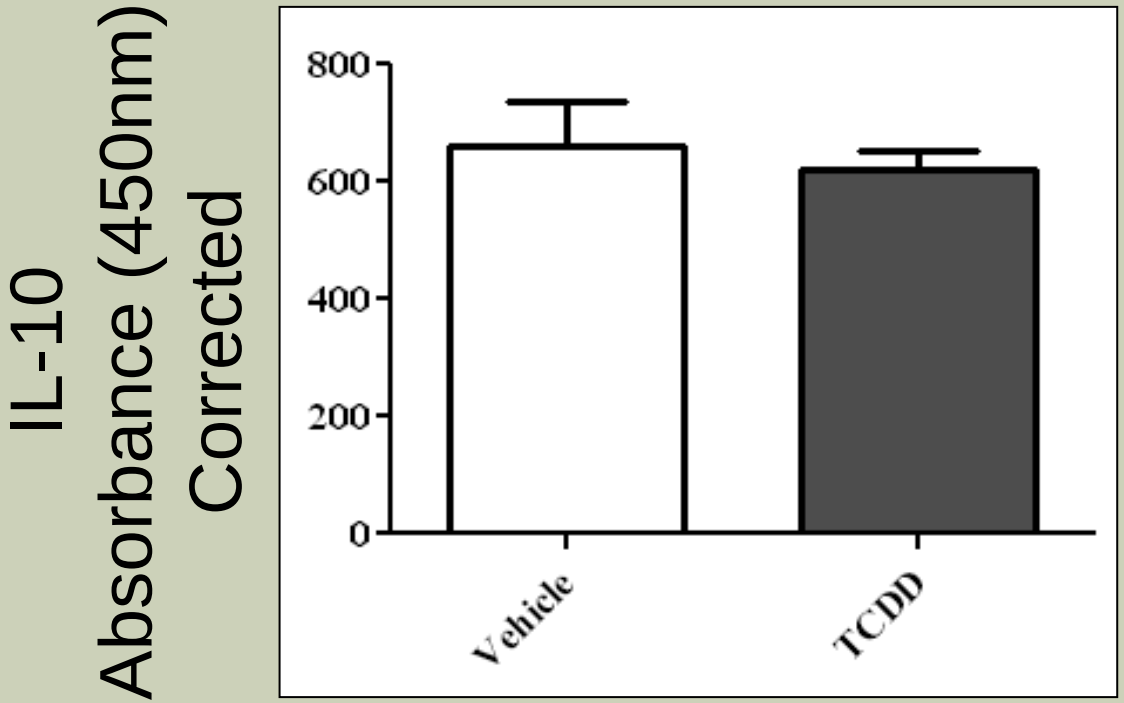
DAY 2 GvH

- Production of IL-10 in both VEH and TCDD were measured on supernatants from a day 2 GvH
- No difference between treatment groups



DAY 3 GvH

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



CONCLUSION

CONCLUSION

- Day 2 GvH production of IFN-ϒ
  - IFN-ϒ protein levels similar between TCDD and VEH-treated mice
- Day 3 GvH production of IFN-ϒ
  - Increased IFN-ϒ protein levels in TCDD-treated compared to VEH
    - Statistically significant (p=0.011)
  - TCDD up-regulated IFN-ϒ production

CONCLUSION

- IFN-ϒ consistent with prior studies
  - IFN-ϒ 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

CONCLUSION

- IL-10 production not influenced by treatment or time point of GvH
- Not consistent with findings in Marshall et al, 2008
  - 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-g
    - No difference on Day 2
    - Significant difference on Day 3, TCDD-treatment induced

FUTURE EXPERIMENTATION

- Look at IL-2 production on day 2 and day 3 GvH
  - IL-2 inhibits IFN-g production

ACKNOWLEDGEMENTS

Thanks to Dr. Nancy Kerkvliet, Diana Rohlman, Kerkvliet lab, the *EHSC Flow Cytometry and Cell Sorting Facility, the BioResource Research Program, and the National Institutes of Health, R01 Grant.

*The EHSCC is funded by grant No.2P30ES000210-42 from the NIEHS.

Credit: Nicole Nasholm

QUESTIONS?

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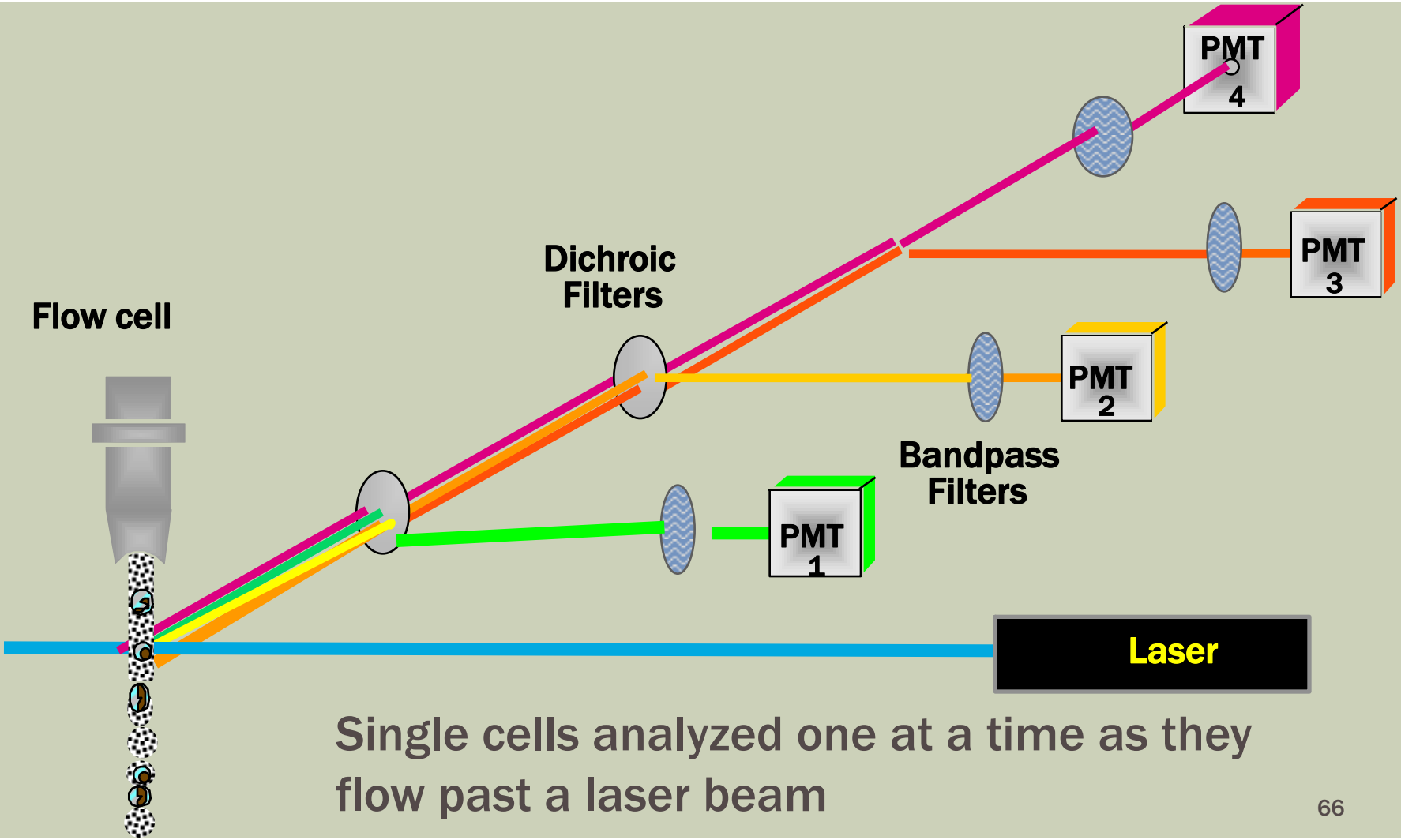
HOW FLOW CYTOMETRY WORKS



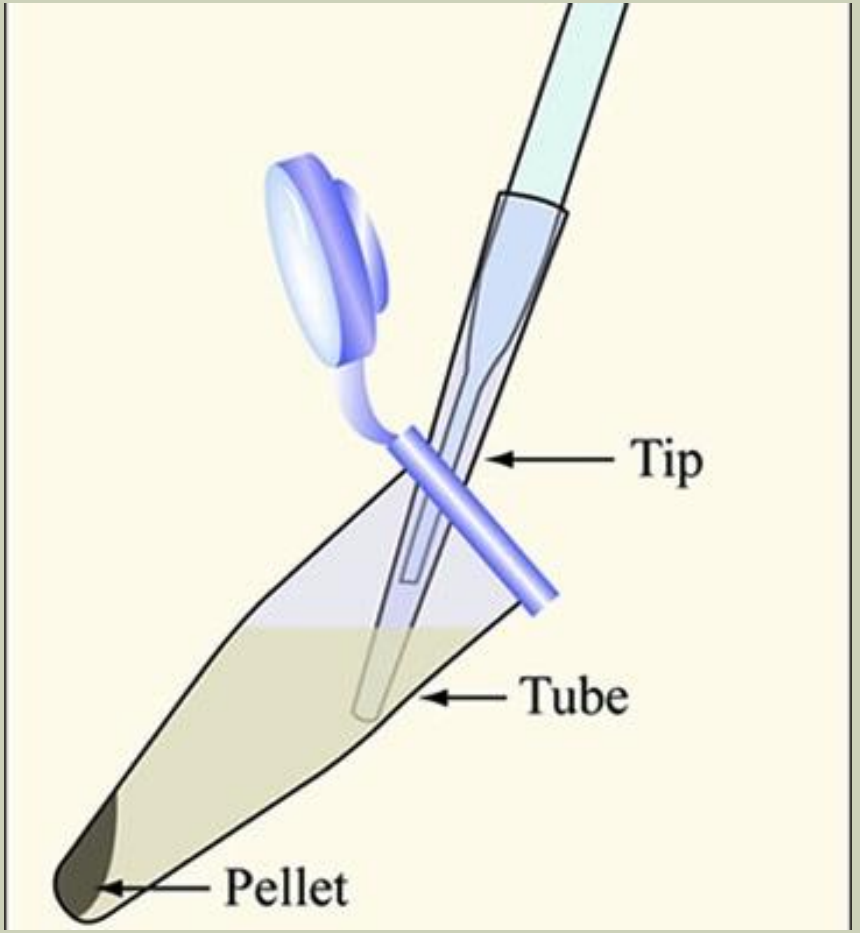
Single cells analyzed one at a time as they flow past a laser beam

OTHER MODELS

- 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

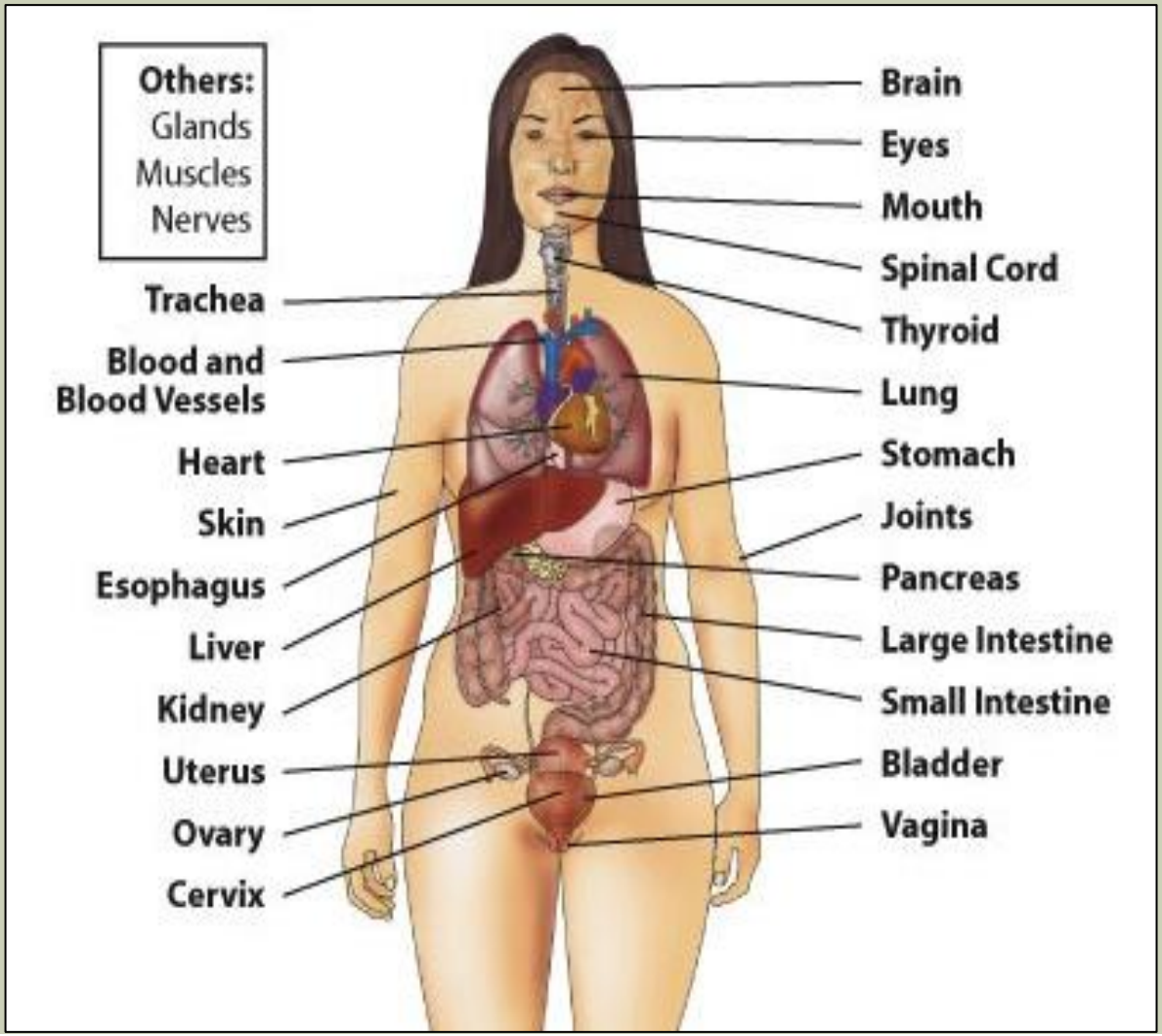
CYTOKINES

- Cytokines
  - located in the supernatant
  - Released by cells
- Supernatant



http://ocw.mit.edu/courses/biological-engineering/20-109-laboratory-fundamentals-in-biological-engineering-fall-2007/labs/examine_clones/

BODY PARTS THAT CAN BE AFFECTED BY AUTOIMMUNE DISEASES



TYPES OF AUTOIMMUNE DISEASES AND THEIR SYMPTOMS

Diseases	Symptoms
Diabetes type 1 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. www.womenshealth.gov	• Being very thirsty • Urinating often • Feeling very hungry or tired • Losing weight without trying • Having sores that heal slowly • Dry, itchy skin • Losing the feeling in your feet or having tingling in your feet • Having blurry eyesight
Addison's Disease 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. www.mayoclinic.com	• Muscle weakness and fatigue • Weight loss and decreased appetite • Darkening of your skin (hyperpigmentation) • Low blood pressure, even fainting • Salt craving • Low blood sugar (hypoglycemia) • Nausea, diarrhea or vomiting • Muscle or joint pains • Irritability • Depression