Epigenetic Modifications After Ambient Air Pollution Exposure

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In collaboration with Fresno Asthma Children’s Environmental Study
Asthma

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

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

• 34 million Americans with asthma

• $20 billion/year spent on asthma care in the U.S.

• Possible Cause:
  – Inappropriate immune response in a genetically susceptible individual driven by environmental exposures

• Asthma can be linked to allergies, viruses, pollution, and other events
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}CD127^{lo} 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?
GENERAL HYPOTHESIS and AIM

Overall: Treg are dysfunctional in subjects 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 developing target-driven treatments to reduce the burden of asthma in children who are exposed to high levels of air pollution.
Methods

Four cohorts chosen:

- Children’s Environmental Study
  - 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, polycyclicaromatic 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 SA: Age matched and sex matched children with no asthma and no allergies (n=30)

Note: Stanford group must live 6 yrs or more at residence in Palo Alto, CA and must live 30 meters outside range of major highway

PAHs about \( \sim 7x \) higher in Fresno Air

Data in children exposed to chronic, high levels of phenanthrene demonstrate a mean serum concentration of 2.0 \( \mu \text{M} \), we chose a 10-fold higher concentration for our \textit{in vitro} work performed over 7 days, similar to other publications.

\begin{table}
\centering
\caption{Comparison of area ambient pollutant concentrations between Fresno and Palo Alto, Calif, based on CARB compliance monitoring for 2008}
\begin{tabular}{|l|c|c|}
\hline
Pollutant & Location of compliance monitor* & \\
 & Redwood City & First Street, Fresno \\
\hline
PAHs, annual average (ng/m\(^3\)) & 0.6 & 4.4 \\
PM\(_{2.5}\), annual average (\(\mu\text{g/m}^3\)) & 10.5 & 21.2 \\
PM\(_{2.5}\), 24-h maximum (\(\mu\text{g/m}^3\)) & 36.0 & 93.0 \\
PM\(_{10}\), 24-h high (\(\mu\text{g/m}^3\)) & 41 & 78 \\
\(\text{O}_3\), highest 8-h average (ppb) & 70 & 132 \\
\(\text{O}_3\), no. of days > state 1-h standard & 0 & 44 \\
\(\text{O}_3\), no. of days > state 8-h standard & 0 & 86 \\
\hline
\end{tabular}
\end{table}

*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.

Outcome Variables

For all 4 cohorts:

• Regulatory T cells (CD4+CD127loCD25^{hi}): molecular analysis and function

• Effector T cells or responder T cells (CD4+CD127+CD25^{neg}): molecular analysis (including epigenetics) 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.
Epigenetic Studies  

Foxp3 CpG regions are hypermethylated in FACES subjects

Schematic View of Human Foxp3 CpG Islands

Upstream CpG islands (-5786 to -5558 bp)  
Promoter islands (-210 to -25) N=8  
Intronic islands (+3826 to +4321) N=13

Binding site to:  
MeCP2, MBD2, DNTM1, DNTM3b  
SP1,AP1, TIEG1, SMAD3, NFAT, STAT5  
STAT5,CREB,ATF

Results

*Treg Foxp3 expression is associated with asthma severity*

A

![Graph showing Treg Foxp3 expression](image)

B

![Graph showing Treg function in FA subjects](image)

C

![Graph showing GINA Symptom Score against Treg expression](image)

D

![Graph showing percent predicted FEV1 against Treg function in FA subjects](image)

Nadeau, et al. 2010
Results

Methylation of CpG sites on Foxp3 locus in Treg is associated with PAH exposure

Nadeau, et al. 2010

\[ P \leq 0.001 \]

\[ P \leq 0.001 \]

\[ P \leq 0.01 \]

\[ R^2 = 0.81 \]
Th2 skewing occurred in FA subjects compared to other groups.

Nadeau, et al. 2010
Ex vivo studies using phenanthrene in culture with Treg
DNMT inhibitor or AhR inhibitor abrogate the effects of Phenanthrene

**B**

Methylated CpG islands in Foxp3 locus

**C**

Foxp3 transcript expression (fold over β-glucuronidase)
Gene Silencing Experiments demonstrate dependency of DNMT1 and 3a expression on presence of AhR

![Graph showing transcript expression](image-url)
Molecular links between pollution, immune system changes and health outcomes

Summary and Next Steps

• Clinical Outcomes (ie. Severity of asthma) correlate inversely with presence of memory Treg with stable Foxp3 expression
• Memory Treg, not Naïve Treg, are most affected by air pollution effects
• Foxp3 is unstable in Treg after methylation of promoter and intronic regions
• ChIP Seq demonstrates AhR-Sp1 binding site in promoter region of DNMT1
• Test in AhR knock out model in mice (Sunwoo lab)
Possible Overall Schematic

Environment Air Pollutant (X)

Aryl hydrocarbon Receptor

1) X causes methylation of promoter and intronic enhancer region

AhR-Arnt binds with Sp1 to DNMT1 promoter

2) Transcription of Foxp3 mRNA and translation of Foxp3 protein is decreased

Foxp3

Treg suppressive function on conventional T effector cells is attenuated leading to dysregulation of Th2 pathways

Treg chemotactic function to airway epithelium is impaired due to CCR8 decreased expression

IL-13, IL-4 \(\rightarrow\) IgE

IL-13, IL-4

Allergy

Health Outcome

Asthma
Thank you

- **Laboratory Team**
  - Nadeau lab: Marco Garcia, Olivier Humblet, Jennifer Jenks, Jing Liu, Takahiro Kanai, Shuchen Lyu, Jiming Rong, Scott Seki

- **Clinical Research Team**
  - Stanford: Tina Dominguez, Daniela Pineda, Margie Woch, Anne Keller, Grace Yu
  - Fresno: Leah Melendez, Cynthia Appel

- **Exposure/Epidemiology/Statistics Team**
  - Berkeley: Fred Lurmann, Katharine Hammond, Alan Hubbard

- **Current Collaborators**
  - Stanford: HIMC, CTRU, Boyd lab, Fire lab, Galli lab, Snyder lab, Sunwoo lab, Gary Shaw
  - Outside: Wesley Burks (Duke), Karagas lab (Dartmouth), Xiu-Min Liu (Mt Sinai), Miller lab (Columbia), Ira Tager (Berkeley), Umetsu lab (Harvard),
CHAPS SJV
Children’s Health & Air Pollution Study
San Joaquin Valley

Funded By:

Berkeley
UNIVERSITY OF CALIFORNIA

STANFORD
UNIVERSITY

EPA
United States Environmental Protection Agency

NIEHS
National Institute of Environmental Health Sciences

AMERICAN LUNG ASSOCIATION®
Back up slides
Sp1 binds as a transcription factor to DNMT1

Binding Site of Sp1 in DNMT1 promoter

-147  TCCCCATCACACCTGAAAGAATGAATGAATGAATGCCTCGGGCACC
-100  TGCCCACCTCCAGCAACCGTGAGCTTGAGACGAGCCACTGCTCCGCG
-50   TGGGGGGGTGTGTGCCCGCCTTGCGCATGCGTGTTCCCTGGGCATGGCC
1     GGCTCCGTTCCAGGATCGCGGCTCCCCCTGCGCCTGCCTGCCTGC
51    ATCCCCCTCCCTCCCCACGCCGGACTGGGTGTAGACGCCCGCTCCGCT
101   CATGCCCTTCCCCATCGTTTCCGCGCGAAAGCCGGGGCGCTTGCGCT
151   GCCGCGCCCGCG

DNA ase I footprinting demonstrates Sp1 binds AhR-Arnt complex

Kobayashi, et al. 1995. JBC
Why focus on Fresno?

State of the Air 2011

<table>
<thead>
<tr>
<th>Metropolitan Area</th>
<th>US Rank 2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bakersfield</td>
<td>1</td>
</tr>
<tr>
<td>Fresno</td>
<td>2</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>4</td>
</tr>
<tr>
<td>Visalia</td>
<td>7</td>
</tr>
<tr>
<td>Hanford</td>
<td>9</td>
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<td>San Diego</td>
<td>15</td>
</tr>
<tr>
<td>Stockton</td>
<td>16</td>
</tr>
<tr>
<td>San Jose – San Francisco – Oakland</td>
<td>24</td>
</tr>
</tbody>
</table>

American Lung Association
Methods

PAH accumulative exposure over 12 months

Legend
11/22/2003, n=269
est.pah456 ng/m3

- 15.1 - 20.0
- 20.1 - 25.0
- 25.1 - 30.0
- 30.1 - 35.0
- 35.1 - 40.0
- 40.1 - 45.0

FSF
Highways
major roads
land use
Urban
Agriculture
Semi-agricultural
Native vegetation
Waterways

0 1 2 4 Miles
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
Technologies available for Immune Monitoring

• In addition to:
  – Proteomics (protein arrays using antibodies or HPLC)
  – Metabolomics (examining small molecules/products in human samples)
  – RNA-Seq (replacing microarrays)
  – Complete Genomic Sequencing
  – Methylomics of whole blood gDNA
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 in small amounts of blood
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.
Phenotypical Studies

Blood

Lymphoid cells

Myeloid cells

T reg
CD4+
CD4
CD25hi
CD127lo

CD4
CD25lo
Or
CD25-

Th2
CD4
CD294

Th17
CD4
IL-17

B cell
CD19
CD20
CD27

NK cell
CD56
CD16

Dendritic Cells
CD123
CD303
CD11c
CD1c

Monocytes
CD14
CD16

Eosinophils
CD66bhi
CD16lo

Basophils
2D7
FcεRI
CD203hi

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
Stimulated by Antigen

Unstimulated

Expression of molecules on the cell surface (expressed by cytoplasmic compartments) for example CD203c or CD63

New Diagnostic Allergy Test based on rapid assessment of blood basophil activation
New Diagnostic Allergy Test
Based on rapid assessment of blood basophil activation

1. Spin 300 x g
2. 0 Breaks
3. Plasma Leukocytes Gradient
4. RBC
5. Antibodies
   1. Anti-CD203c
   2. Anti-CD63
   3. Anti-CD123
   4. HLA-DR

Abs diluted in 1% BSA, 0.05% NaN₃ in PBS

Wash And Flow Cytometry

TIME To RESULT: ~ 1 HOUR

• No RBC Lysis
• Activation stop during the staining with 1% BSA, 0.05% NaN₃
• Various Allergens/Patients can be tests at the same time
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:

pre
Detection of Clonal Sequences: post
Fluidigm Biomark Platform for Single Cell qPCR on Many Genes
Systems Biology Approach to Immune Monitoring in Human Samples

Summary and Next Steps

• High Throughput sequencing on small amts of blood
• Detailed phenotypical and functional studies on small amts of blood
• Single cell PCR will allow detection of critical changes in specific cell populations

Next steps

• Application of use to samples (thousands)
• Determine correlation with immune development and risk assessments in human development to toxic can exposure