Molecular and Genetic dissection of Chronic Inflammatory Diseases
Discovery of novel pathogenic genes and/or pathways

Rheumatoid Arthritis

- Chronic inflammation
- Persistence of cytokine networks
- Immune perturbations
- Effector cells: Fibroblasts
- Deregulated ECM homeostasis
- Local, tumor-like behavior

Idiopathic Pulmonary Fibrosis

- Chronic inflammation
- Persistence of cytokine networks
- Immune perturbations
- Effector cells: Fibroblasts
- Deregulated ECM homeostasis
- Local, tumor-like behavior

Common characteristics

- Polygenic, multi-factorial nature
- Pathogenic complexity and redundancy
- Pathogenic mechanisms not completely understood

Discovery of novel pathogenic genes and/or pathways

Approach Outline

Discovery Driven (Top down)
- High throughput methodologies
- Sequenced genomes
- Bioinformatics

Hypothesis Driven (Bottom up)
- Molecular & Cellular methodologies
- Functional assays
- Genetic modification of mice
- Animal modeling

Expression profiling of Rheumatoid Arthritis (RA)
Discovery of novel pathogenic genes and/or pathways
**RHEUMATOID ARTHRITIS (RA)**

- Common human disease (1% prevalence)
- Abnormal immune response
- Chronic inflammation of the joints
- Prominent development of new vessels
- Synovial hyperplasia
- Bone & cartilage destruction

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**Animal models of arthritis**

**Induced:**
- Collagen Induced Arthritis (CIA)
- Adjuvant Arthritis (AA)
- Antigen Induced Arthritis (AIA)

**Spontaneous:**
- Tg197 hTNF transgenic
- mTNF^∆ARE^ knock in

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**Expression profiling of RA - Animal Models**

- Tg197
- mTNF^∆ARE^ knock in

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**Expression profiling of RA - RNA samples isolation**

- Total RNA: RA WJ, RA pSF, wt WJ, wt pSF

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**Expression profiling of RA - Platforms**

**I.**
- Subtractive libraries
  - cDNA libraries
  - Library normalization
  - Library subtraction
  - Large scale sequencing
  - BLAST
  - Statistical analysis

**II.**
- DNA microarrays
  - cDNA samples - labeling
  - Chip hybridization
  - Data pre-processing
  - Statistical analysis

Cross platform comparison
Differentially Expressed Genes (DEGs)

Meta-analysis
Confirmation - Validation
Expression profiling of RA – I. Subtractive libraries

- 1st strand cDNA synthesis at 56°C in the presence of trehalose & sorbitol
- 2nd strand cDNA synthesis (SSLLM)
- Biotin/Streptavidin CAP selection of full length cDNAs
- Cloning in Phagemid λ-FLCII
- Solid phase amplification
- In vivo cre-mediated plasmid bulk excision from Phagemid λ-FLCII
- Single stranded plasmid preparation

Expression profiling of RA – I. Subtractive libraries

L0= Tg197 SFs minus wt SFs : Upregulated genes in RA SFs
L1= wt SFs minus Tg197 SFs : Downregulated genes in RA SFs
L2= wt WJs minus Tg197 WJs : Downregulated genes in RA WJs
L7= Tg197 WJs minus wt WJs : Upregulated genes in RA WJs
L8= Tg197 WJs minus wt WJs

Expression profiling of RA – I. Subtractive libraries

Final Results

<table>
<thead>
<tr>
<th>Code</th>
<th>Results</th>
<th>Clusters</th>
<th>Redundancy</th>
<th>New genes (%)</th>
<th>New at RIKEN (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L0</td>
<td>7939</td>
<td>4097</td>
<td>346 (8.4)</td>
<td>856 (20.9)</td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>8602</td>
<td>4186</td>
<td>226 (5.4)</td>
<td>601 (15.6)</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>7753</td>
<td>3331</td>
<td>392 (11.8)</td>
<td>844 (25.3)</td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>3286</td>
<td>1740</td>
<td>120 (3.9)</td>
<td>283 (14.3)</td>
<td></td>
</tr>
<tr>
<td>L8</td>
<td>6486</td>
<td>1997</td>
<td>123 (6.2)</td>
<td>293 (14.7)</td>
<td></td>
</tr>
</tbody>
</table>

Expression profiling of RA – I. Subtractive libraries

Statistical analysis of differential expression

Differential expression likelihood R
(based on clone abundance in each library)

Estimation of R-value cutoffs
(identification of statistically significant thresholds)

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Average Cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td>L0 vs L1</td>
<td>0.939333</td>
<td>0.933333</td>
<td>2.581159</td>
</tr>
<tr>
<td>L2 vs L7</td>
<td>0.939333</td>
<td>0.933333</td>
<td>0.743888</td>
</tr>
<tr>
<td>L2 vs L8</td>
<td>0.939333</td>
<td>0.933333</td>
<td>2.439847</td>
</tr>
<tr>
<td>L7 vs L8</td>
<td>0.939333</td>
<td>0.933333</td>
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Expression profiling of RA - Platforms

I. Subtractive libraries
   - cDNA libraries
   - Library normalization
   - Library subtraction
   - Large scale, Sequencing
   - BLAST
   - Statistical analysis

II. DNA microarrays
   - cDNA samples - labeling
   - Chip hybridization
   - Data pre-processing
   - Statistical analysis

Cross platform comparison
Differentially Expressed Genes (DEGs)

↓

Meta - Analysis
Confirmation - Validation

Expression profiling of RA - II. DNA microarrays

Affymetrix Mu11K (A+B) chipset

In situ synthesis on glass
1.28x1.28 cm²
20 25mers/gene
13000 genes/ESTs
Fluorescent RNA labelling (PE)
One RNA population/chip

Expression profiling of RA - II. DNA microarrays

multiple DNA chip hybridizations
(In duplicates)

<table>
<thead>
<tr>
<th>Tg197</th>
<th>mTNF/ΔARE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole joints, ex vivo SFs, tsSFs</td>
<td>Whole joints, ex vivo SFs, tsSFs</td>
</tr>
</tbody>
</table>

Expression profiling of RA - II. DNA microarrays

Data analysis Outline
- Microarray intensities of 20 PM & 20 MM oligonucleotides per gene
- Affymetrix MAS
- Detection call & Signal intensity (background subtracted, average)
- Affymetrix MAS Scaling
- Bioconductor
- Quantile normalization
- Gene Spring
- Empirical filtering
- Fold Change Model
- Differentially Expressed genes (at 95, 95% significance levels)

Expression profiling of RA - II. DNA microarrays

Final Results

<table>
<thead>
<tr>
<th>Tg197</th>
<th>mTNF/ΔARE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSF</td>
<td>182 (60)</td>
</tr>
<tr>
<td></td>
<td>657 (325)</td>
</tr>
<tr>
<td>WJ</td>
<td>113 (211)</td>
</tr>
<tr>
<td></td>
<td>413 (409)</td>
</tr>
</tbody>
</table>

Specificity 95 (90) %
Expression profiling of RA - Cross-platform comparison - DEGs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Expression</th>
<th>Libraries</th>
<th>Microarrays</th>
<th>Common</th>
</tr>
</thead>
<tbody>
<tr>
<td>WJ</td>
<td>Up</td>
<td>(50) 214</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>(127) 133</td>
<td>439</td>
<td>11</td>
</tr>
<tr>
<td>SF</td>
<td>Up</td>
<td>(120) 117</td>
<td>150</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Down</td>
<td>(120) 234</td>
<td>127</td>
<td>7</td>
</tr>
</tbody>
</table>

High statistical importance (p<0.001) DEG list

Expression profiling of RA - Platforms

I. **Subtractive libraries**
   - cDNA libraries
   - Library normalization
   - Library subtraction
   - Large scale Sequencing
   - BLAST
   - Statistical analysis

II. **DNA microarrays**
   - cDNA samples - labeling
   - Chip hybridization
   - Data pre-processing
   - Data normalization
   - Statistical analysis

Cross platform comparison
Differentially Expressed Genes (DEGs)

Meta - Analysis
Confirmation - Validation

Expression profiling of RA - Meta - Analysis

- Automated text mining/Literature validation
- Semi quantitative RT-PCRs in mouse samples
- Real-Time RT-PCRs in mouse samples
- Cross referencing with genetic linkage
- Real-Time RT-PCRs in human samples

Expression profiling of RA - Functional validation

Additional prioritization
Differentially Expressed Genes (DEGs)

Differentially Expressed Functions (DEFs)
(As encoded by Gene Ontology (GO) terms)

Expression profiling of RA - Meta - Analysis

Prioritized DEGs

Expression profiling of RA - Functional validation

Grouping of Gene Ontology terms
Statistical analysis (hypergeometric distribution)
Discovery Driven Hypothesis generation:
Do Arthritic Synovial Fibroblasts (SFs) have a Re-arranged actin cytoskeleton?

- wt SFS
- RA SFS

Expression profiling of RA - Functional validation
Arthritic Synovial Fibroblasts:
- have a re-arranged actin cytoskeleton with pronounced stress fibers and exhibit more pronounced FAK and Tyrosine phosphorylation, suggesting exaggerated signaling from the membrane.
- Adhere stronger to the ECM in vitro, exhibiting a more elongated, flattened shape in vivo

Which is the major gene among the prioritized DEGs which is involved in actin cytoskeleton Dynamics?

- Actin binding protein with filament severing properties
- Downregulated in a variety of tumors
- Downregulated in RA (in two animal models and in human patients)
- Maps to CIA QTL (genetic linkage studies)
- Gelsolin-/- fibroblasts exhibit stress fibers very similar to RA-SFs

So does gelsolin ablation exacerbate the disease?

Tg197^-/-  ×  Gelsolin +/-  RA?
Gelsolin deficiency promotes RA pathogenesis, mainly through synovial membrane hyperplasia.

Arthritic Synovial Fibroblasts:
- have a re-arranged actin cytoskeleton with pronounced stress fibers and exhibit more pronounced FAK and Tyrosine phosphorylation, suggesting exaggerated signaling from the membrane.
- Adhere stronger to the ECM (in vitro), exhibiting a more elongated, flattened shape.

Which other gene among the DEGs which is involved in actin cytoskeleton dynamics?

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- have a re-arranged actin cytoskeleton with pronounced stress fibers and exhibit more pronounced FAK and Tyrosine phosphorylation, suggesting exaggerated signaling from the membrane.
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- Adhere stronger to the ECM (in vitro), exhibiting a more elongated, flattened shape.

Which other gene among the DEGs which is involved in actin cytoskeleton dynamics?
Autotaxin (ATX/Enpp2)
Generation of a conditional ATX knockout mice
(Poster 30)

- Heterozygous ATX+/- mutant mice are healthy and fertile.
- Homozygous ATX-/- mutant mice die at E9.5 dpc.
- ATX-/- mutant mice exhibit defective angiogenesis.
- ATX-/- mutant mice have malformations of the nervous system including swollen allantois, open neural tube, asymmetric headfolds while they exhibit no axial turning and aberrant dorsal ventral patterning.
- The above results suggest that ATX is essential for neuronal development and proper angiogenesis.

Autotaxin (ATX/Enpp2)
Generation of the complete ATX knockout mice
(Poster 30)

- Heterozygous ATX+/-. mutant mice are healthy and fertile.
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- ATX-/- mutant mice have malformations of the nervous system including swollen allantois, open neural tube, asymmetric headfolds while they exhibit no axial turning and aberrant dorsal ventral patterning.
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Autotaxin (ATX/Enpp2)
ATX involvement in various Chronic Inflammatory Diseases

- RA
- IPF
- MS

Expression profiling of Pulmonary Fibrosis (IPF)
Discovery of novel pathogenic genes and/or pathways

(Idiopathic) Pulmonary Fibrosis
Cryptogenic fibrosing alveolitis

- Progressive dyspnea and worsening of lung function.
- Radiographic evident diffuse pulmonary infiltrates (honeycombing).
- Histological pattern of Usual Interstitial Pneumonia (UIP): Varying degrees of inflammation, fibrosis, or both.
- Fibroblast foci and exuberant deposition of ECM.
- Secondary to a variety of autoimmune disorders, including RA.

(Idiopathic) Pulmonary Fibrosis
Etiology

Fibroblast activation
Contraction and remodeling of ECM
Experimental procedure

- 5 mice /group /time point
- 100 mg/kg of body weight (1/3 LD)
- Sacrifice 7, 15 and 23 days
- Record weight of animal
- Perform lung lavage (BAL)
- Cytospin - total and differential cell counts
- BAL fluid – 70°C
- Perfusion via the heart

Histopathology

- Total soluble collagen content
- RNA

Total & differential cell counts
- Inflammatory index
- Fibrotic index

Animal model of IPF (BLM induced)

Differentially Expressed Genes (DEGs)

Meta - Analysis
Confirmation - Validation

Expression profiling of IPF - Platforms

I. CAGE
   (Cap Analysis of Gene Expression)

II. DNA microarrays
   - cDNA samples - labeling
   - Chip hybridization
   - Data pre-processing
   - Data normalization
   - Statistical analysis

Cross platform comparison
Differentially Expressed Genes (DEGs)

Expression profiling of IPF - cDNA microarrays

- Riken chip
- Indirect Labelling
- 28 Grids
- 441 genes/grid
- 21,148 genes/chip

- RNA extraction
- RNA Purification (single passage through an RNeasy column)
- RNA Integrity (electrophoresis on denaturing 1.2% agarose/formaldehyde gel)
- RNA quantity/quality (OD readings at 260/280 nm)

Expression profiling of IPF - cDNA microarrays

- Image extraction and quantification,
  (signal 80–95% light intensity, background 5–20% light intensity)
- Pre-processing - Filtering
- Normalization
- Statistical selection
  - One way Anova

Expression profiling of IPF - cDNA microarrays

Data analysis Outline

- Statistical analysis
- Gene Ontology analysis
- Text mining analysis
- Differentially expressed genes & pathways
- TNF related genes
- Gelsolin and actin binding proteins
- Hypoxia related (inducing or induced)
Expression profiling of IPF – Validation

**TNF a pleiotropic cytokine**

Beneficial functions
- Resistance to infections
- Humoral and cellular responses
- Immune homeostasis

Detrimental functions
- Inflammation
- Direct cytotoxicity
- Metabolic disturbances

• TNF is initially produced as a membrane-associated 26kd form
• TmTNF is enzymatically cleaved by TACE into the soluble 17.5kd cytokine
• Both forms of TNF has been shown to be functionally active
• solTNF is regarded as the main ligand for p55TNFR
• TmTNF is superior to solTNF in activating p75TNFR

**Expression profiling of IPF – Validation**

TNF & IPF
- Increased levels of TNF in BronchoAveolarLavage fluid of human patients with IPF
- Increased levels of TNF in BronchoAveolarLavage fluid of various animal models of IPF
- TNF polymorphisms have been associated with an increase risk of developing the disease
- Controversial role

**Expression profiling of IPF – Validation**

Bleomycin induced animal model of IPF
- Genetically modified animals lacking components of TNF/TNFR signaling

<table>
<thead>
<tr>
<th></th>
<th>wt</th>
<th>tnf-/-</th>
<th>tnfR-/-</th>
<th>tnfR+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>YES</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>

Complementation with aerosolized recombinant TNF restores disease potential!!!

**Expression profiling of IPF – Validation**

TNF & IPF

Conclusions
- TNF is necessary for the development of PF
- Inflammatory responses elicited by TmTNF do not support the development of PF
- Redundant TNFR signaling in PF
Disease induction was always associated with increased lymphocyte accumulation which was severely abrogated in animal lacking soluble TNF. Complementation with recombinant TNF, that restores disease potential, resulted in massive lymphocytes accumulation.

**Conclusions**
- TNF is necessary for the development of PF
- Inflammatory responses elicited by tmTNF do not support the development of PF
- Redundant TNFR signaling in PF
- Lymphocytes are necessary for sol-TNF driven PF

**Identification of possible cellular sources of TNF**
- Bone marrow transfer experiments
- i.v. injection of ID8 cells
- Absence of hematopoietic compartment
- Chimeric-reconstituted mouse

"Pathogenic" TNF is secreted from apoptosing epithelial cells.
Expression profiling of IPF – Validation

TNF & IPF

Conclusions
- TNF is necessary for the development of PF
- Inflammatory responses elicited by tmTNF do not support the development of PF
- Redundant TNFR signaling in PF
- Lymphocytes are necessary for sol-TNF driven PF
- Stromelysin derived TNF is sufficient for disease development
- TNF is expressed from apoptosing epithelial cells
- Redundant TNFR signaling in PF
- TNF is necessary for TGF induction and fibrogenesis

Expression profiling of IPF – II. DNA microarrays

Gene Ontology analysis
Text mining analysis
Differentially expressed genes & pathways
TNF related genes
Gelsolin and actin binding proteins
Hypoxia related (inducing or induced)

Expression profiling of IPF – Confirmation

Gelsolin & IPF

cDNA microarrays
Real Time RT-PCR

Expression profiling of IPF – Validation

Gelsolin & IPF

Identification of the cellular types involved in disease protection

MPO assay
Total neutrophils

Impaired infiltration of neutrophils in the lung of gsn-/- mice upon BLM or LPS injection

Expression profiling of IPF – Validation

Gelsolin & IPF

Complete protection from disease induction

Histopathology
Inflammatory Index
Fibrotic Index

Reduced lung damage and KC mRNA expression in gsn-/- mice (epithelial cells)
Gelsolin & IPF

Identification of the cellular types involved in disease protection

Expression profiling of IPF - Validation

Gelsolin & IPF

Reduced apoptosis in gsn⁻/⁻ epithelial cells and MEFs

Bone marrow transfer experiments

gsn⁻/⁻ on wt

wt on gsn⁻/⁻

gsn deficiency in stromal cells is responsible for disease protection

Conclusions

gsn deficiency in epithelial cells results in defective epithelial apoptosis, reduced expression of chemotactants and reduced neutrophil infiltration which confer resistance to IPF

Rheumatoid Arthritis

Idiopathic Pulmonary Fibrosis

Common characteristics

- Chronic inflammation
- Persistence of cytokine networks
- Immune perturbations
- Effector cells: Fibroblasts
- Deregulated ECM homeostasis
- Local, tumor-like behavior

Additional common characteristics

- Presence of myofibroblasts and cytoskeletal rearrangements
- TNF involvement
- Gsn involvement (with different, opposing functions)
- ATX over expression

Molecular and Genetic dissection of Chronic Inflammatory Diseases

Discovery of novel pathogenic genes and/or pathways

Discovery driven Title
Stromal cells in the pathogenesis of Chronic Inflammatory Diseases
Discovery of novel pathogenic genes and/or pathways
Hypothesis driven Title