Pediatric Allergy and Immunology Development:

Molecular Mechanisms on Effects of Environmental Exposure

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In collaboration with Fresno Asthma Children's Environmental Study

STANFORD HOSPITAL & CLINICS
Stanford University Medical Center

Lucile Packard Children's Hospital
STANFORD UNIVERSITY MEDICAL CENTER

Objectives

- Provide an example of immune monitoring studies which led to improved understanding of toxicant mechanism
- Discuss a Systems Biology Approach and new applications in immune monitoring
- Examine sensitive and insensitive time points in pediatric immune development
- Discuss Potential Framework to examine relative risks to the immune system in children

The problem

The overall rate of asthma is currently estimated to be 7.85 percent of the population, an increase of about 0.5 percent every three years

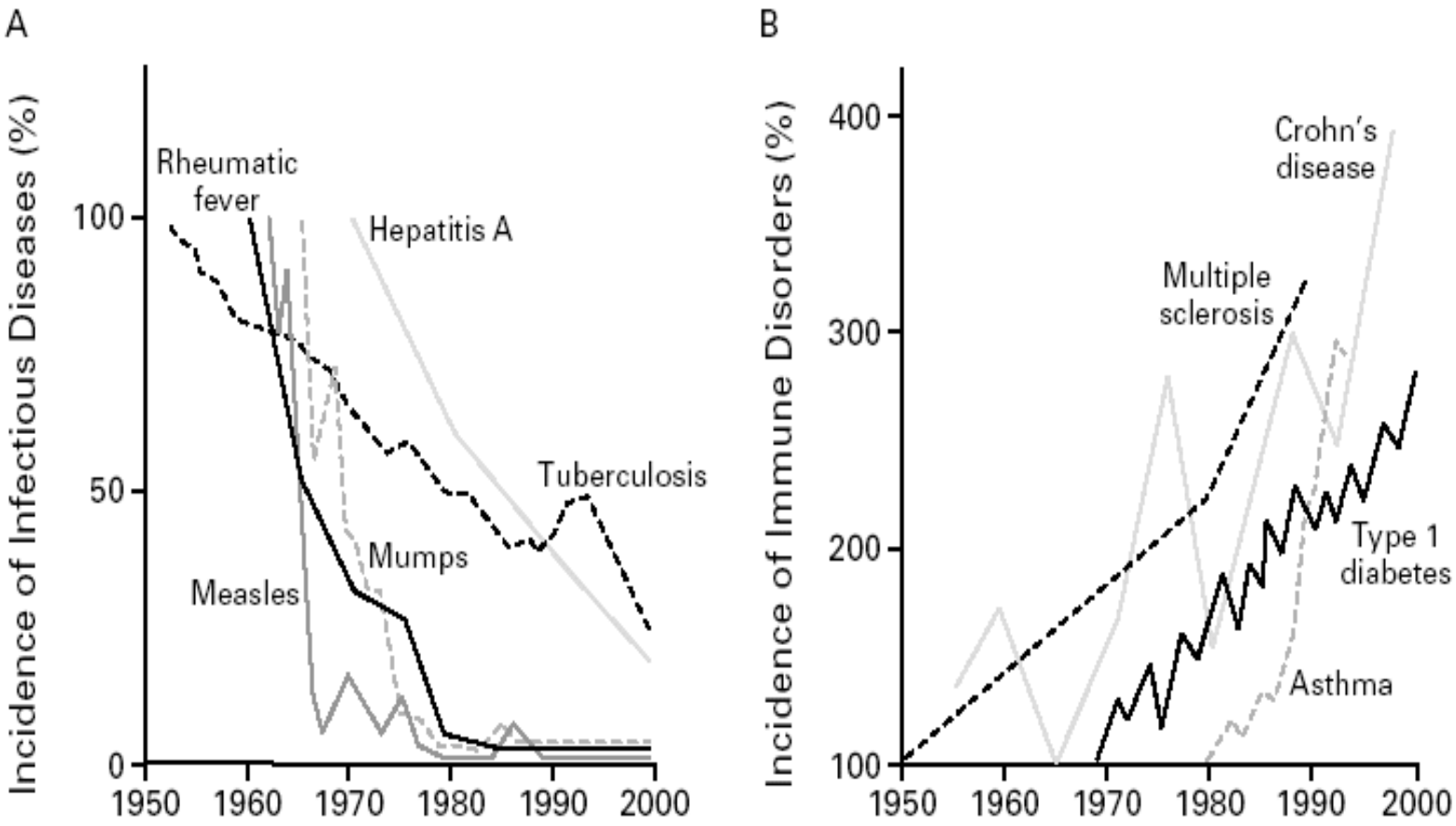
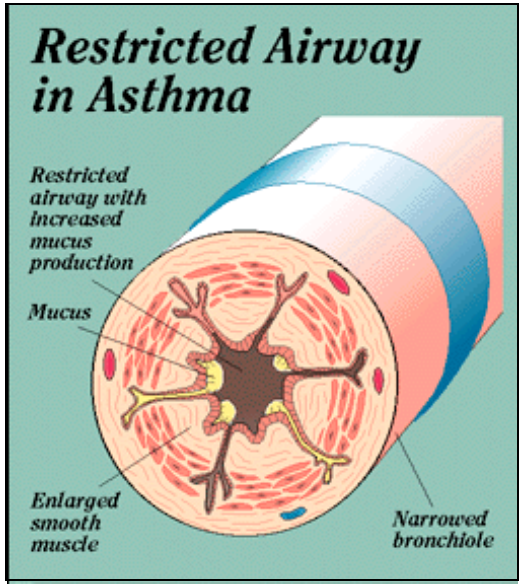


Figure 1. Inverse Relation between the Incidence of Prototypical Infectious Diseases (Panel A) and the Incidence of Immune Disorders (Panel B) from 1950 to 2000.

Up to 70% of asthma and up to 80% of allergies begin before age of 21 yrs.

Background

Asthma



- Dramatic increase in the prevalence of asthma worldwide in industrialized countries

- Chronic multifactorial disease characterized by :
  - Airway obstruction
  - Airway hyperresponsiveness (AHR)
  - Airway inflammation
  - Airway remodeling

- Pathophysiology :

Inappropriate immune response
in a genetically susceptible individual
driven by environmental exposures

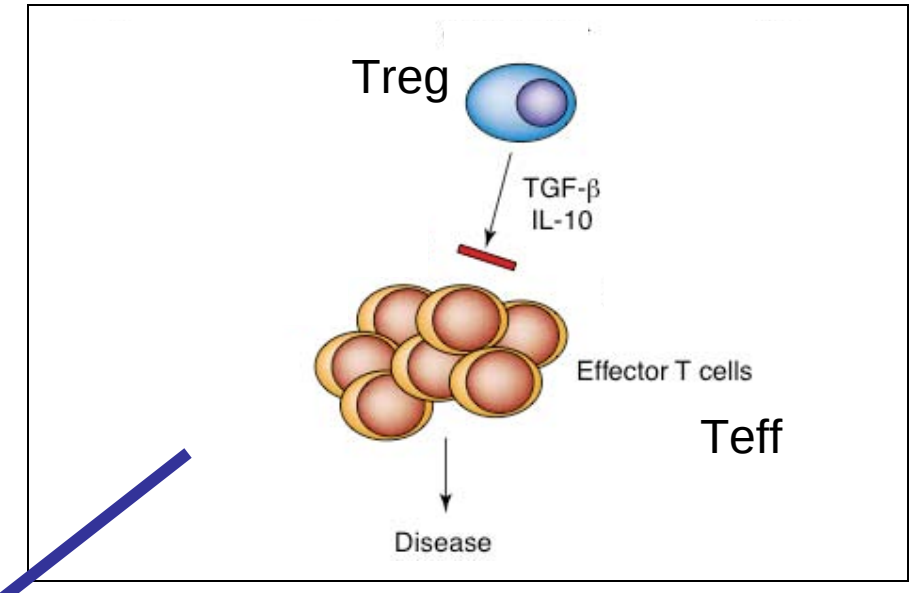
- Our research is a prototype study to:

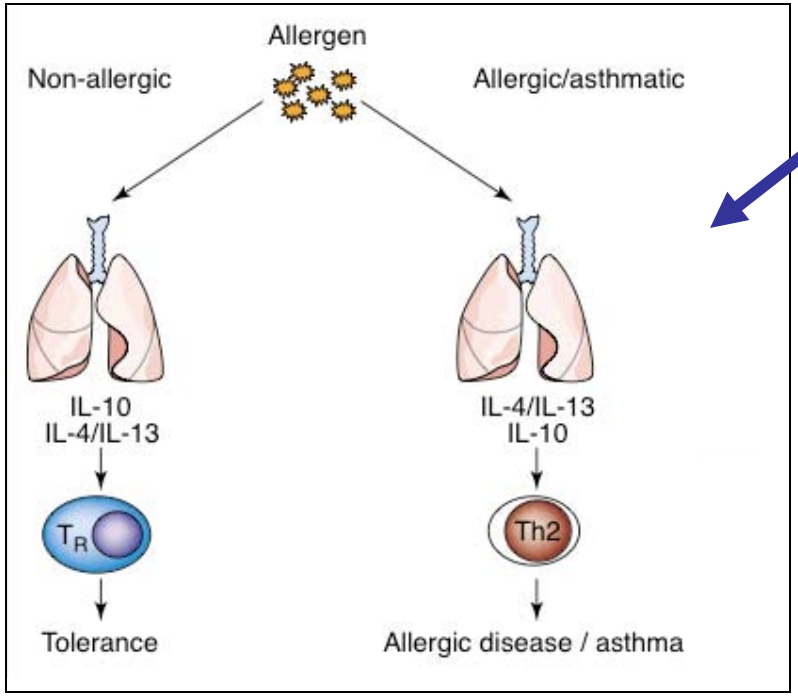
Dissect mechanisms of disease by examining associations of environmental exposures to molecular events at the DNA level to cellular changes in the Immune system which lead to specific health outcomes

Background

Regulatory T cells in Asthma

- Cells, which control or suppress the function of other cells
- **Foxp3** transcription factor associated with Treg
- Children lacking Foxp3 have severe allergies, asthma, GI disease, and diabetes type I.
- CD4+CD25^{hi}CD127lo cells can inhibit effector T cells





- **Natural Treg(CD4+CD25^{hi}) suppress effector T cells**
- **What Environmental Factors worsen Treg function in asthma? Do they affect suppressive function? Is Foxp3 expression altered? If so, how? By epigenetics of CpG islands in Foxp3 locus?**

GENERAL HYPOTHESIS and AIM

Overall: Treg are dysfunctional in children exposed to high levels of ambient air pollution exposure

Aim: The research aims to help elucidate the key role of air pollution in asthma, a link which is theoretically understood, circumstantially clear, but not yet proven.

Rationale: Understanding biological mechanisms is an important step towards decreasing the risk and developing target-driven treatments to reduce the burden of asthma in children who are exposed to high levels of air pollution.

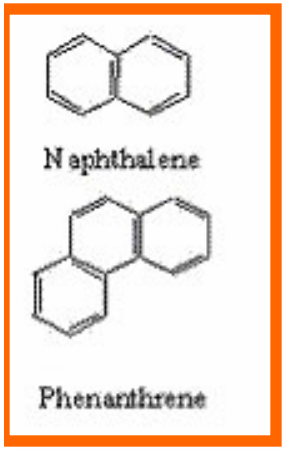
Four cohorts chosen:

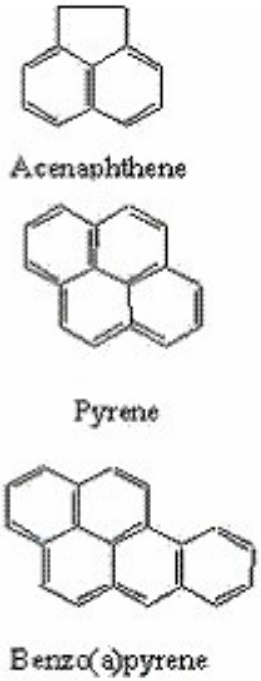
- Children's Environmental Study in the Central Valley of CA.
  - 9 years of exposure data
  - Children 8-12 yrs with asthma (n=71, **FA**)
  - 315 families
  - Serial PFTs and clinical outcome score
  - Individual Estimates of Exposure to:
    - Elemental carbon, $PM_{2.5}$, PM_{10}, metals, ozone, polycyclic aromatic hydrocarbons, endotoxin, pollens, carbon monoxide
- Fresno control or FC: Age matched and sex matched children with no asthma and no allergies (n=40)
- Stanford Asthma or SA: Age matched and sex matched children with asthma (same clinical outcomes and PFTs used) (n=30)
- Stanford control or SC: Age matched and sex matched children with no asthma and no allergies (n=30)

Nadeau, et al. JACI 2010.

PAHs about ~7x higher in Fresno Air

Volatile to semivolatile compounds





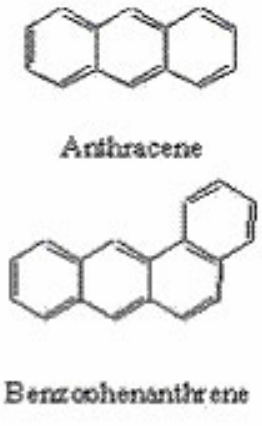


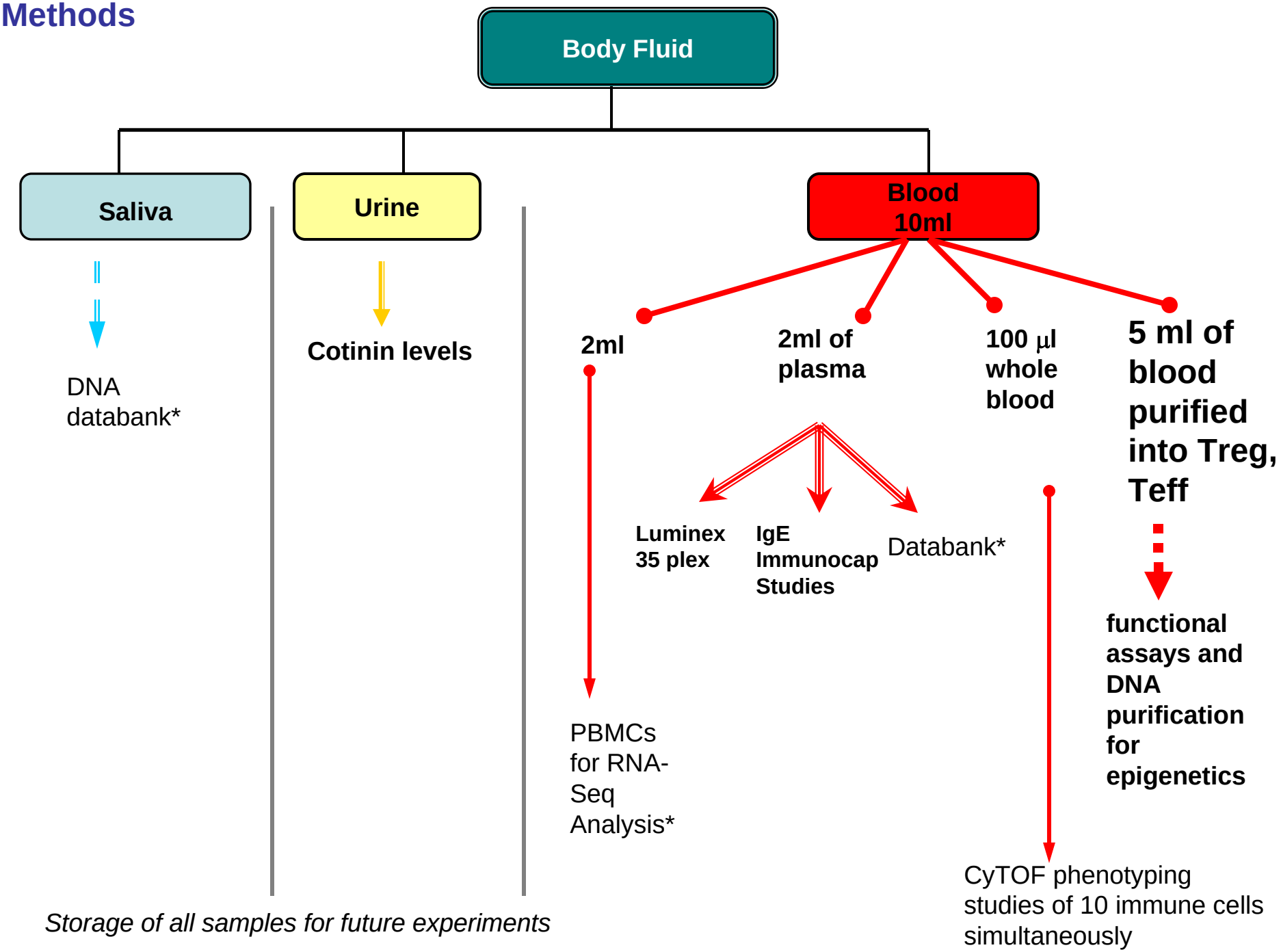
TABLE I. Comparison of area ambient pollutant concentrations between Fresno and Palo Alto, Calif, based on CARB compliance monitoring for 2008

Pollutant	Location of compliance monitor*	
	Redwood City	First Street, Fresno
PAHs, annual average (ng/m^3)	0.6	4.4
$PM_{2.5}$, annual average (μg/m^3)	10.5	21.2
$PM_{2.5}$, 24-h maximum (μg/m^3)	36.0	93.0
PM_{10}, 24-h high (μg/m^3)	41	78
O_3, highest 8-h average (ppb)	70	132
O_3, no. of days > state 1-h standard	0	44
O_3, no. of days > state 8-h standard	0	86

PM_{10}, Particulate matter with aerodynamic diameter ≤10 μm.
*Redwood City is the compliance monitor within 4.7 km of the Palo Alto residences of the Stanford cohort. All FA subjects live within a circle with the First Street monitor as its center and a radius of 20 km.

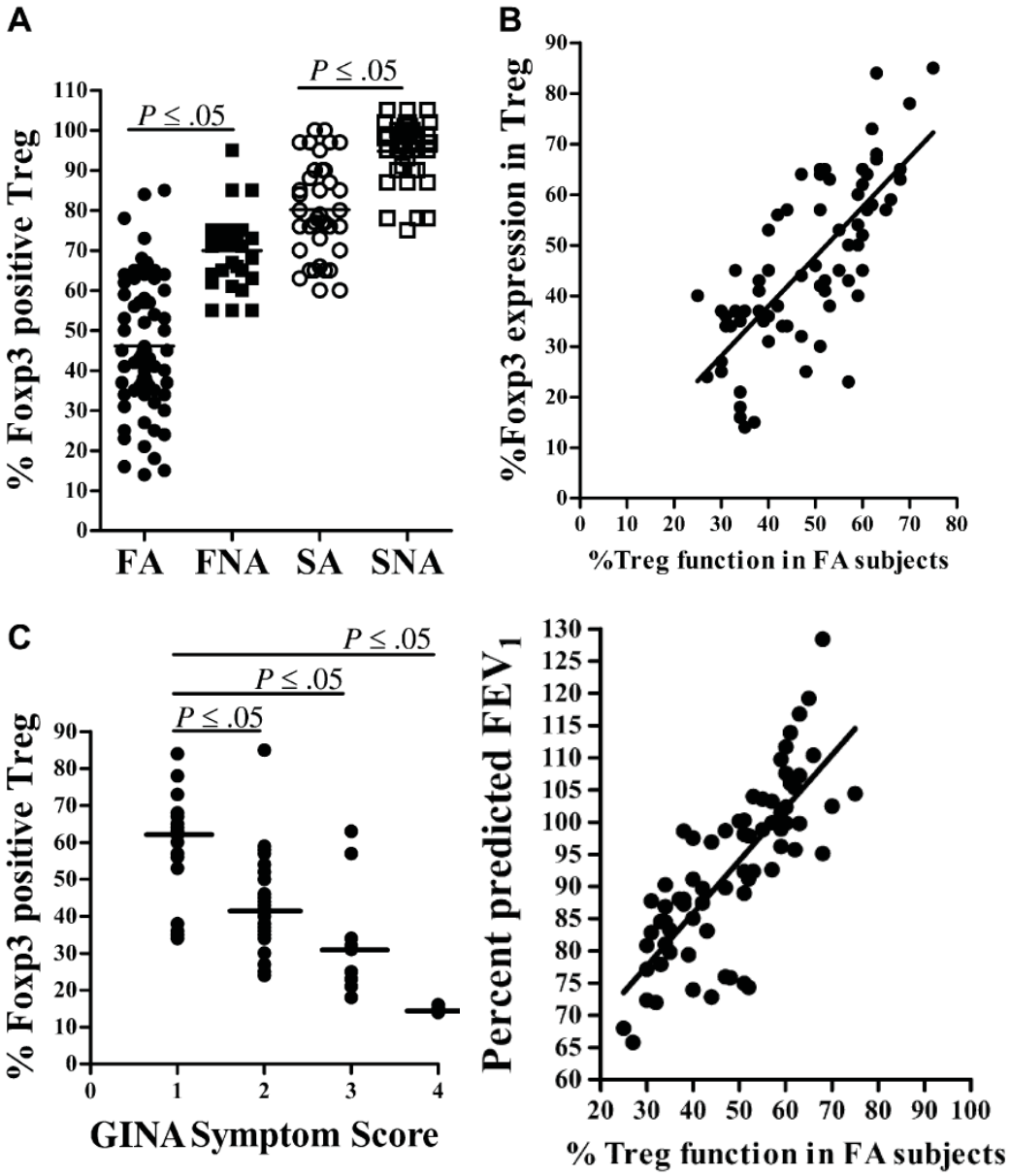
Nadeau, et al. JACI 2010.

Methods


Body Fluid
Saliva
Urine
Blood 10ml
DNA databank*
Cotinin levels
2ml
2ml of plasma
100 µl whole blood
5 ml of blood purified into Treg, Teff
Luminex 35 plex
IgE Immunocap Studies
Databank*
functional assays and DNA purification for epigenetics
PBMCs for RNA-Seq Analysis*
CyTOF phenotyping studies of 10 immune cells simultaneously
Storage of all samples for future experiments

Results

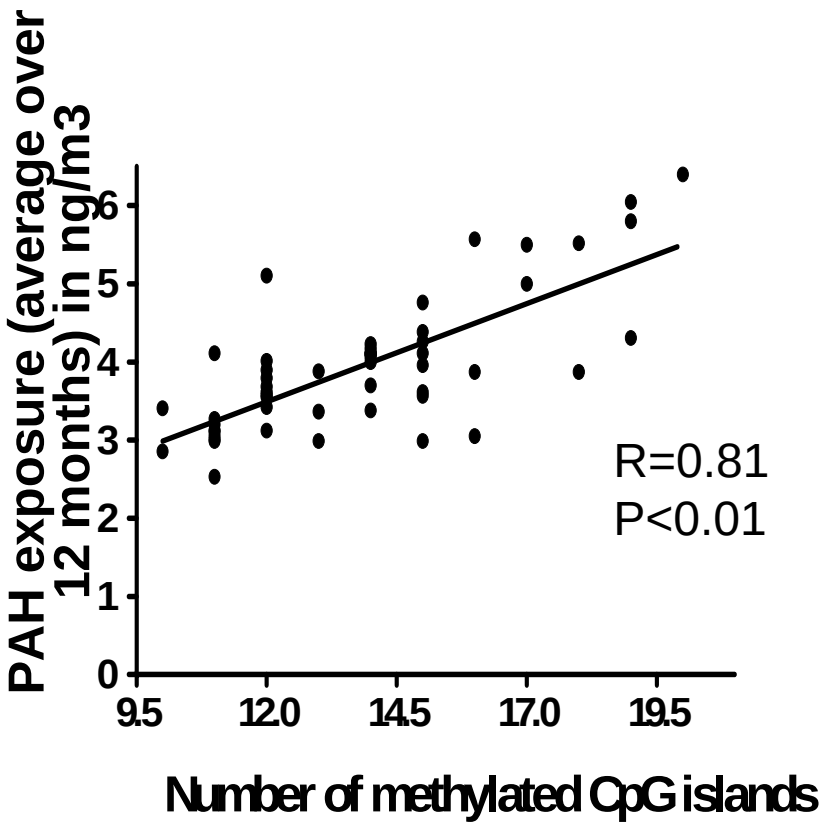
Treg Foxp3 expression is associated with asthma severity

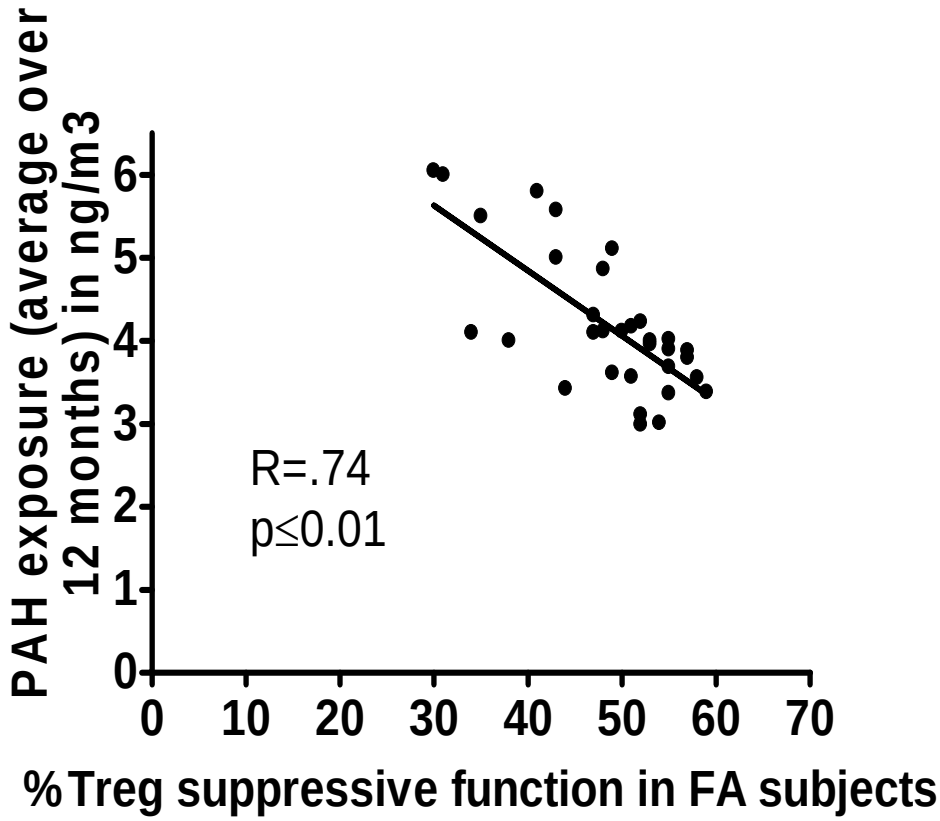


Nadeau, et al. 2010

Epigenetic Studies and PAH Exposure Data

Possible association between PAH exposure (2006) and Methylation of Foxp3 locus in FA subjects





Note: Blood collected 3 yrs after exposure assessments were done

Nadeau, et al. JACI 2010.

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Technologies available for Immune Monitoring

- In addition to:
  - Proteomics (protein arrays using antibody capture)
  - Metabolomics (examining small molecules/products)
  - RNA-Seq (replacing microarrays)
  - Complete Genomic Sequencing
  - Methylomics of whole blood gDNA vs purified cells gDNA
- …..There are:

  CyTOF (Mass Spec on cell phenotyping)

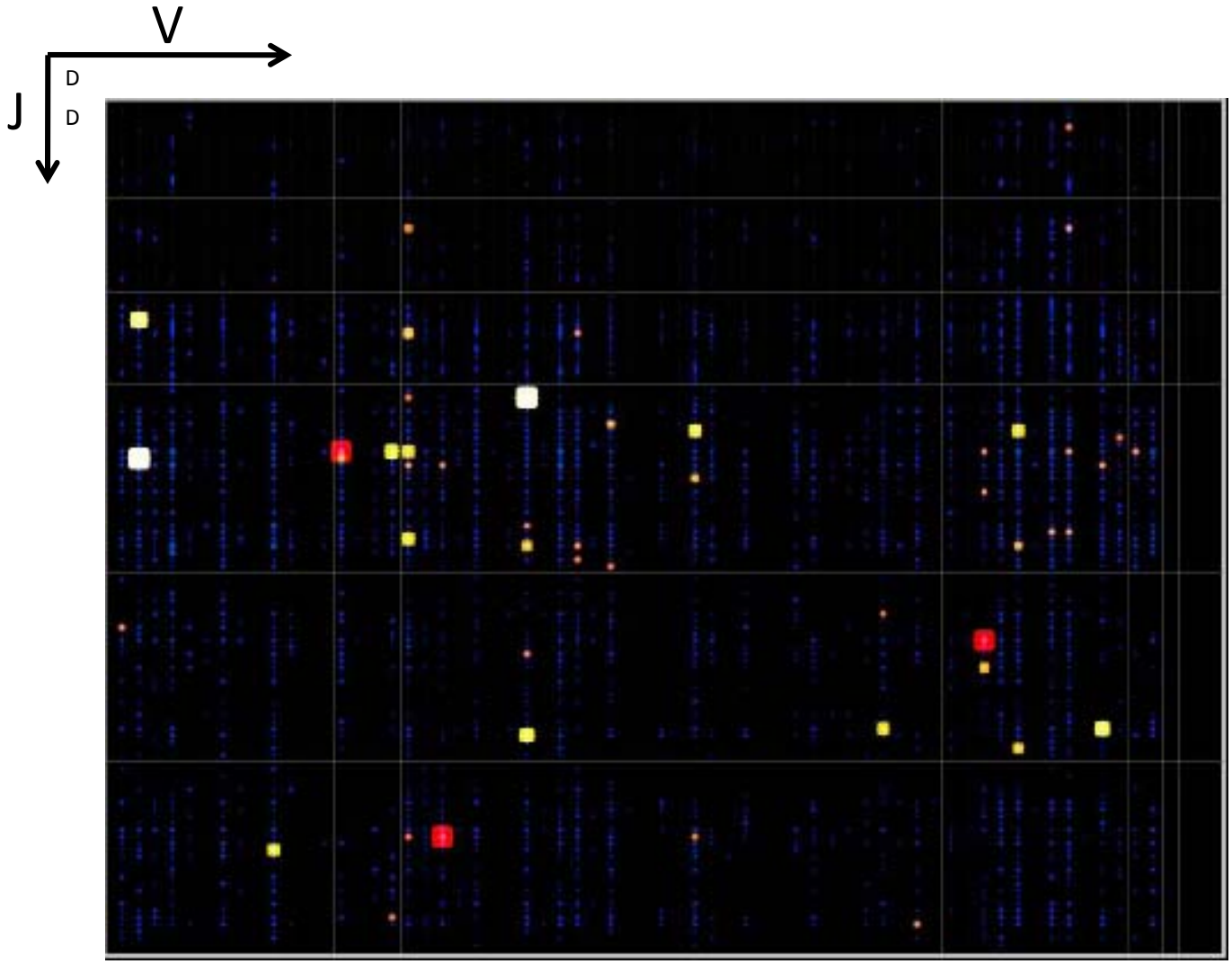
  New Blood Drop Allergy Test

  High Throughput Sequencing of Immune Cells

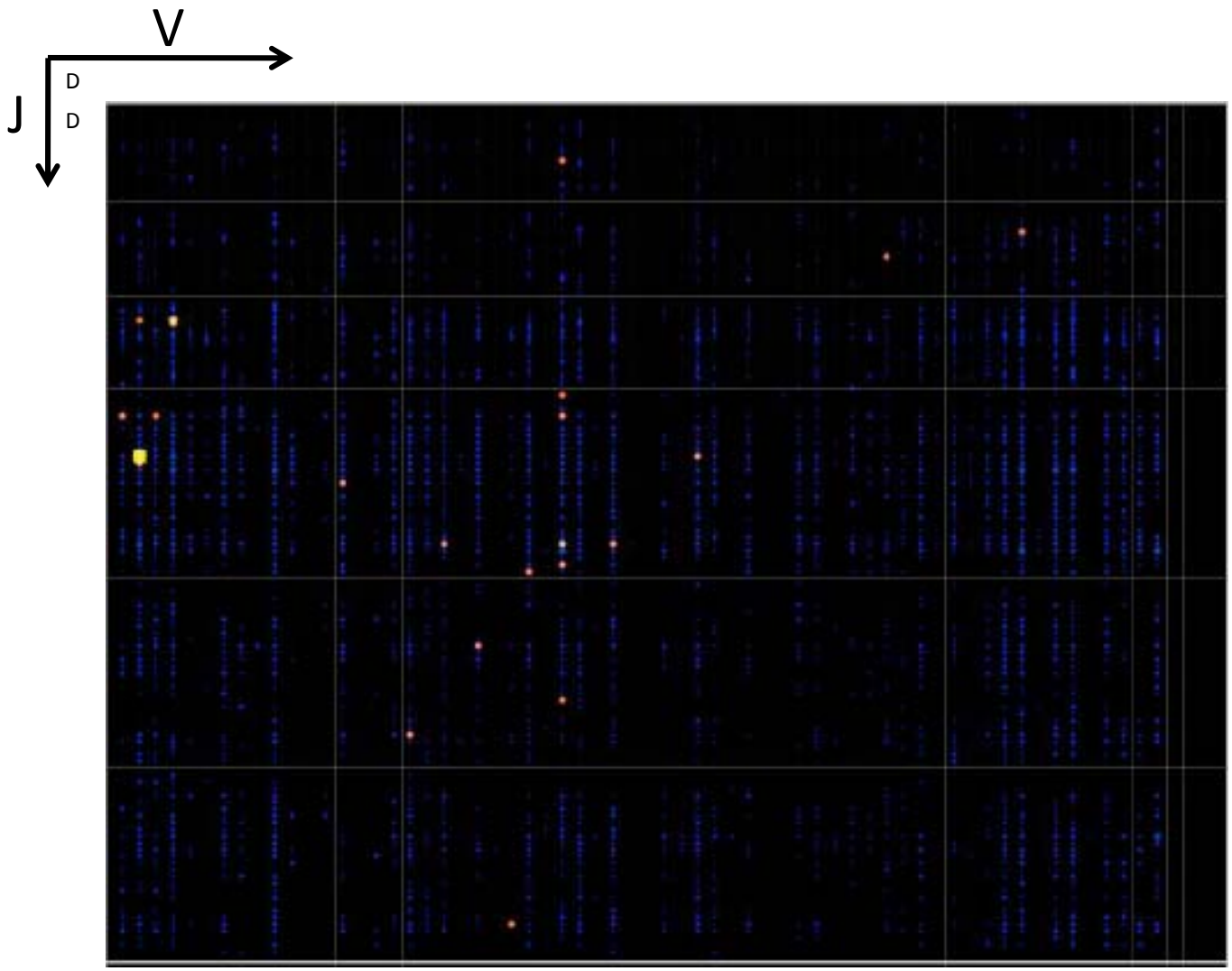
Immune Cell High Throughput Sequencing

- Hypothesis-based
- Certain VDJ recombinations are associated with allergies or neoplasia
- Can we detect these early before manifestation of disease?

Detection of Clonal Sequences:



Detection of Clonal Sequences:



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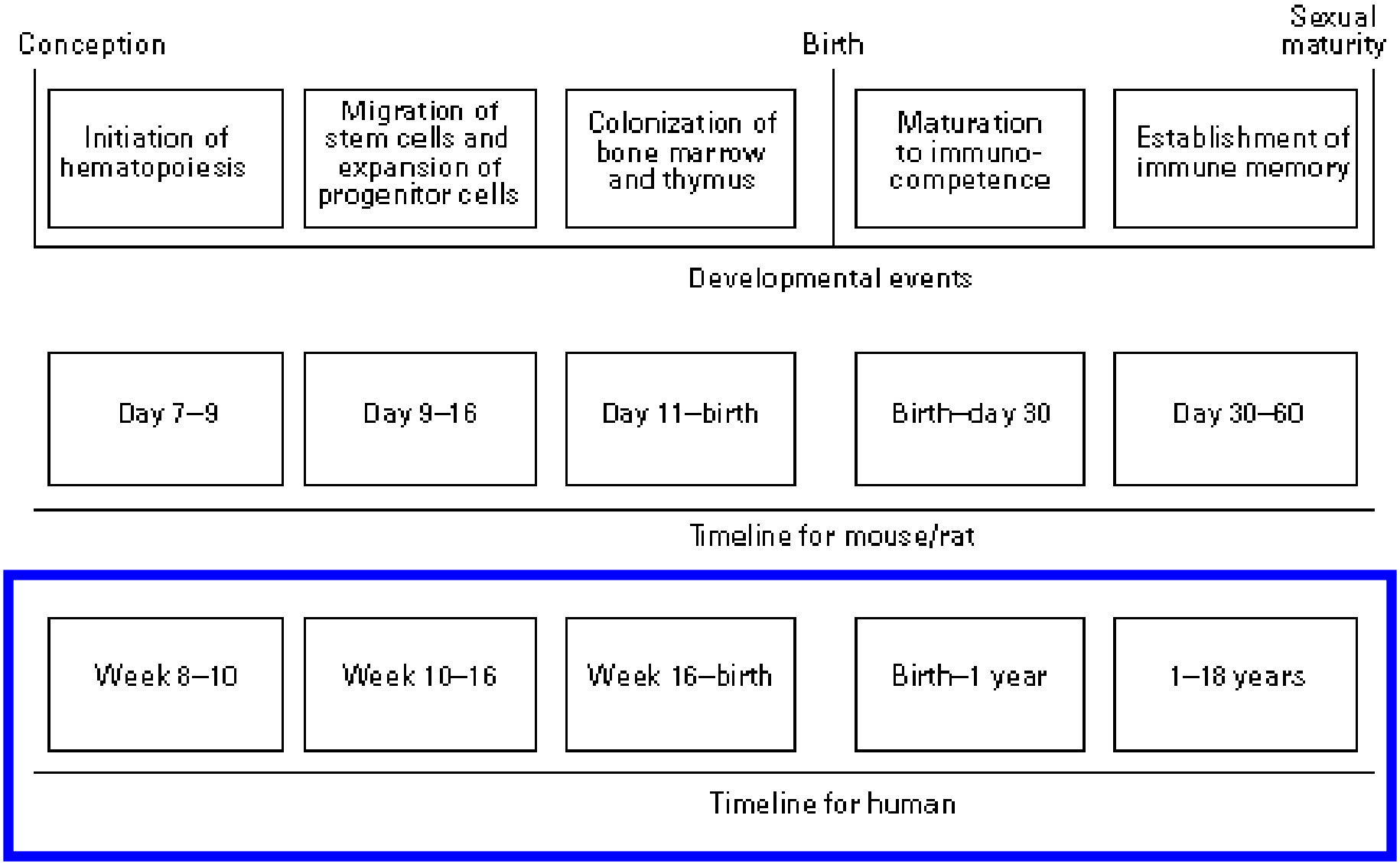
Critical Sensitive Periods in Immune Development



Human Immune System Development

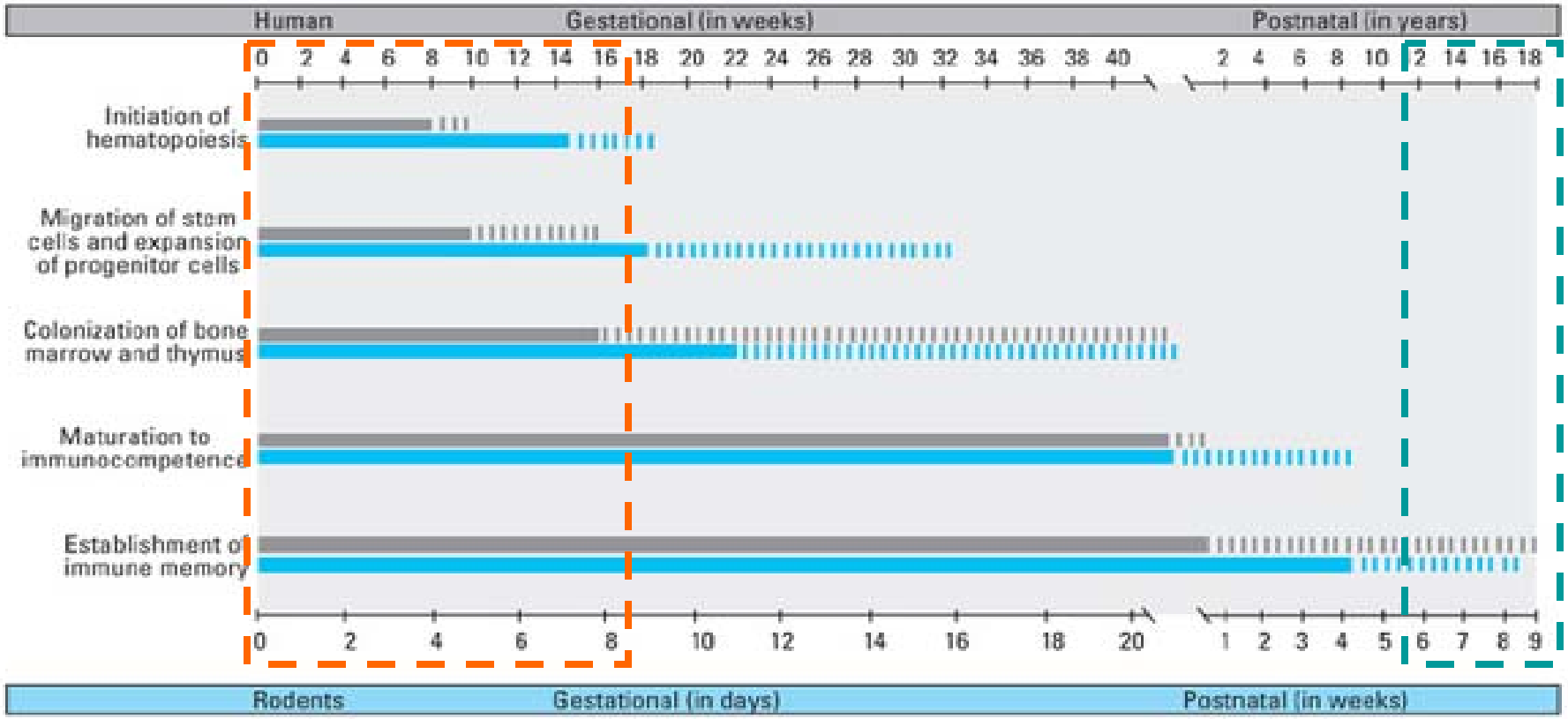


Figure 6. Immune system development in humans and rodents. A comparison of critical stages for immune system development is shown for humans and rodents. Hashed bars represent discrete development steps in the formation of the mature immune system and represent periods where differential susceptibilities to immunotoxicants could be expected (Holladay and Smialowicz 2000). Solid bars show time to "sensitive window" in each species. Figure reproduced from Faustman et al. (2003) and reprinted with permission of the Institute for Risk Analyses and Risk Communication at the University of Washington.

A Framework for Assessing Risks to Children from Exposure to Environmental Agents. Daston, et al. EHP. 2004

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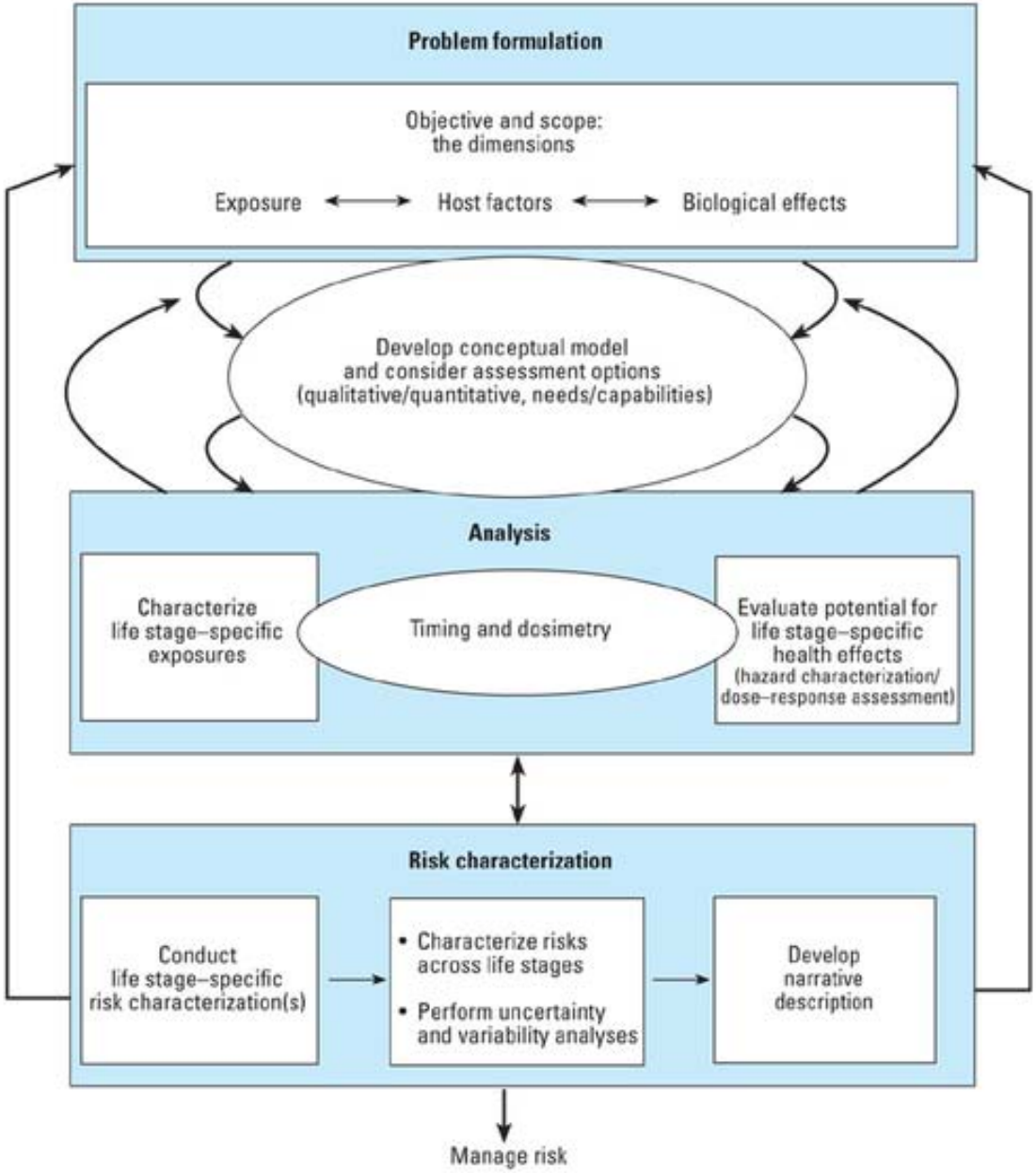
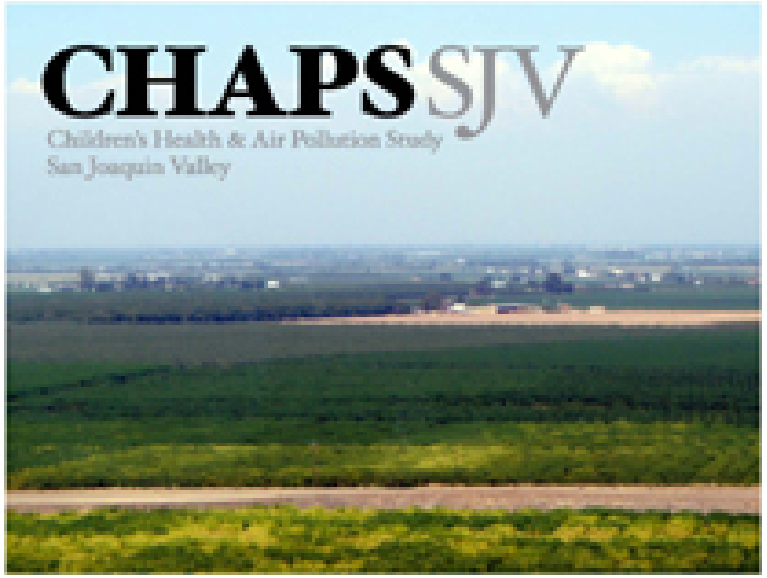


Figure 1. Proposed framework for assessing risks to children from exposure to environmental agents.

A Framework for Assessing Risks to Children from Exposure to Environmental Agents. Daston, et al. EHP. 2004

Thank you

University of California, Berkeley
(Drs. Tager, Hammond, Balmes)
The Fresno Community
The Fresno Asthma Coalition



FACES
Fresno Asthmatic Children's Environment Study

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NIEHS, EPA, ALA, NHLBI, Mickey Leland Grant, Austin Foundation, Westly Foundation

Contact information: knadeau@stanford.edu

- Back up Slides

Outcome Variables

For all 4 cohorts:

- Regulatory T cells (CD4+CD127loCD25^{hi}): molecular analysis (including epigenetics of candidate gene, Foxp3) and function
- Effector T cells or responder T cells (CD4+CD127+CD25^{neg}): molecular analysis and function
- Assays of interaction between the two above cell types: suppression assays, cell death assays
- Subject CBC with differential, physical findings and symptoms, pulmonary function and allergy tests.

Table 1 - Traffic-related air pollution exposure and childhood asthma

Approach	Study design	Cohort	Exposure(s)	Results
Measured traffic-related pollutants	Longitudinal birth cohort	Aged 0-4 years in Sweden	NO_x and PM_{10}	NO_x and PM_{10} during first year of life associated with increased sensitization, wheeze, and lower lung function at an age of 4 years
	Population-level time series	Aged 0-17 years in Baltimore, MD	Ambient $PM_{2.5}$-Zinc	Increases in ambient zinc associated with increase in ED asthma visits or hospital admissions
	Exposure assessment	Homes in Boston, MA	$PM_{2.5}$, NO_x, (EC)	PM2.5, NO_x, and EC predicted by roadway length
GIS	Cross-sectional analysis	Aged 0-1 years in Cincinnati, Ohio	EC	EC concentrations derived from LUR models associated with wheeze among infants
	Case-control study	Aged 0-17 years in Puerto Rico	Proximity to point emission sources	Symptomatic children more likely to reside in proximity to point emission sources
	Longitudinal birth cohort	Aged 0-1 years in Munich, Germany	Proximity to a major road	Infants living within 50 m of a main road had increased odds of runny nose and sneezing.
	Longitudinal birth cohort	Aged 0-6 years in Munich, Germany	Proximity to a major road	Residence within 50 m of a main road associated with increased odds of sensitization at an age of 4-6 years
	Spatial analysis	Towns or census tracts in Massachusetts	DEP	Towns containing major highway corridors had higher levels of DEP and asthma incidence
Biomarkers of airway inflammation	Panel study	Nonasthmatic and asthmatic children aged 5-10 years in Tokyo, Japan	$PM_{2.5}$, NO, NO_x	eNO levels correlated with $PM_{2.5}$, NO, NO_x and organic black carbon
	Cross-sectional analysis	Aged 9-11 years in Windsor, Ontario	Proximity to roadway	Residence closer to any roadway associated with higher eNO levels
	Panel study	Asthmatic children with mean age of 9 years in Mexico City	$PM_{2.5}$, ozone	Increases in 8-h moving averages of $PM_{2.5}$and ozone associated with increases in EBC malondialdehyde
	Panel study	Nonasthmatic and asthmatic males aged 12-18 years in Christchurch, New Zealand	PM_{10}, $PM_{2.5}$, $PM_{1.0}$	Increases in PM had no effect on exhaled biomarkers

DEP, diesel exhaust particle; EBC, exhaled breath condensate; EC, elemental carbon; ED, emergency department; eNO, exhaled nitric oxide; LUR, land-use regression; NO, nitric oxide; PM, particulate matter

Air pollution and childhood asthma: recent advances and future directions.
Patel, Molini; Miller, Rachel
Current Opinion in Pediatrics. 21(2):235-242, April 2009.
DOI: 10.1097/MOP.0b013e3283267726

Table 2 - Host characteristics, air pollution and pediatric asthma

Host characteristic	Study design	Cohort	Exposure(s)	Results
Exposure to violent events	Longitudinal birth cohort	Aged 0-18 years in Boston, MA	NO_2	Higher levels of lifetime NO_2 associated with asthma only among those exposed to violent events
Trimester of exposure	Retrospective analysis	Asthmatic children aged 6-11 yrs. in San Joaquin Valley, CA	O_3, PM_{10}, CO, NO_2	Prenatal and early-life exposures to CO, PM_{10}, and NO_2 associated with decreased lung function
Chronic stress	Cross-sectional analysis	Mean age 12.8 yrs. in Vancouver, BC	NO_2	Effects of chronic stress on airway inflammation, symptoms higher among children with lower NO_2 exposures
Poverty	Cross-sectional analysis	Aged 1-65+ years	Residential traffic density	Effects of residence in high-traffic-density areas larger among individuals living in poverty
CD14	Panel study	Asthmatic; Chapel Hill, North Carolina	$PM_{2.5}$, $PM_{10\text{-}2.5}$	CD14 expression on neutrophils correlated with FEV1 in response to increases in $PM_{2.5}$ and $PM_{10\text{-}2.5}$
GSTP1	Longitudinal cohort	Aged 10-18 years in Southern California	Residential proximity to a major road	GSTP1 Val/Val associated with increased lifetime asthma of living less than 75 m from a major road
TGFβ-1	Longitudinal cohort	Aged 10-18 years in Southern California	Residential proximity to a major road	TGFβ -509 TT associated with asthma if living within 500 m of a freeway
GSTP1	Longitudinal birth cohort	Aged 0-4 years in Stockholm, Sweden	NO_x	Interaction observed between GSTP1 Ile/Val and Val/Val and NO_x on sensitization; 3-way interactions observed among NO_x, GSTP1 polymorphisms, and TNF -308 GA/AA genotypes

FEV1, forced expiratory volume in 1 s; GSTP1, glutathione-*S*-transferase P1; NO_x, nitrogen oxides; PM, particulate matter; TGFβ, transforming growth factor β; TNFα, tumor necrosis factor α

The problem

People

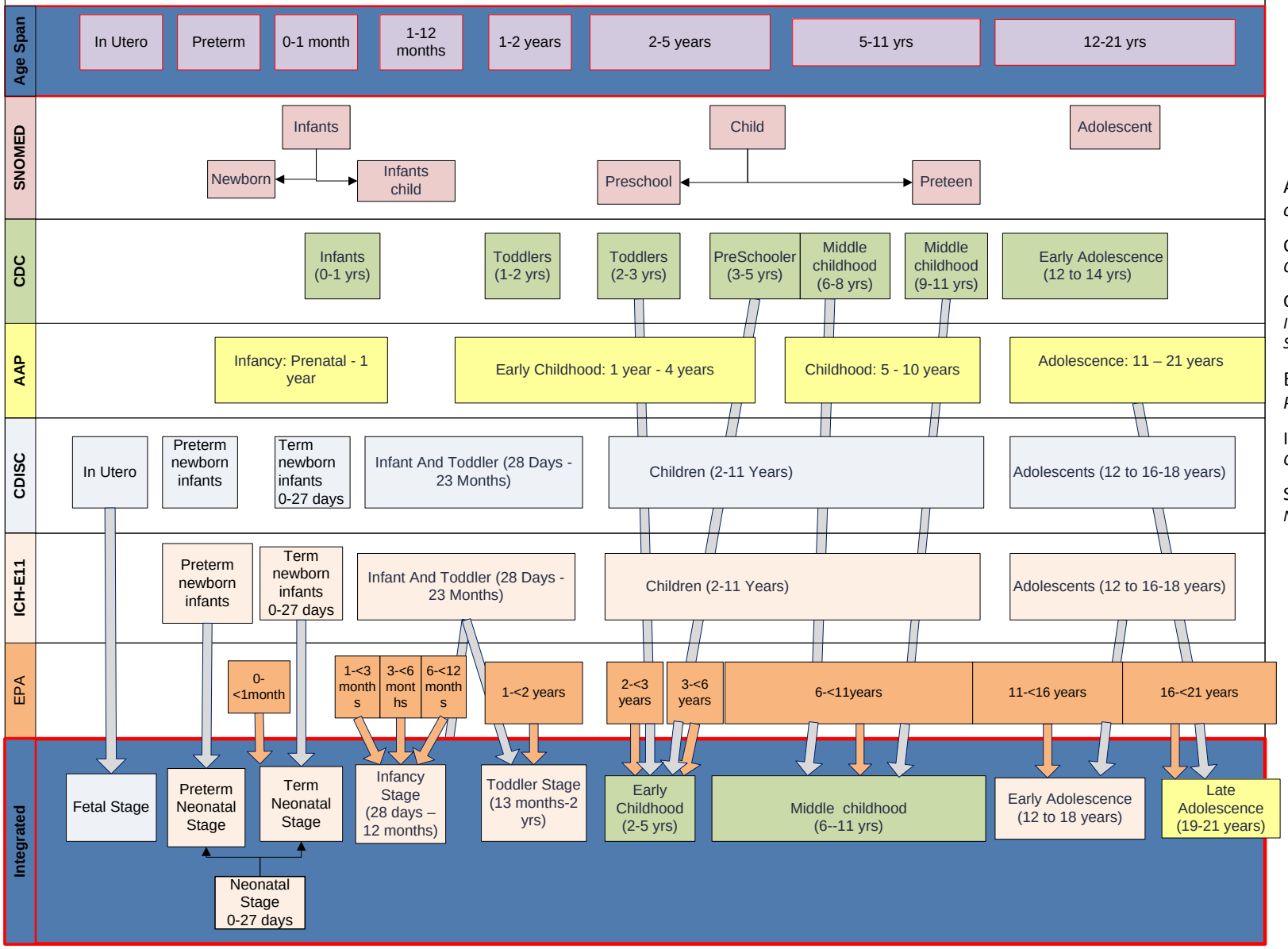
CA cities rank 8 out of 10 highest ambient air pollution with increased health risk

2011 Rank[1]	Metropolitan Statistical Areas	Total Population[2]	Under 18[3]	65 and Over[3]	Pediatric Asthma[4,8]	Adult Asthma[5,8]	Chronic Bronchitis[6,8]	Emphysema[7,8]	Poverty[9]
1	Los Angeles-Long Beach-Riverside, CA	17,820,893	4,682,410	1,902,902	310,610	1,030,481	552,457	257,170	2,579,016
2	Bakersfield-Delano, CA	807,407	250,561	72,666	16,621	43,747	23,012	10,309	170,614
3	Visalia-Portervillo, CA	429,668	141,279	40,393	9,372	22,622	11,998	5,494	97,542
4	Fresno-Madera, CA	1,053,899	319,551	104,947	21,198	58,379	30,977	14,213	221,348
5	Sacramento—Arden-Arcade—Yuba City, CA-NV	2,436,109	607,251	300,098	40,307	143,692	79,445	39,034	320,925
6	Hanford-Corcoran, CA	148,764	41,081	11,466	2,725	8,468	4,221	1,721	24,546
7	San Diego-Carlsbad-San Marcos, CA	3,053,793	739,625	347,859	49,063	181,385	97,908	46,204	372,782
8	Houston-Baytown-Huntsville, TX	5,968,586	1,693,708	507,966	138,409	275,407	177,262	78,010	897,732
9	Merced, CA	245,321	78,461	24,167	5,205	13,076	6,948	3,210	59,349
10	Charlotte-Gastonia-Salisbury, NC-SC	2,389,763	615,854	263,236	52,818	139,028	75,668	35,810	332,654
11	San Luis Obispo-Paso Robles, CA	266,971	49,825	39,636	3,305	16,962	9,572	4,880	33,198
12	Dallas-Fort Worth, TX	6,772,276	1,884,196	607,900	153,975	314,809	202,280	89,746	950,677
13	El Centro, CA	166,874	51,337	17,578	3,405	9,042	4,822	2,259	35,368
14	Modesto, CA	510,385	149,225	53,538	9,899	28,322	15,287	7,192	85,583
14	Washington-Baltimore-Northern Virginia, DC-MD-VA-WV	8,394,115	2,017,092	913,919	217,649	558,279	272,776	128,313	700,129
16	Cincinnati-Middletown-Wilmington, OH-KY-IN	2,214,954	543,893	270,380	51,168	166,495	72,691	35,624	272,692
17	New York-Newark-Bridgeport, NY-NJ-CT-PA	22,232,494	5,171,357	2,905,795	513,309	1,559,643	744,517	370,377	2,721,910
18	Knoxville-Sevierville-La Follette, TN	1,053,627	231,414	158,809	19,847	66,813	36,686	19,121	162,410
19	Phoenix-Mesa-Glendale, AZ	4,364,094	1,187,246	496,355	96,895	347,250	133,817	63,556	643,772
20	Philadelphia-Camden-Vineland, PA-NJ-DE-MD	6,533,122	1,535,672	869,965	158,452	438,946	219,155	109,986	760,156
21	Birmingham-Hoover-Cullman, AL	1,212,848	291,846	160,168	25,030	70,273	40,311	20,201	177,638
22	Chico, CA	220,577	46,201	33,001	3,065	13,594	7,643	3,920	39,717
23	Atlanta-Sandy Springs-Gainesville, GA-AL	5,831,778	1,573,677	513,199	155,122	296,754	177,090	78,367	802,336
24	Pittsburgh-New Castle, PA	2,445,117	495,068	422,943	51,002	174,497	89,288	48,733	290,876
25	Las Vegas-Paradise-Pahrump, NV	1,947,068	510,425	214,427	35,278	127,748	60,786	28,636	240,066

Notes:

1. Cities are ranked using the highest weighted average for any county within that Combined or Metropolitan Statistical Area.
2. **Total Population** represents the at-risk populations for all counties within the respective Combined or Metropolitan Statistical Area.
3. Those **18 and under** and **65 and over** are vulnerable to $PM_{2.5}$ and are, therefore, included. They should not be used as population denominators for disease estimates.
4. **Pediatric asthma** estimates are for those under 18 years of age and represent the estimated number of people who had asthma in 2009 based on state rates (BRFSS) applied to population estimates (U.S. Census).
5. **Adult asthma** estimates are for those 18 years and older and represent the estimated number of people who had asthma during 2009 based on state rates (BRFSS) applied to population estimates (U.S. Census).
6. **Chronic bronchitis** estimates are for adults 18 and over who had been diagnosed in 2009, based on national rates (NHIS) applied to population estimates (U.S. Census).
7. **Emphysema** estimates are for adults 18 and over who have been diagnosed within their lifetime, based on national rates (NHIS) applied to population estimates (U.S. Census).
8. Adding across rows does not produce valid estimates, e.g., summing pediatric and adult asthma and/or emphysema and chronic bronchitis.
9. **Poverty** estimates come from the U.S. Census Bureau and are for all ages.

Integrated child-life stages for NICHD Pediatric Terminology as mapped to existing medical terminologies



AAP: *American Academy of Pediatrics*

CDC: *Centers for Disease Control and Prevention*

CDISC: *Clinical Data Interchange Standards Consortium*

EPA: *Environmental Protection Agency*

ICH-E11: *International Conference on Harmonisation*

SNOMED: *Systematized Nomenclature of Medicine*

Lung Development in Humans

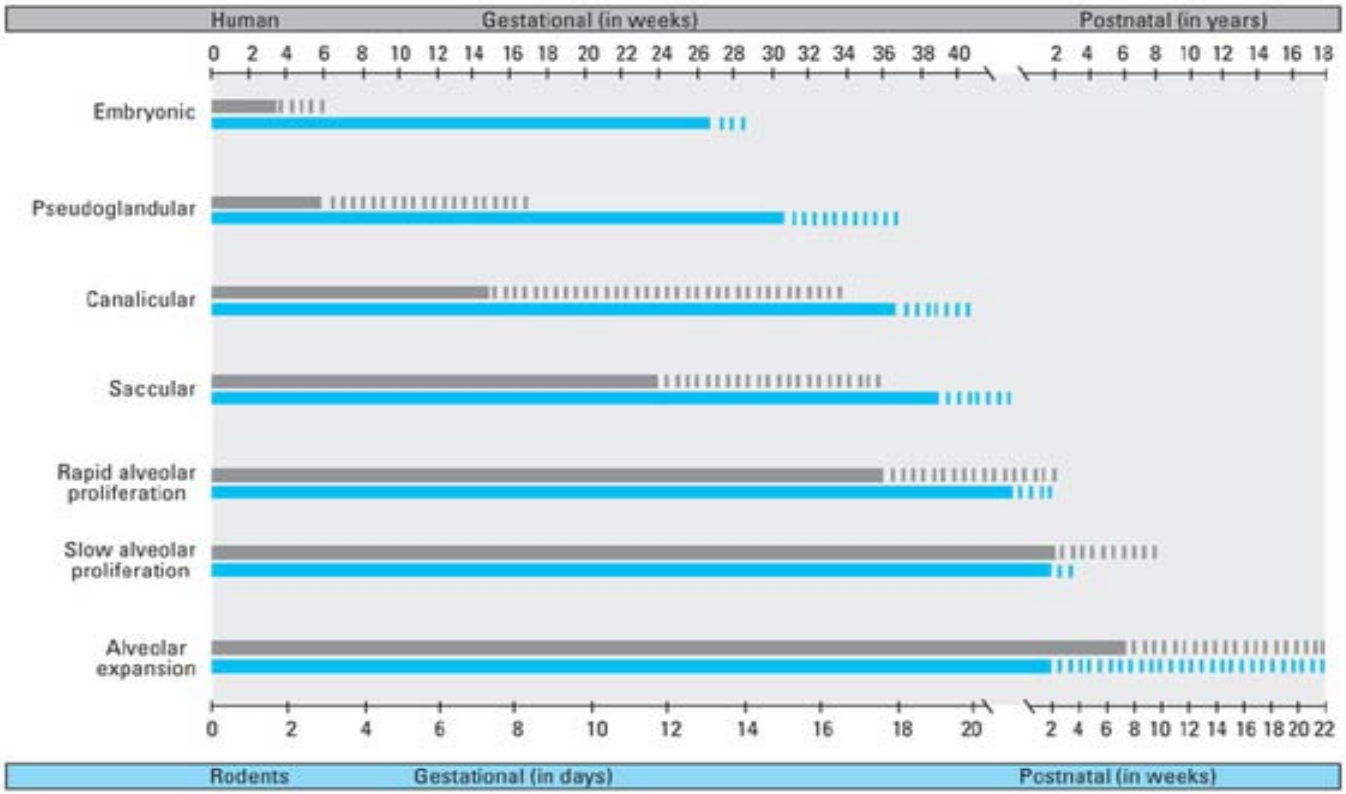


Figure 5. Respiratory system development in humans and rodents. Discrete maturational windows for prenatal and postnatal development are shown using hashed bars and compared for humans and rodents. These discrete maturational windows represent periods in respiratory system development that may have differential vulnerabilities to respiratory toxicants (Dietert et al. 2000; Pinkerton and Joad 2000). The solid bars represent time to these initial maturational stages. Figure reproduced from Faustman et al. (2003) and reprinted with permission of the Institute for Risk Analyses and Risk Communication at the University of Washington.

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CyTOF Technology

- Mass Spectrometry based cytometer
- Link 32 colors to be able to display many immune cells, intracellular products and activation pathways simultaneously
- Allows for functional studies to occur

Organ System Development (Grey= Human)

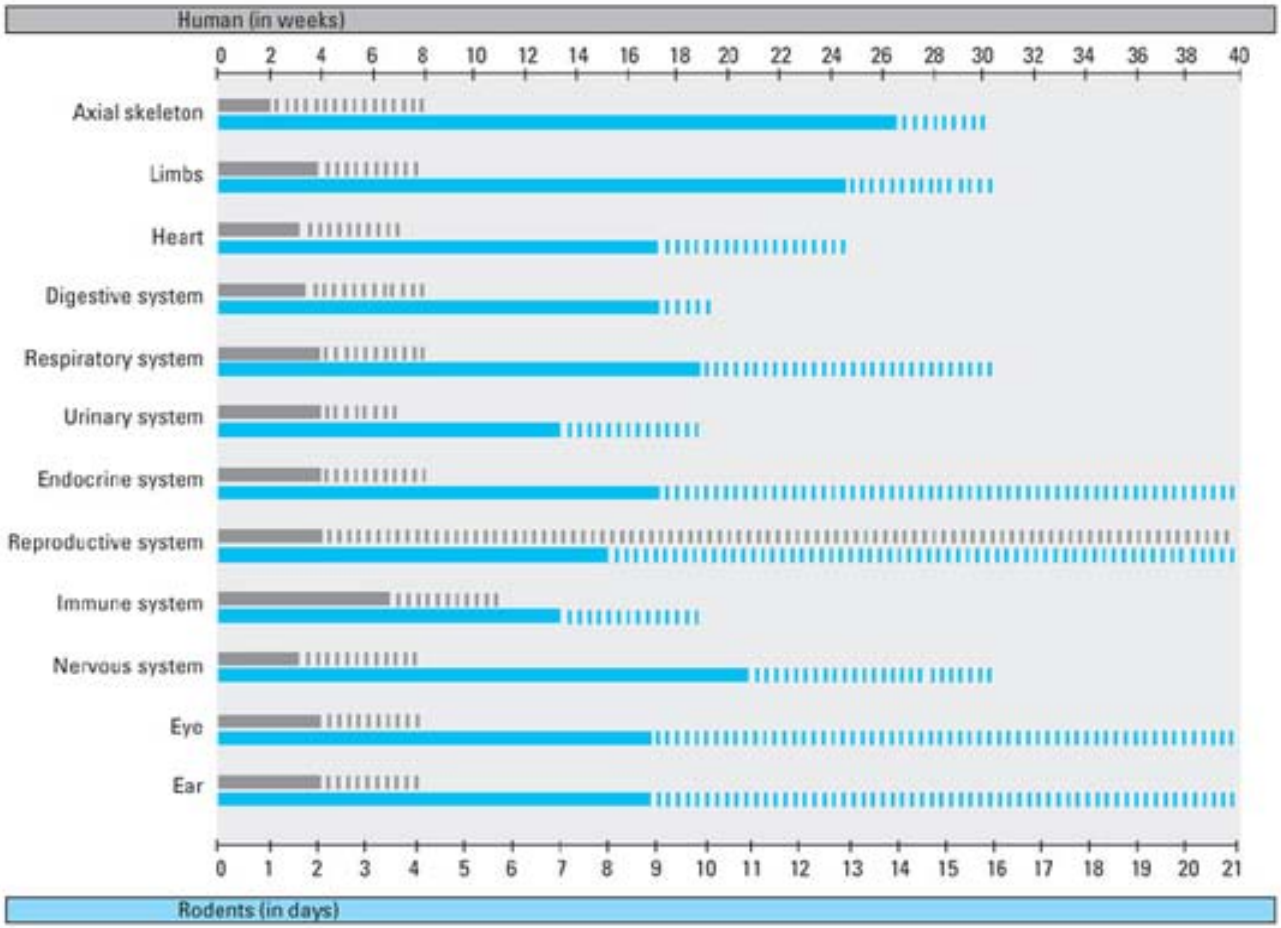

Figure 4. Initial appearance of organ systems during gestation in humans and rodents. A comparative timeline for the initial appearance of cellular structure of the various components of each organ system during gestation is given for humans and rodents. The solid bars represent time to initial appearance, and the hashed bars represent the time window of appearance of these initial organ systems. Note that for some complex organ systems, the appearance of many initial structures within that organ may be over an extended period of development. The connections and maturation for most of these systems continues until after birth for both species. Figure reproduced from Faustman et al. (2003) and reprinted with permission of the Institute for Risk Analyses and Risk Communication at the University of Washington.

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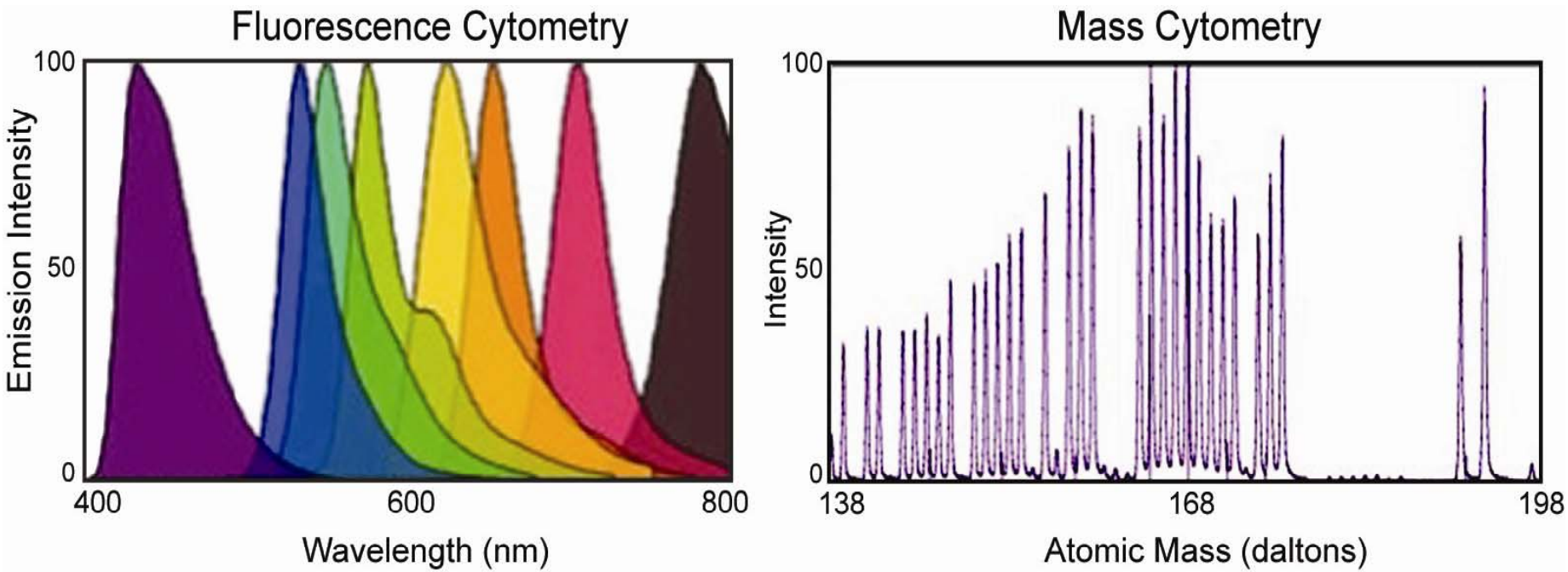
Mass Cytometry (CyTOF) Accommodates More Antibody Specificities without Overlap, Greatly Improving Immunophenotyping of Cells involved in Allergy



The DVS Sciences (Toronto, CA) has produced inductively coupled plasma mass spectrometer (ICP-MS), known as CyTOF (for Cytometry by Time-of-Flight Mass Spectrometry). In the Stanford HIMC (Director, Holden Maecker, PhD) we have been able to detect up to 35 different parameters in a single sample.

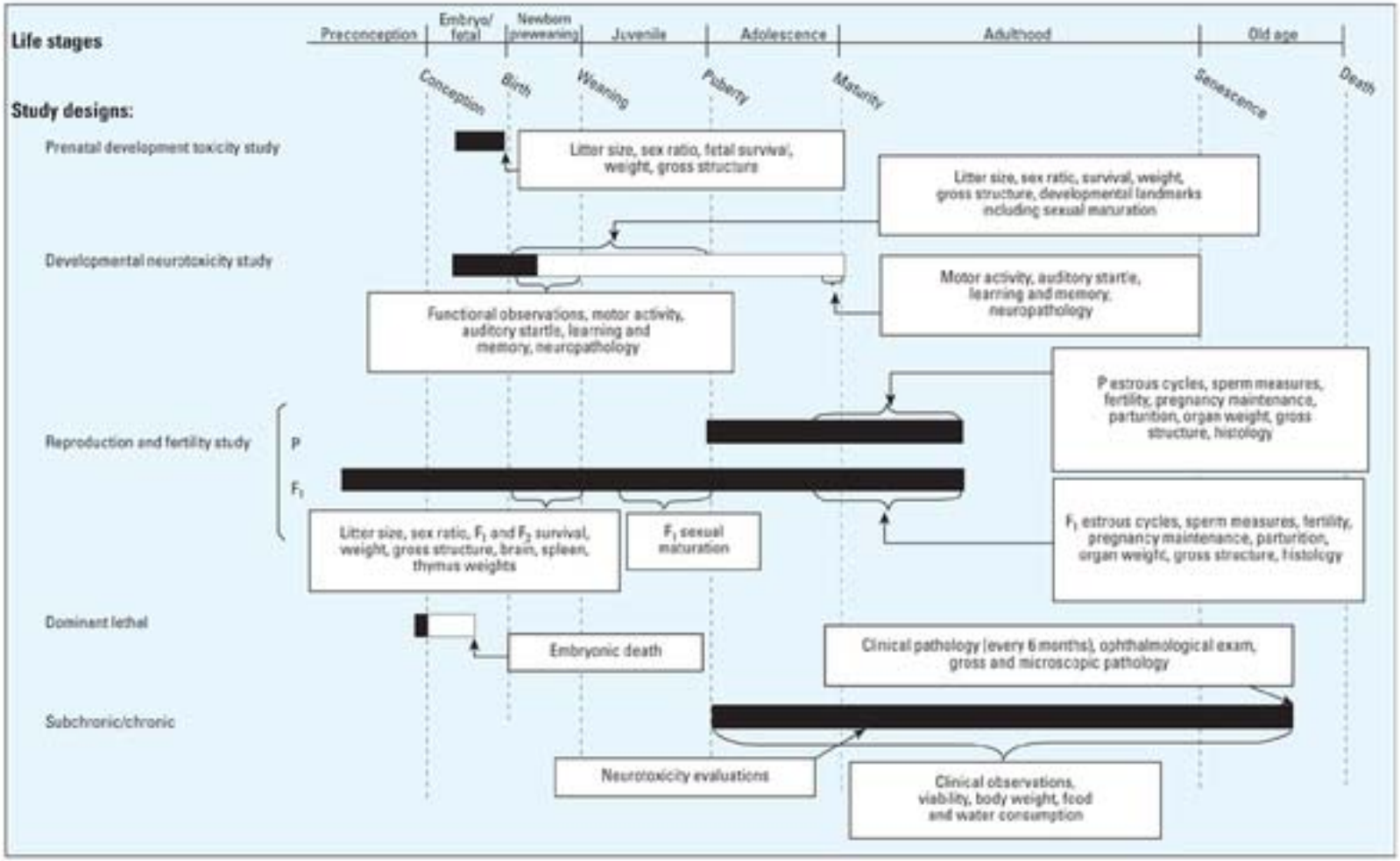
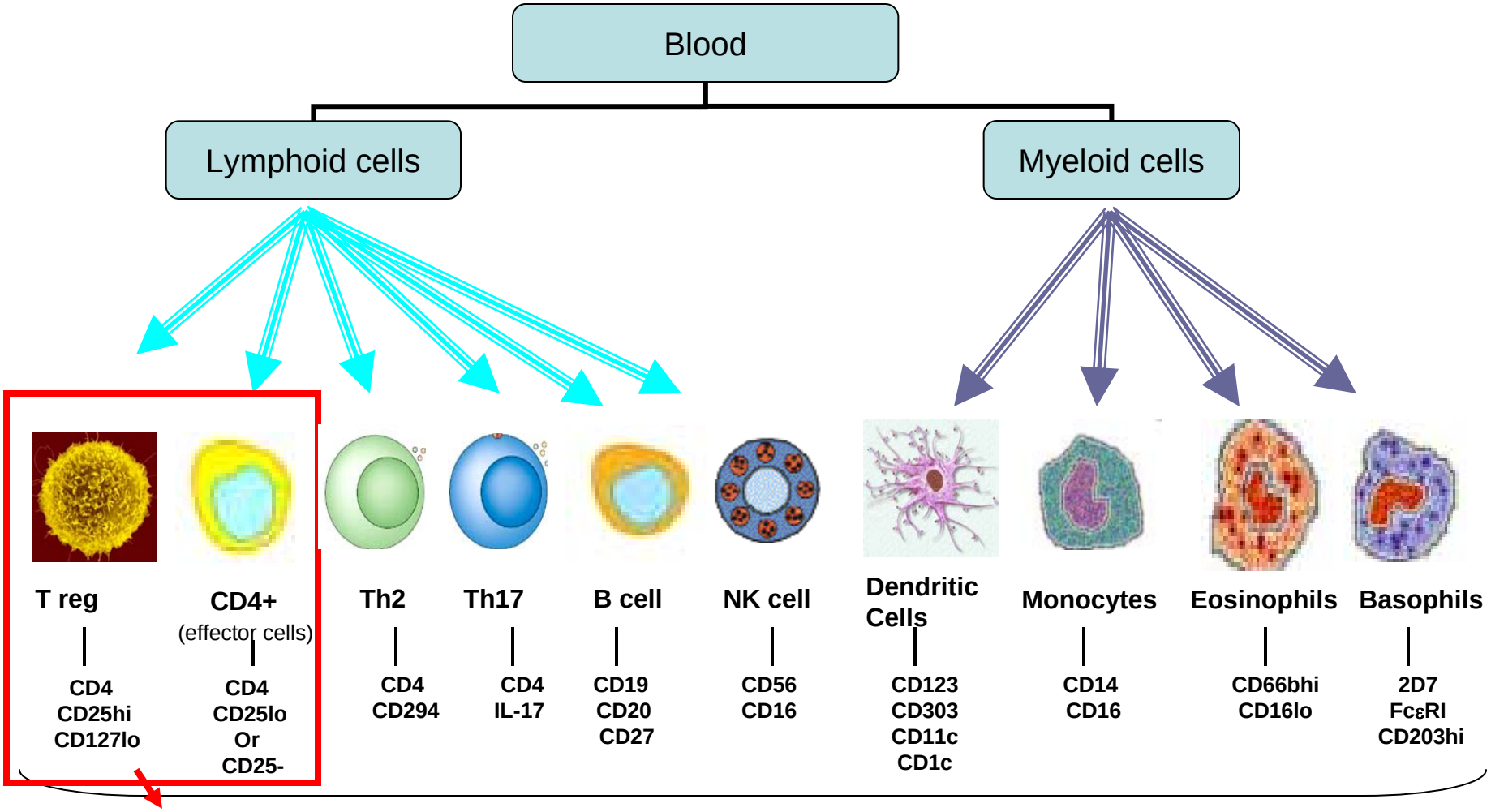


Figure 3. Exposures and end points related to developmental toxicity evaluations. Figure adapted from Kimmel (2001).

Phenotypical Studies



Molecules expressed on cell surface with CCR4, CCR8, CD45RO, CD45RA

Intracellular markers to be stained: Foxp3, pSTAT5, CCL1, CCL22, CCL17, IL-4, IL-13, IL-10, TGF-β, IL-17, IFN-γ

HUMAN IMMUNE MONITORING CENTER, STANFORD UNIVERSITY

Allergy Diagnosis

- Atopy risk vs Atopy
- IgE is not predictive
- Skin testing is not predictive
- Basophil activation test is being used currently as an improvement for allergy testing
- Gold Standard still remains the challenge test

Possible Method to Determine Effects of Toxicant on Development

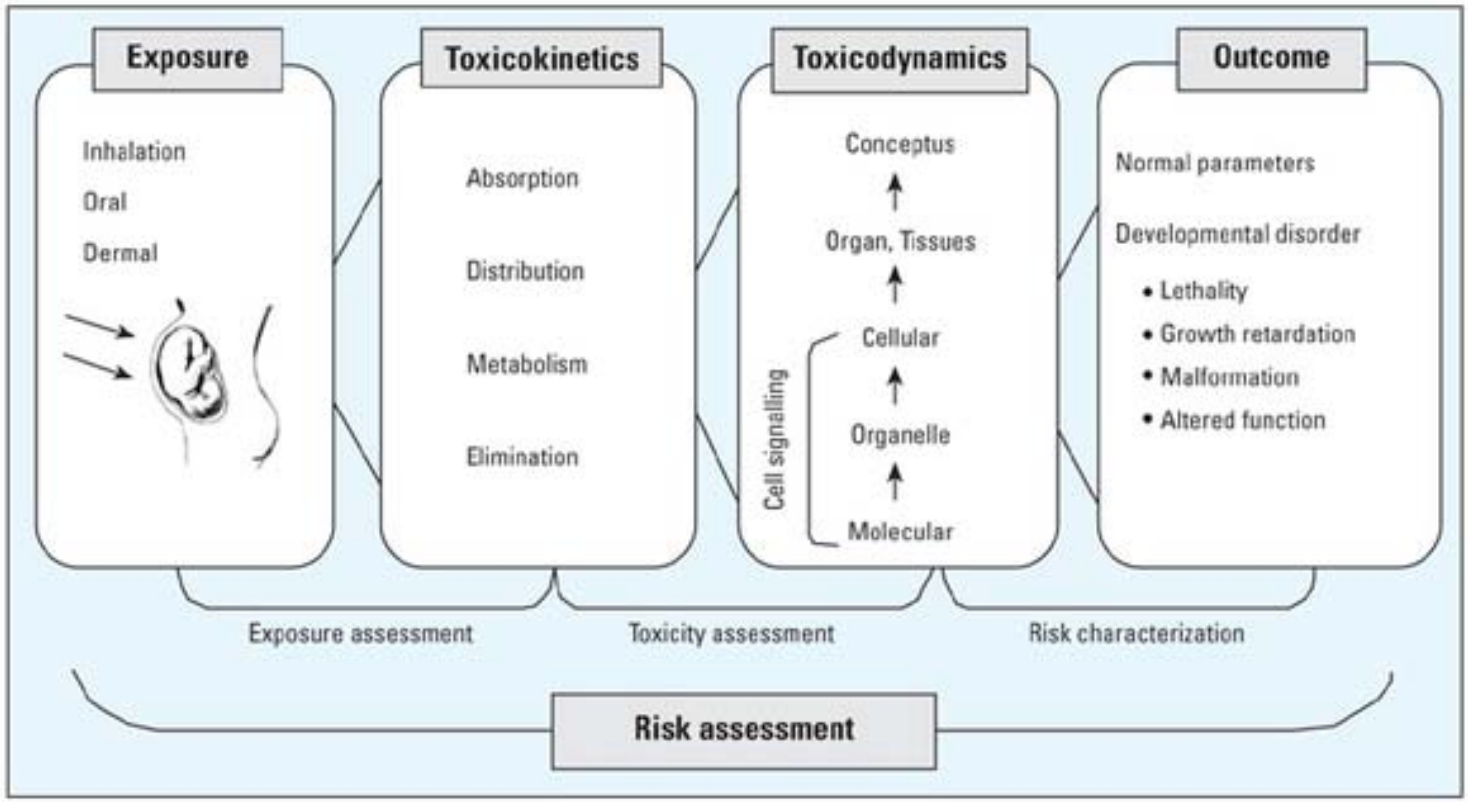


Figure 9. Levels of mechanistic inquiry for assessing the effects of a toxicant on development. Figure reproduced from NRC (2000) and reprinted with permission by the National Academy of Sciences, courtesy of the National Academies Press.

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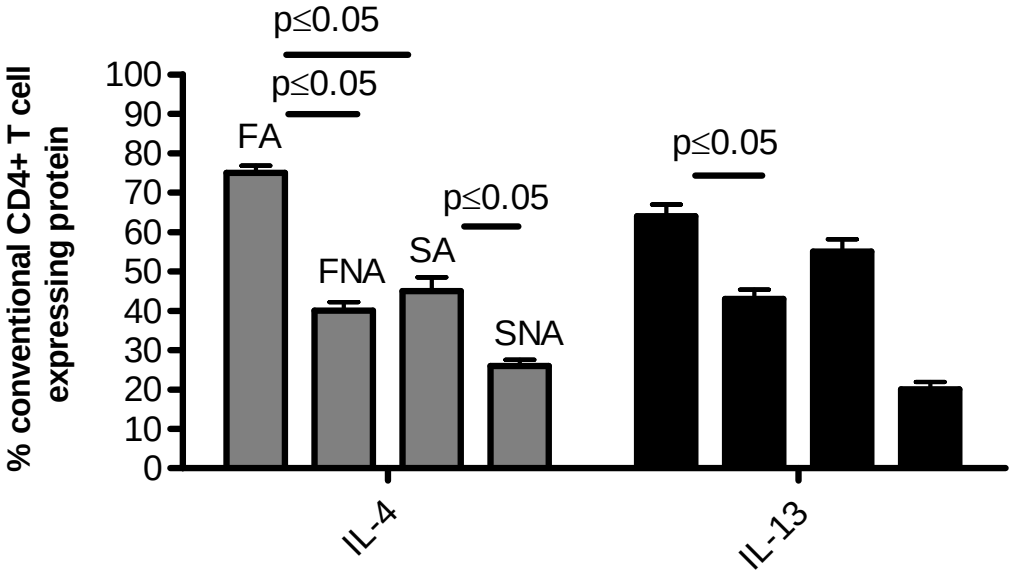
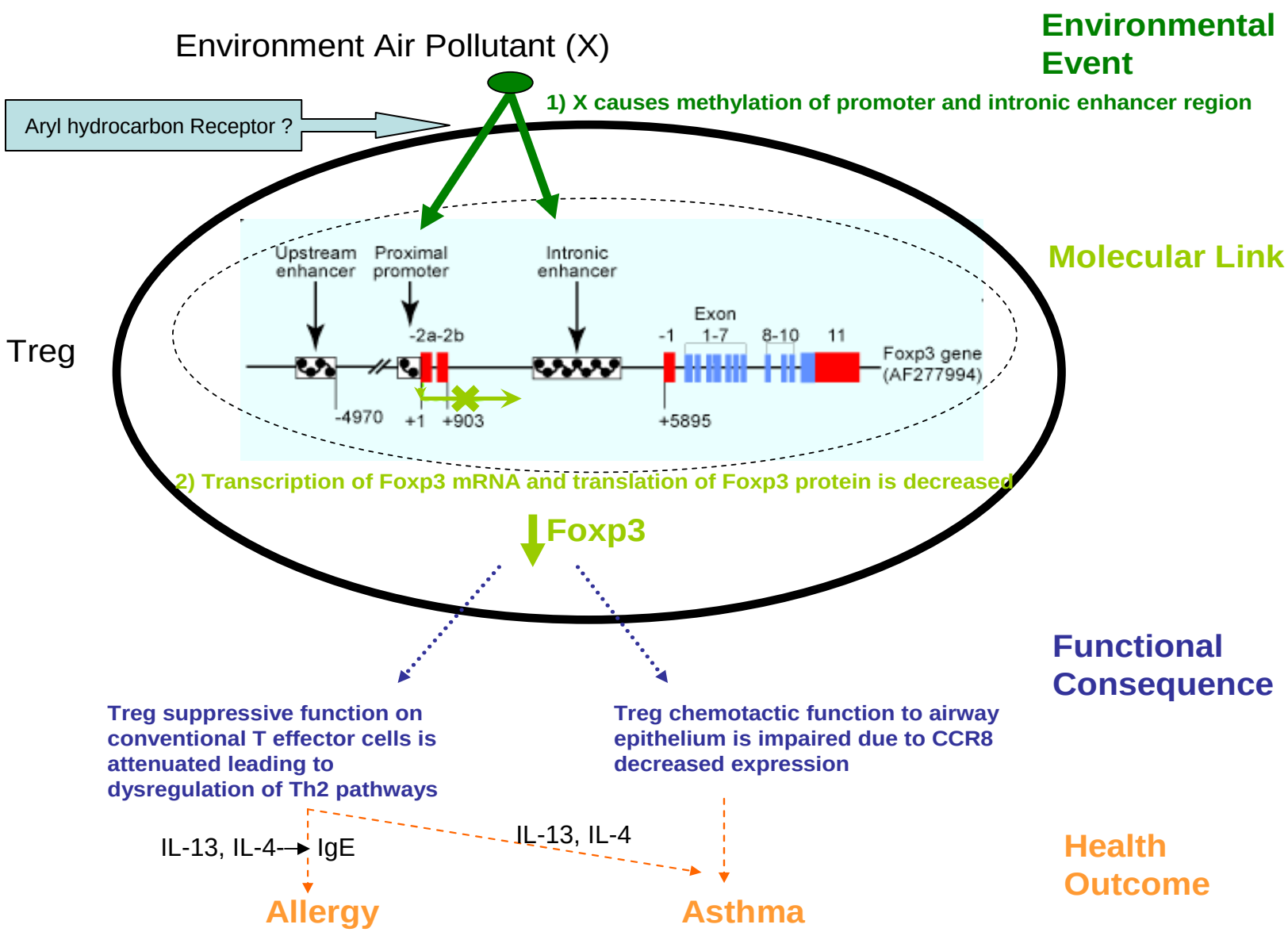
Enhanced Th2 skewing in FA subjects



Multicolor flow cytometry stained for CD4, CD25, IL-4, and IL-13 (FA, n=71, FNA, n=30, SA, n=40, SNA, n=40)

Possible Overall Schematic