Identification of synoviocyte activation inhibitors by parallel biomarker profiling

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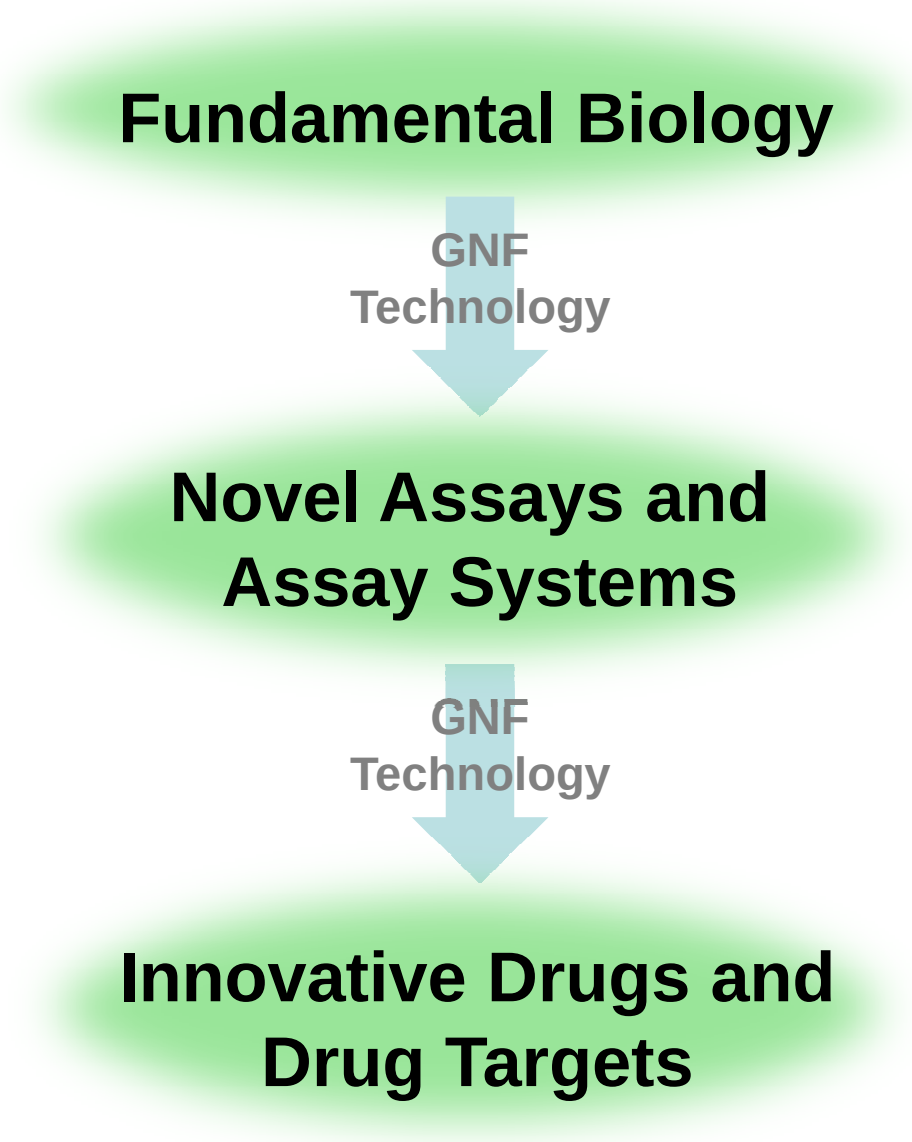
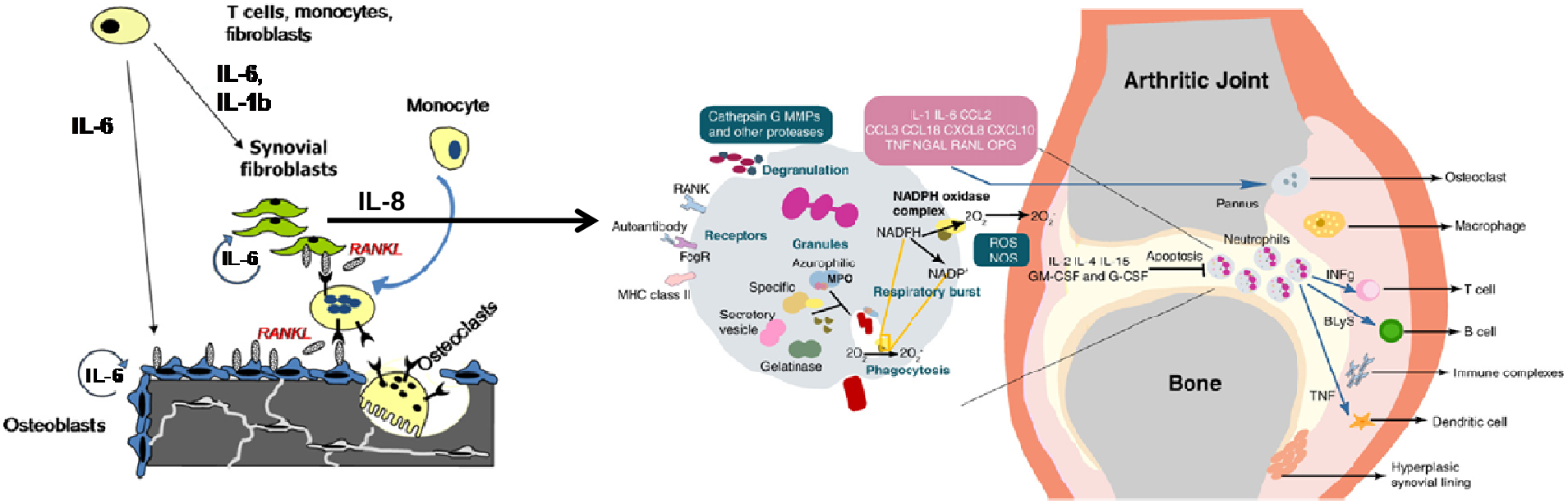
GNF Paradigm for Discovery



Synoviocyte Cytokine Secretion Screen

- Rationale:
  - Synovial fibroblasts contribute to the pathology of rheumatoid arthritis through hyperproliferation and cytokine secretion (IL-6, IL-8, MCP-1, TNFa).
  - Compounds that enhance or inhibit inflammatory cytokine secretion from RA synoviocytes could help identify novel targets and therapeutics for RA.
- Approach:
  - Expose cultured primary RA synoviocytes (Asterand) to purified natural product compounds, then stimulate with IL-1b.
  - Measure both IL-8 and IL-6 in supernatants by HTRF, looking for enhancers and inhibitors. Measure cell viability in parallel to eliminate toxic compounds/proteins



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Synoviocyte Natural Product screen: Workflow

Seed 1K cells/40uL/well in 384 (A)

Transfer 100nL compounds (B)

Transfer 5uL IL-1b stimulus (A)

Incubate o/n at 37 degrees (C)

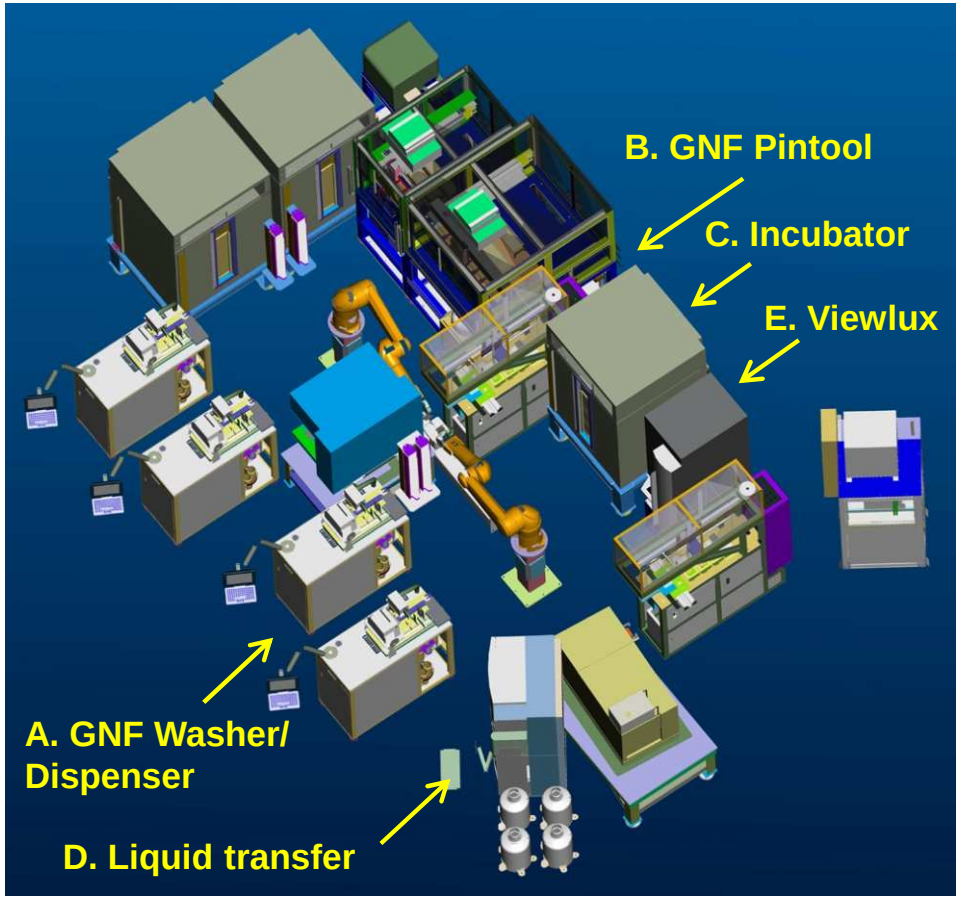
Transfer 2 uL/well sup into 4 wells of 1536-well plate containing 2uL/well media (D)

Add 20uL/well Cell Titer Glo to cell plate (A)

Add 4uL/well of IL-6 (odd rows) or IL-8 (even rows) HTRF reagent (A)

Read luminescence on Viewlux (E)

Incubate 2-3hrs at RT, read on Viewlux (E)

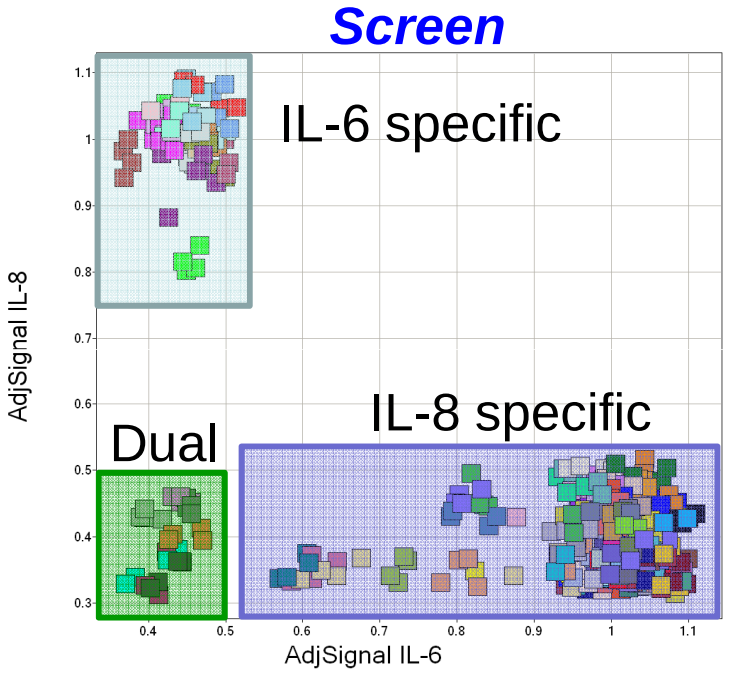


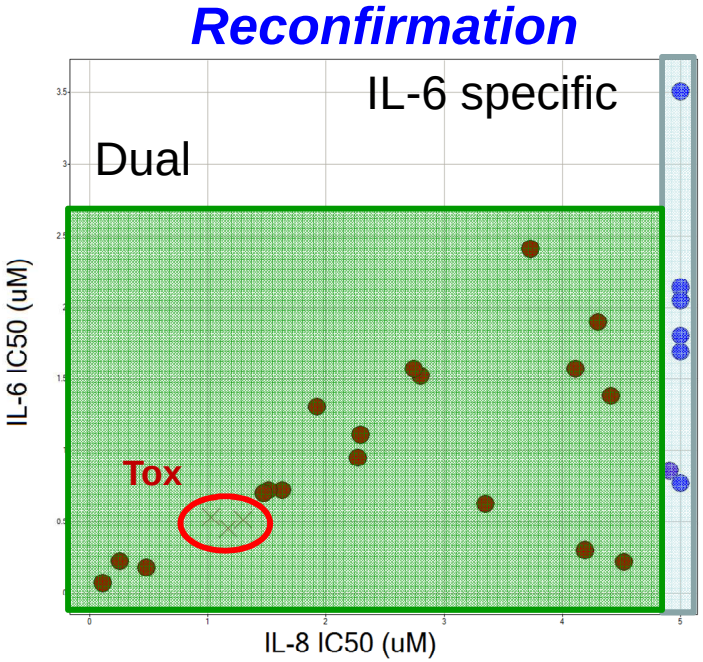
GNF Systems– the Enabling Technology

Parallel biomarker measurements and cell viability readout

Synoviocyte Natural Products Screen: Summary

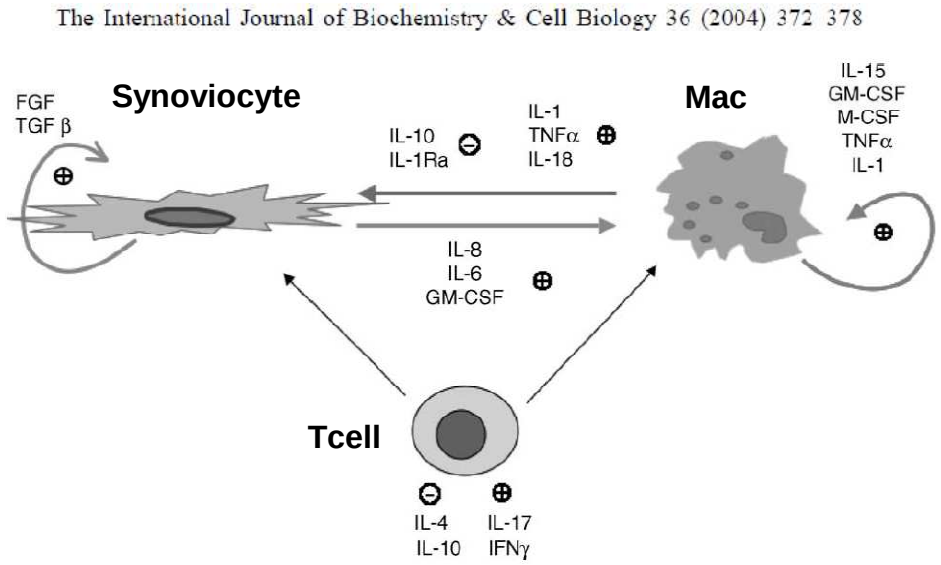
- Hits were chosen based on the following criteria:
  - At least 50% inhibition in all 4 replicates of either IL-6, IL-8, or both readouts
  - Less than 25% inhibition of cell titer glo signal
- 125 interesting hits were identified
  - 10 compounds with significant effect in both cytokine readouts
  - 96 compounds with significant effect in IL-8 readout only
  - 19 compounds with significant effect in IL-6 readout only
- Reconfirmation identified 23 compounds that showed inhibition in either IL-6, IL-8 or both without significant toxicity.
  - 19 compounds with dual activity
  - 6 compounds with IL-6 specific activity, 3 of which were also IL-6 specific in the original screen.

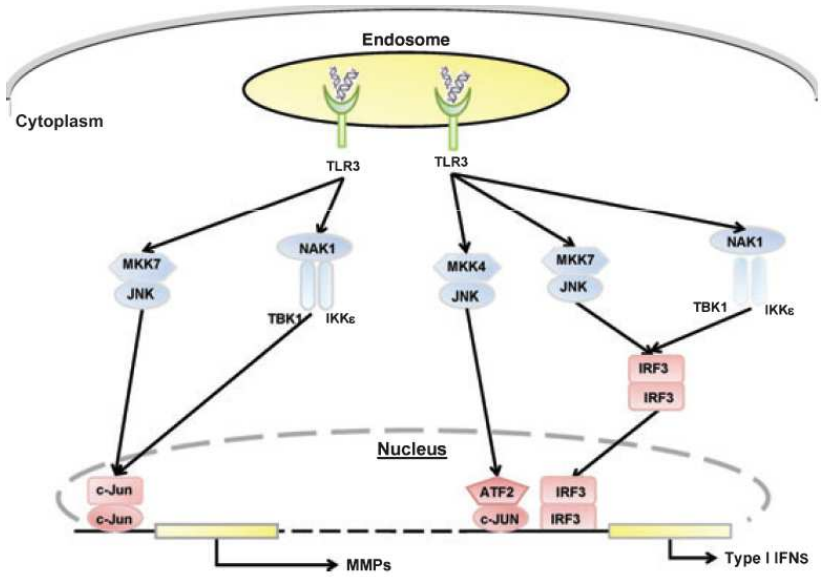




Synoviocyte Activation in RA

- Both IL-1b and TNFa stimulate pathogenic cytokine, chemokine, MMP, and adhesion molecule expression, but not all genes are expressed the same by both stimuli.
- Synovial fibroblasts express TLRs (2, 3, 4), and TLR activation is implicated in RA pathogenesis.
- Key questions: Do our natural product hits affect…
  - cytokines production following alternate stimuli (TNFa, LPS)?
  - MMP production from synoviocytes?
  - integrin expression in synoviocytes?
  - which signaling pathway?





Immunological Reviews 2010
Vol. 233: 233–255

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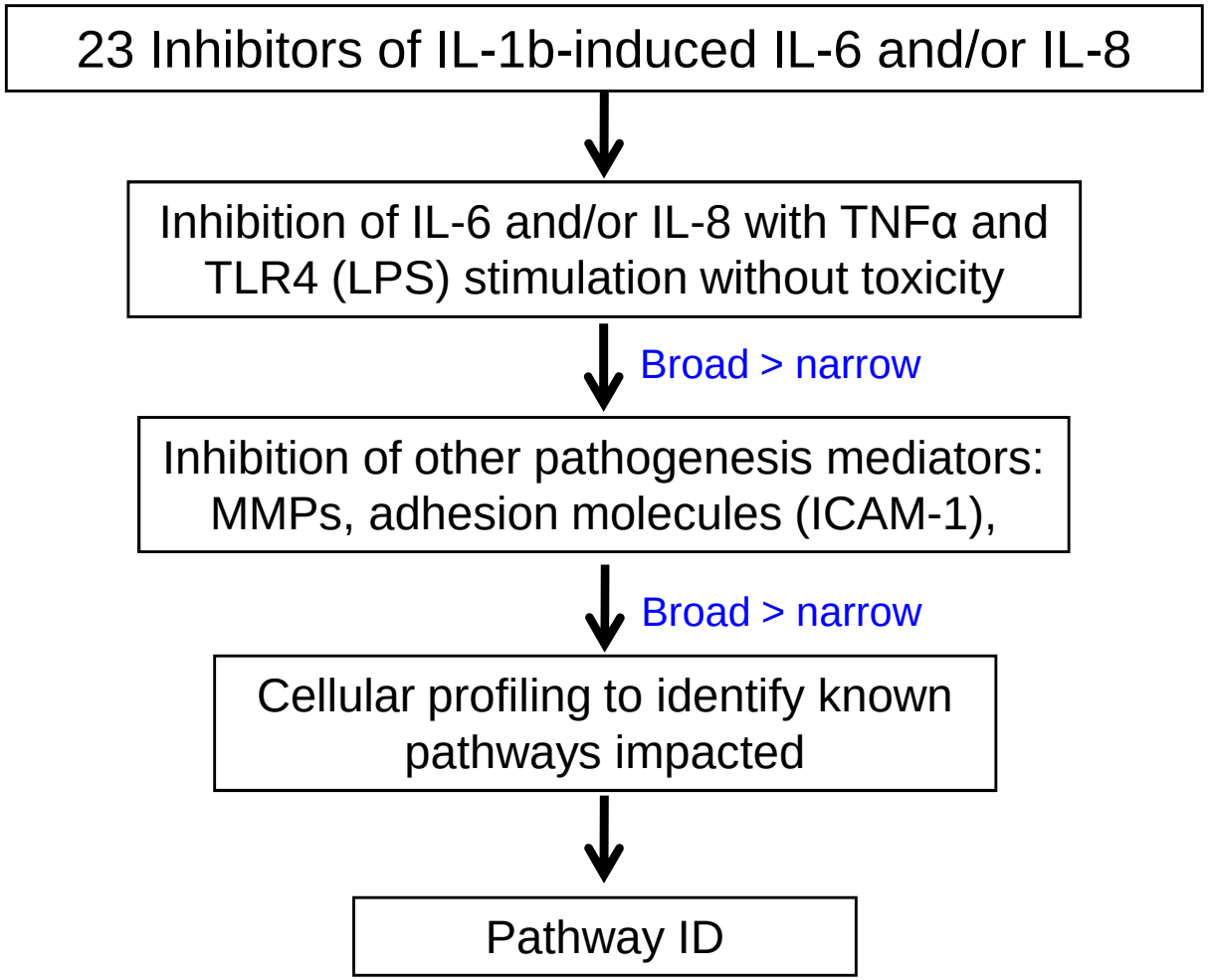
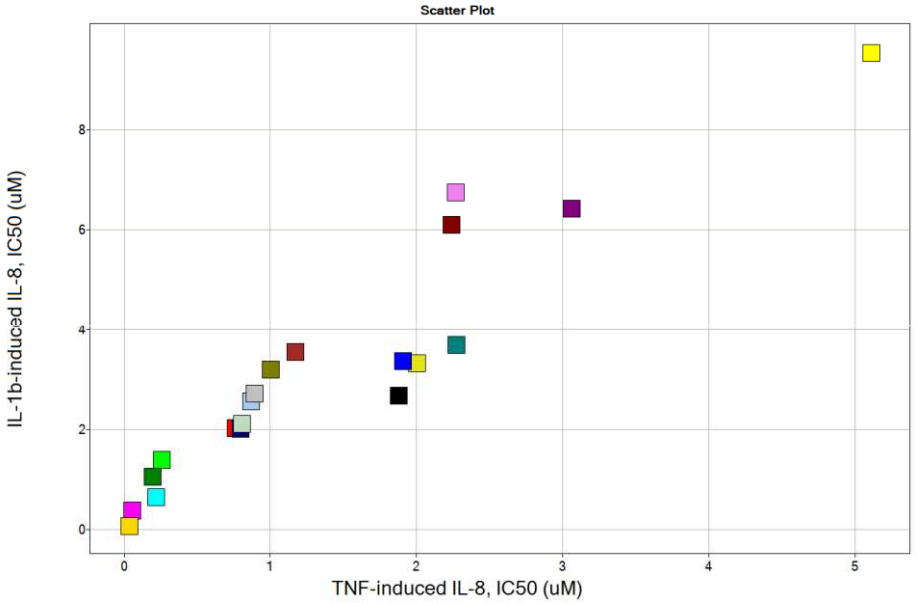
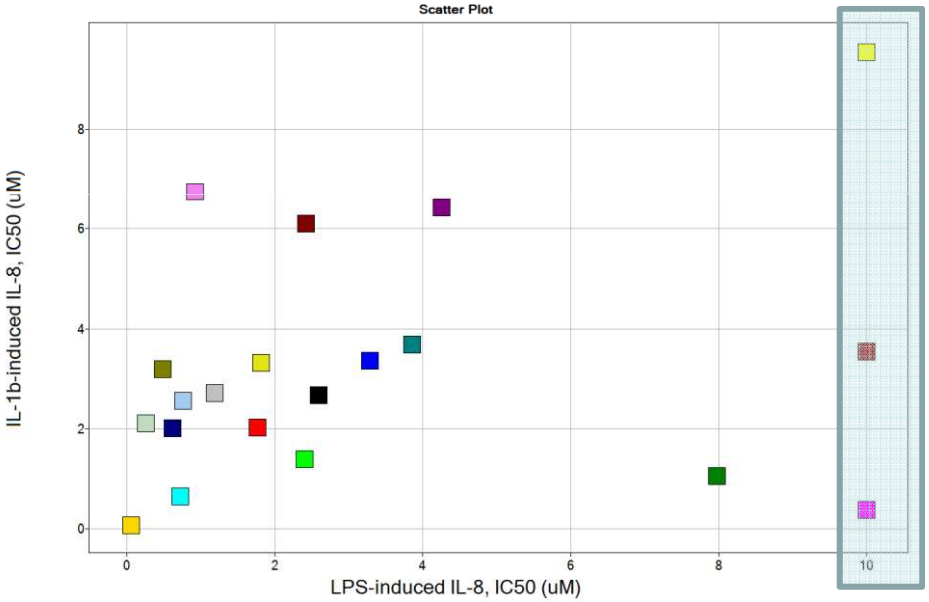
Synoviocyte Natural Products: Follow-up plan



Natural Product hits impact diverse stimuli

- Following input from the natural products unit on past history of the compounds, 20 of the 23 reconfirmed hits were profiled against additional stimuli
  - TNFa induced IL-8
  - LPS induced IL-8 and IL-6
  - Compounds were also repeated against IL-1b and in cytotoxicity assays
- 15/20 compounds were active against all stimuli
  - High IC50 correlation between IL-1b and TNF induced IL-8 (R^2 = 0.88)
- 3 compounds were inactive against LPS-induced cytokine secretion
- 2 compounds showed toxicity upon repeat and were eliminated at this stage.





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Natural Products SAR by inventory

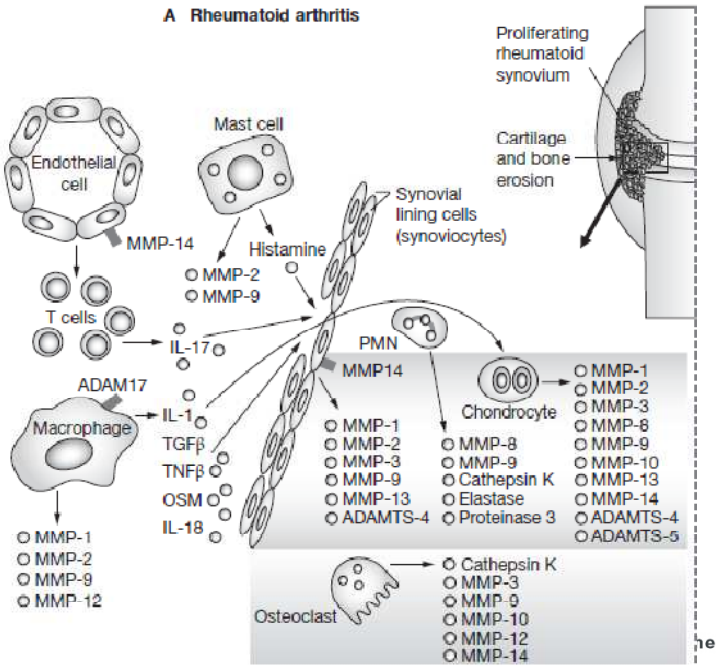
Compound ID	IL-1b-induced IL-8, IC50 (uM)	IL-1b-induced IL-8, %Eff vs Dex	IL-1b-induced IL-6, IC50 (uM)	IL-1b-induced IL-6, %Eff vs Dex	LPS-induced IL-8, IC50 (uM)	LPS-induced IL-8, Fold Change	TNF-induced IL-8, IC50 (uM)	TNF-induced IL-8, %Eff vs Dex	Synoviocyte toxicity CTG, IC50 (uM)	Synoviocyte toxicity CTG, %Eff vs BVS321	# active analogs / # analogs tested
A	2.022	145	1.142	111	1.766	0.80	0.760	164	>10	18	2/4
B	2.020	145	0.720	107	0.620	0.80	0.795	161	>10	0	1/2
C	2.118	141	1.052	114	0.254	0.81	0.805	159	>10	9	1/3
D	2.681	138	1.192	114	2.594	0.81	1.877	164	>10	5	2/3
E	3.362	139	1.527	111	3.287	0.81	1.909	165	>10	13	2/3
F	9.527	76	1.397	83	>10	0.00	5.113	114	>10	8	1/2

- Analogs were available for 18 of the initial 23 hits – profiled against the same 3 stimuli and cytotoxicity
- Scaffolds were ranked based on the activity of the analogs, any known activities of the parent compound in other programs
- 6 scaffolds were chosen for further profiling, 5 of which are broadly inhibitory and one lacks activity against LPS.

Top 6 natural products: MMP production

Compound ID	LPS-induced IL-8, IC50 (uM)	LPS-induced IL-8, Fold Change	TNF-induced IL-8, IC50 (uM)	TNF-induced IL-8, %Eff vs Dex	IL-1b-induced IL-6, IC50 (uM)	IL-1b-induced IL-6, %Eff vs Dex	IL-1b-induced IL-8, IC50 (uM)	IL-1b-induced IL-8, %Eff vs Dex	Synoviocyte toxicity CTG, IC50 (uM)	Synoviocyte toxicity CTG, %Eff vs BVS321	IL-1b-induced MMP1, IC50 (uM)	IL-1b-induced MMP1, %Eff vs Dex	IL-1b-induced MMP3, IC50 (uM)	IL-1b-induced MMP3, %Eff vs Dex	IL-1b-induced MMP9, IC50 (uM)	IL-1b-induced MMP9, %Eff vs Dex
F	>10	0.00	5.11	114	1.04	82	6.88	63	>10	4	0.26	101	0.16	104	0.72	109
C	0.25	0.81	0.80	159	0.93	107	2.51	121	>10	5	1.15	105	0.37	108	0.22	122
A	1.77	0.80	0.76	164	1.14	111	2.32	145	>10	18	3.92	110	2.01	108	2.23	116
B	0.62	0.80	0.80	161	0.71	104	1.53	122	>10	0	6.32	94	4.43	108	5.21	105
E	3.29	0.81	1.91	165	0.88	97	4.55	120	>10	31	>10	29	4.17	96	3.12	112
D	2.59	0.81	1.88	164	0.94	100	5.49	83	>10	2	>10	45	>10	99	5.27	101

- Parent scaffold hits were tested for inhibition of IL-1b induced MMP production
  - Multiplex MMP-1, 3, 9 ELISA
  - MMP-1, 3, 9 are elevated in the serum of RA patients
- 4 of the 6 parent scaffold hits showed significant inhibition of all 3 MMPs



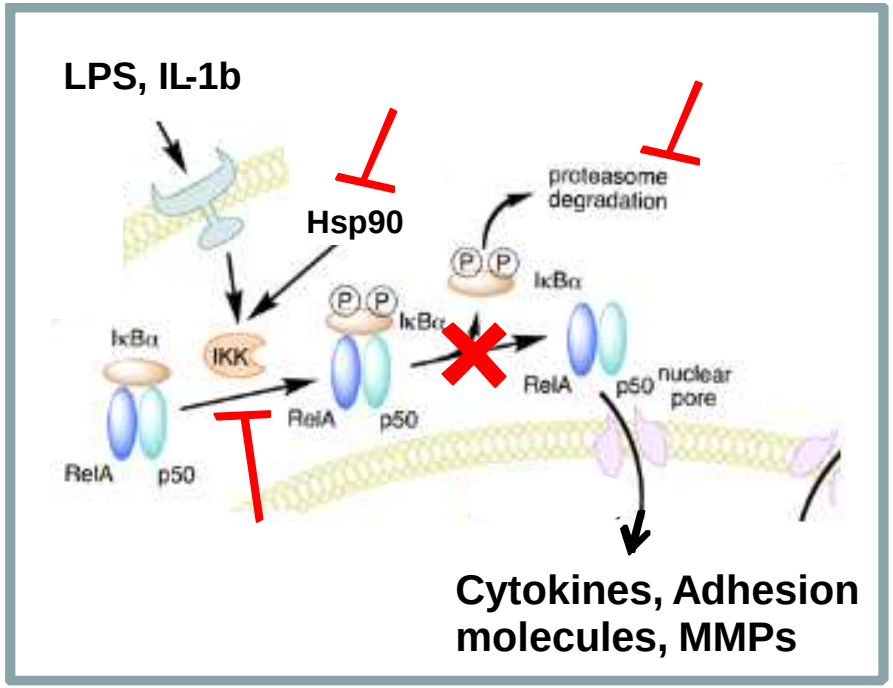
Top 6 natural products: Adhesion molecules

Compound ID	IL-1b-induced IL-8, IC50 (uM)	IL-1b-induced IL-8, %Eff vs Dex	LPS-induced IL-8, IC50 (uM)	TNF-induced IL-8, IC50 (uM)	IL-1b-induced MMP1, IC50 (uM)	IL-1b-induced MMP1, %Eff vs Dex	IL-1b-induced MMP3, IC50 (uM)	IL-1b-induced MMP3, %Eff vs Dex	IL-1b-induced MMP9, IC50 (uM)	IL-1b-induced MMP9, %Eff vs Dex	IL-1b-induced ICAM, IC50 (uM)	IL-1b-induced ICAM, %Eff vs Dex	IL-1b-induced VCAM, IC50 (uM)	IL-1b-induced VCAM, FC
C	2.51	121	0.25	0.80	1.15	105	0.37	108	0.22	122	2.89	132	>10	0.4
B	1.53	122	0.62	0.80	6.32	94	4.43	108	5.21	105	3.51	122	>10	0.1
A	2.32	145	1.77	0.76	3.92	110	2.01	108	2.23	116	4.82	130	>3.333	0.2
E	4.55	120	3.29	1.91	>10	29	4.17	96	3.12	112	3.59	135	6.43	0.5
D	5.49	83	2.59	1.88	>10	45	>10	99	5.27	101	6.17	124	1.97	0.4
F	6.88	63	>10	5.11	0.26	101	0.16	104	0.72	109	>10	5	>10	Stim!

- Parent scaffold hits were tested for inhibition of IL-1b induced ICAM and VCAM expression by FACs
  - Increased expression of adhesion molecules is associated with increased invasiveness and immune cell infiltration.
  - More baseline VCAM expression, stronger induction of ICAM with IL-1b
- 5 of the 6 parent scaffold hits showed significant inhibition of ICAM
  - 2 of these also showed weak inhibition of VCAM
  - Compound F increased VCAM expression – not desired.

Conclusions / Next steps

- Pathway profiling of top synoviocyte activation inhibitors suggests they all act on the NFkB pathway
  - Pathway strongly implicated in arthritis, involved in production of inflammatory cytokines, adhesion molecules and MMPs downstream of multiple stimuli
  - Compound activity may be through Hsp90, proteasome, or other modulation of IKK
- Due to toxicity, difficult to pursue a broad NFkB pathway modulator for RA
- Perform limited follow-up studies to clarify the target, especially for those compounds without previous annotated function (A, B)



GNF Systems

- GNF Systems is a Division of the Genomics Institute of the Novartis Research Foundation (GNF)

- Aims:
  - To make GNF technology available to the life sciences research community
  - To develop new and improved research technologies through interaction with commercial and academic development partners
  - To fund future cycles of technology development

Acknowledgements

Christina Galang
Sabiha Abbas
Laura Bordone
Paul Anderson
John Joslin
Doug Selinger
Philipp Krastel
Dan Sipes
Jennifer Harris
Martin Seidel