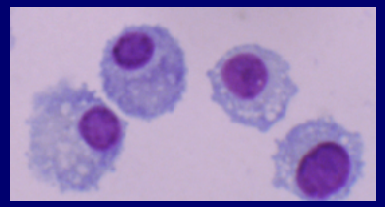
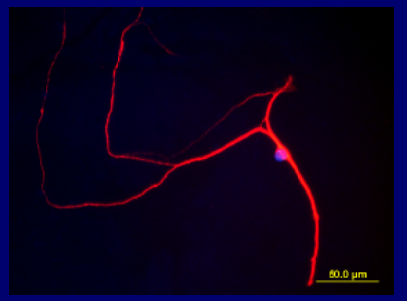
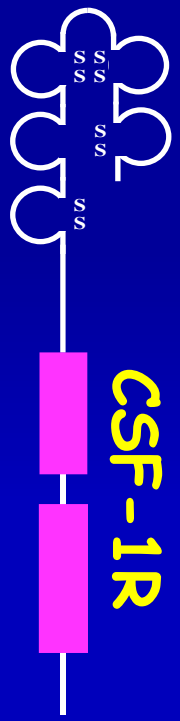




Dissecting signal transduction pathways that regulate macrophage production

Angel W. Lee, M.D., Ph.D.



Research Overview

growth factors (CSF-1)

↓

receptor tyrosine kinases (CSF-1R)

autoregulatory mechanisms
PNAS 87:7270, 1990
JBC 267:16472, 1992
JBC 279:43448, 2004

↓

signaling cascades

scaffolding proteins
MCB 20:6779, 2000
JCB 170:305, 2005

Signaling cross talk
Blood 93:537, 1999
MCB 20:6779, 2000

signal threshold & the Warburg effect
Cell Death Differ 13:1900, 2006

↓

post-translational modifications, gene expression

↓

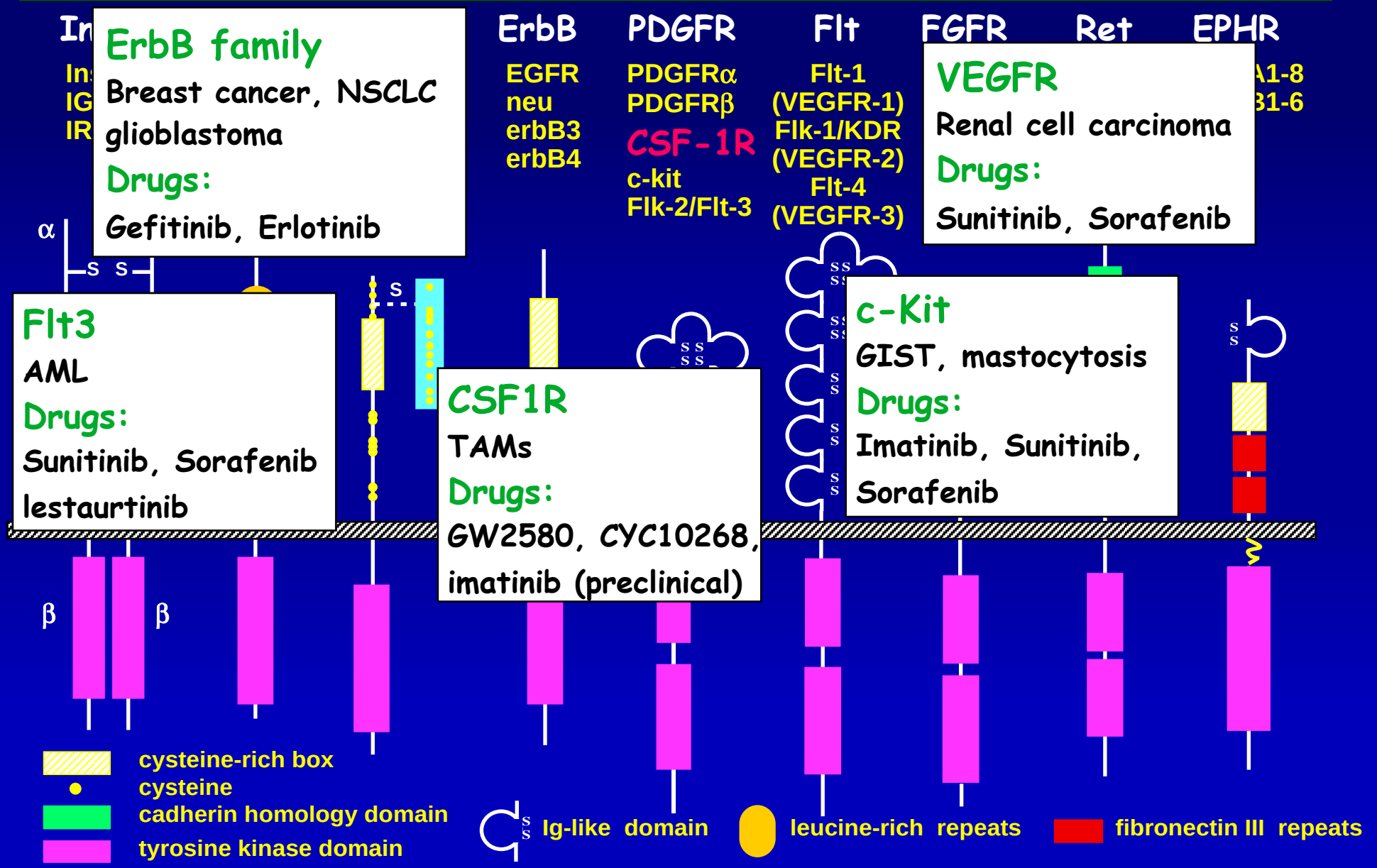
cell cycle progression | growth | survival | differentiation | metabolism

CSF1R in Breast cancer
Oncogene 18:7475, 1999

FGFR in Neural stem cell self renewal & differentiation
JCB 170:305, 2005

Gab2 in CSF-1 dependent MNP production

CSF-1R is a Receptor Tyrosine Kinase



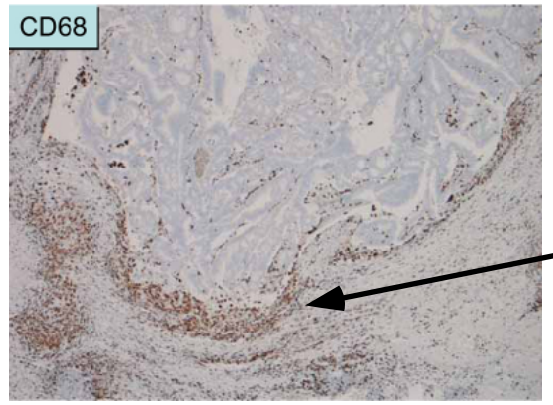
CSF-1 & CSF-1 Receptor

HEMATOPOIESIS - *primary* regulator of proliferation, differentiation and survival of cells of the monocyte-Mϕ lineage; absence of Langerhans cells in CSF-1R-/- mice

BONE REMODELLING - required for osteoclast differentiation and survival

REPRODUCTION - CSF-1-/- (*op/op*) in male and female reproductive
- — Mϕ in reproductive tissues
- — microglia in neuroendocrine

ONCOGENESIS - deregulation of th
- — activating mutations (myeloc
- — aberrant overexpression in s as well as in prostate cancers



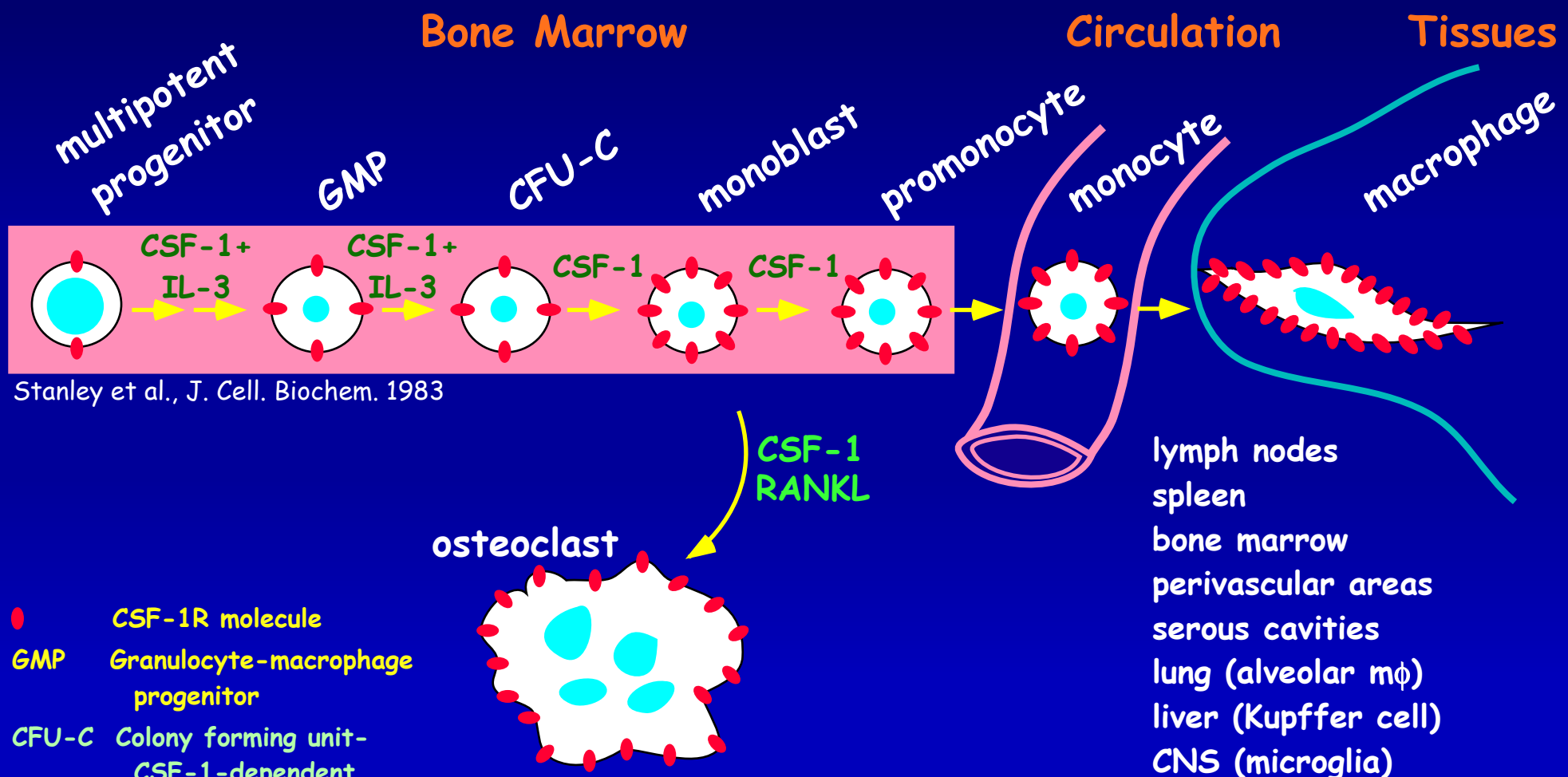
Human colorectal cancer

TAMs

Ohtani, Cancer Immunity, 7:4, 2007

INFLAMMATION
- — CSF-1 primes Mϕs for the inflammatory response
- — increased # of Mϕs in cancers (tumor associated Mϕs or TAMs), arthritis, atherosclerosis, obesity, allografts, brain disorders (e.g. tumors, Alzheimer's)
- — mouse breast CA model: CSF-1 has a paracrine role and important for the angiogenic switch

CSF-1 & the CSF-1R are the Primary Regulators of Mononuclear Phagocyte (MNP) Production



CSF-1R molecule

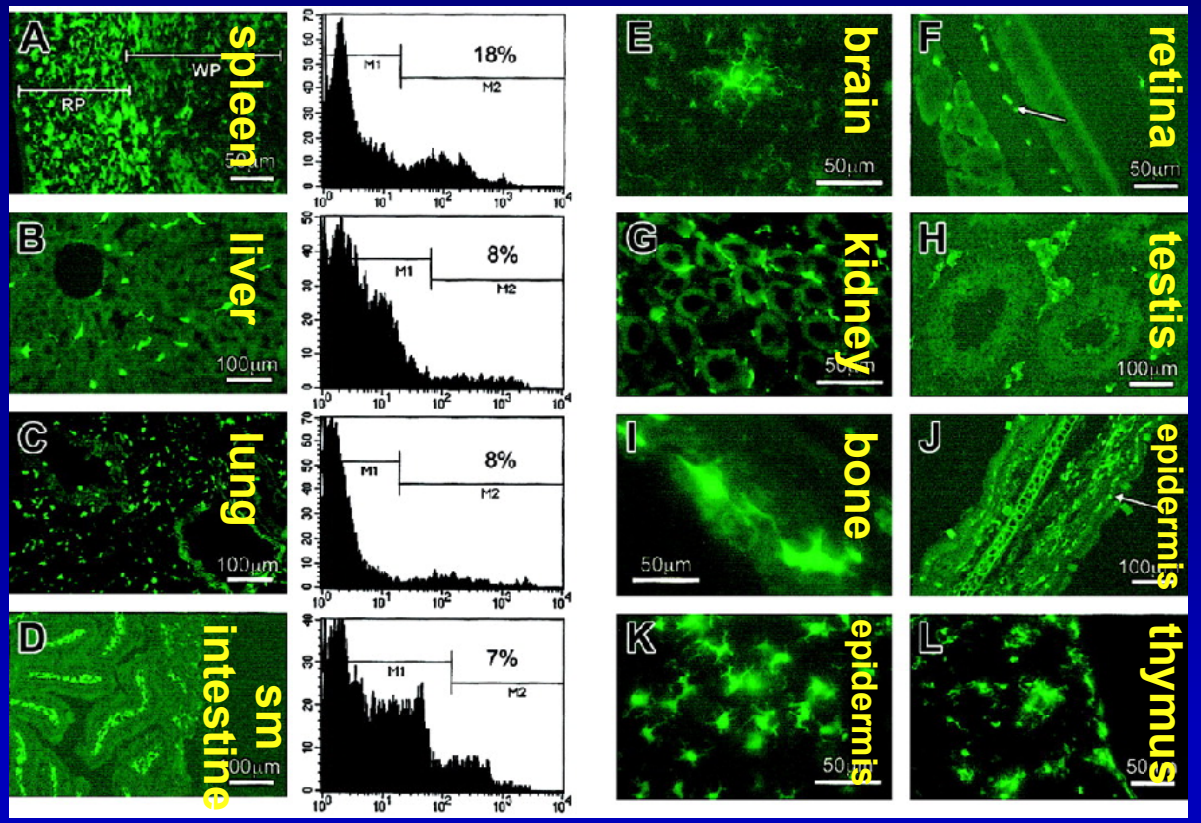
GMP Granulocyte-macrophage progenitor

CFU-C Colony forming unit-CSF-1-dependent

CSF-1 Colony Stimulating Factor-1

Mononuclear phagocytes (MNPs)

"MacGreen Mouse" - Mϕs are everywhere!



Sasmono, R. T. et al. Blood 2003;101:1155-1163

MNPs

- monocytes, tissue Mϕs, dendritic cells, microglia, osteoclasts

Effectors of innate immunity

(germline-encoded molecules that recognize many pathogens)

- recognize and kill microbes: phagocytosis, secretion of cytokines, ROS, NO

Tissue homeostasis

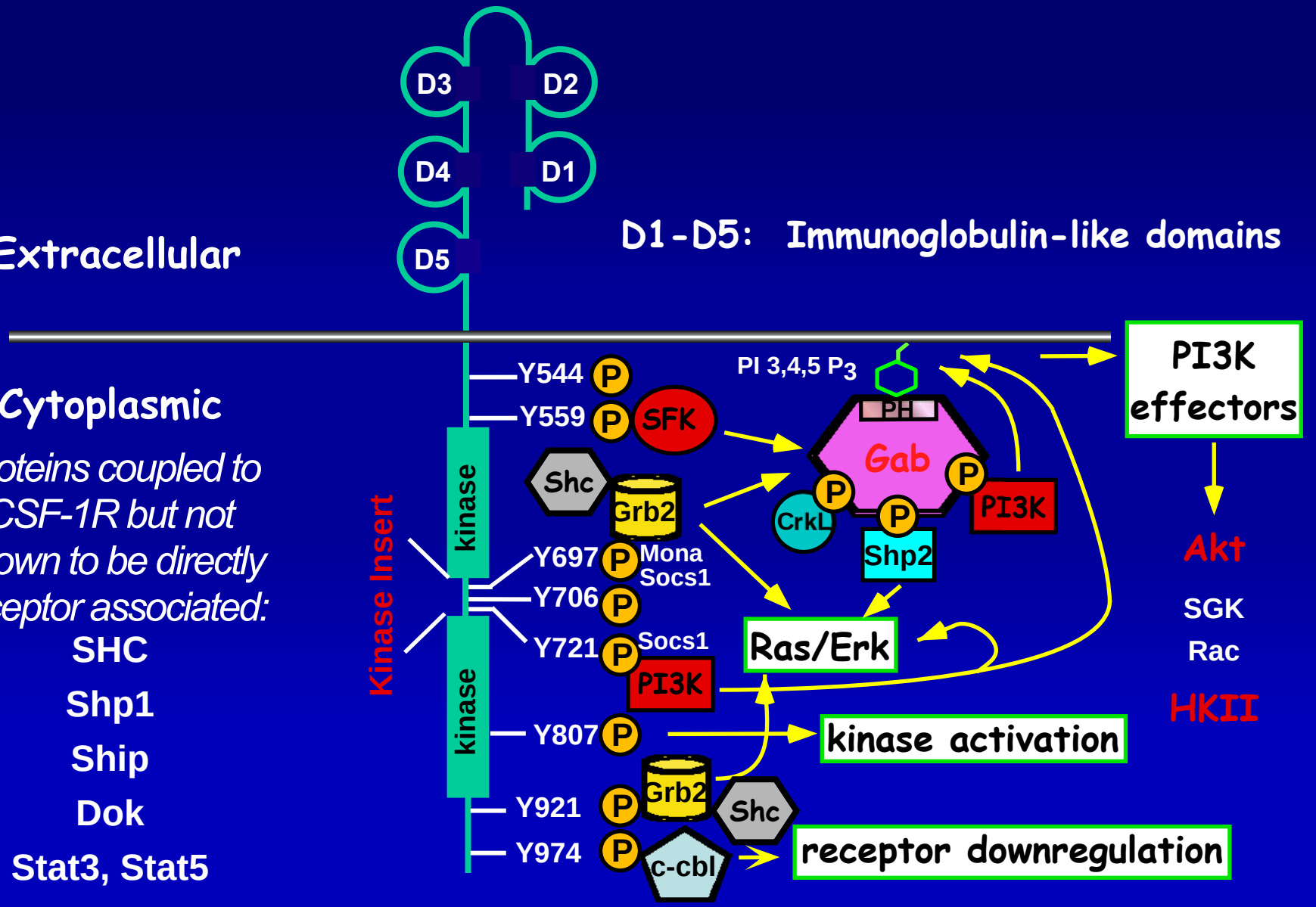
- tissue remodelling, development, ingestion of apoptotic cells

Chronic inflammatory diseases

- tumors (TAMs), adipose tissue, atheromata, neurodegenerative lesions, arthritis

What are the key signaling pathways utilized by the CSF-1R to regulate MNP production ?

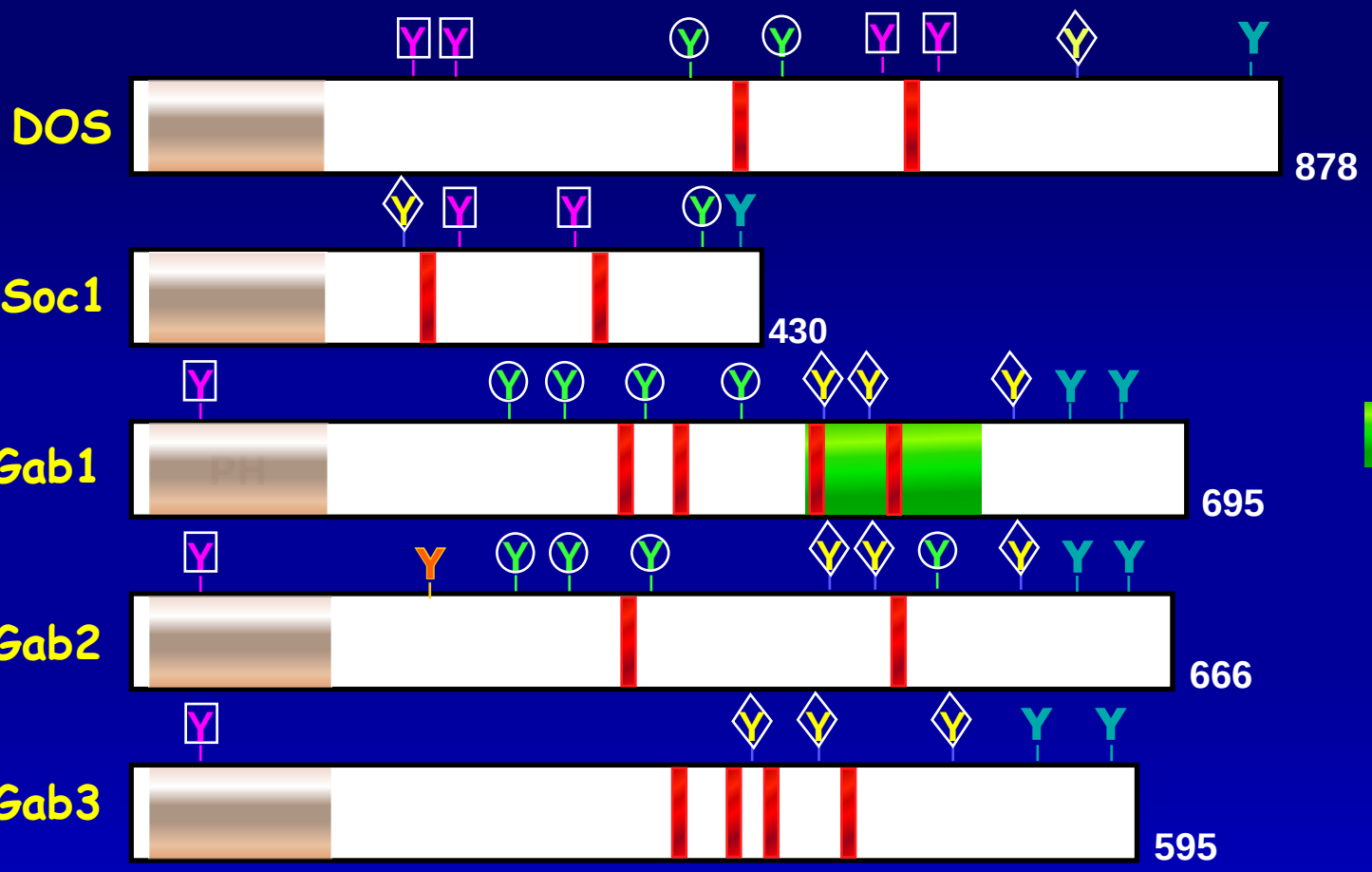
Signaling downstream of the CSF-1R (2008)



CSF-1 regulated pathways that promote macrophage production:

The role of the scaffold, Gab2

Schematic of Gab proteins



PH pleckstrin homology

c-Met binding domain

PXXP motifs

Overall sequence identity	
Gab1 vs Gab2	41 %
Gab2 vs Gab3	37 %
Gab1 vs Gab3	33 %

(proven) p85/PI3K binding site

(proven) SHP2 binding site

(proven) Stat3 binding site

(predicted) Grb2 binding site

(predicted) Crk/CrkL binding site

Gab2 in human disease

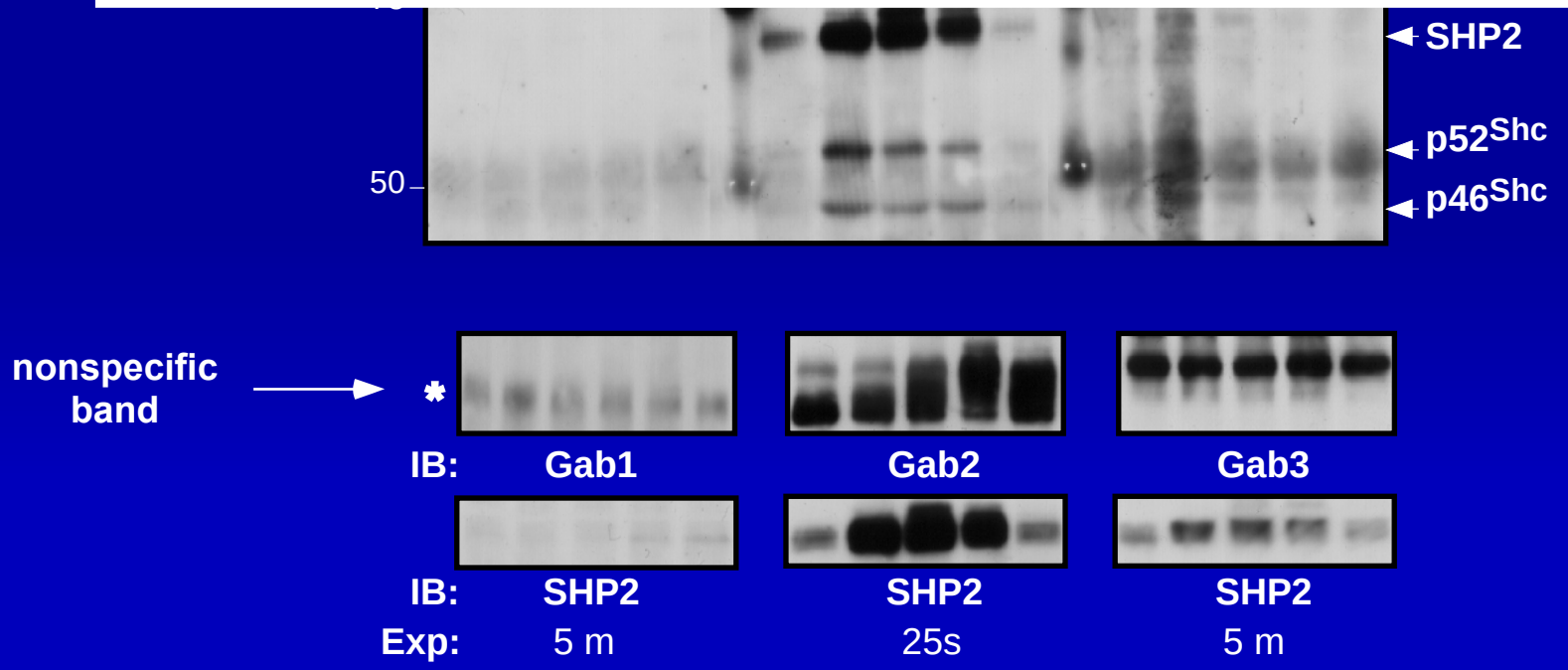
- Gab2 maps to 11q13.5, frequently amplified in human cancer e.g. AML/MDS, breast cancer (Zatkova 2004) and ovarian cancer (Brown 2008)

- Gab2 is overexpressed in human breast cancer and may be regulated by estradiol (Daly 2002; Bentires-Alj 2005)

- Gab2 may be a modifier of late onset Alzheimer's disease risk in APOE ε4 carriers (Reiman 2007) although this link is being debated (Chapuis 2008)

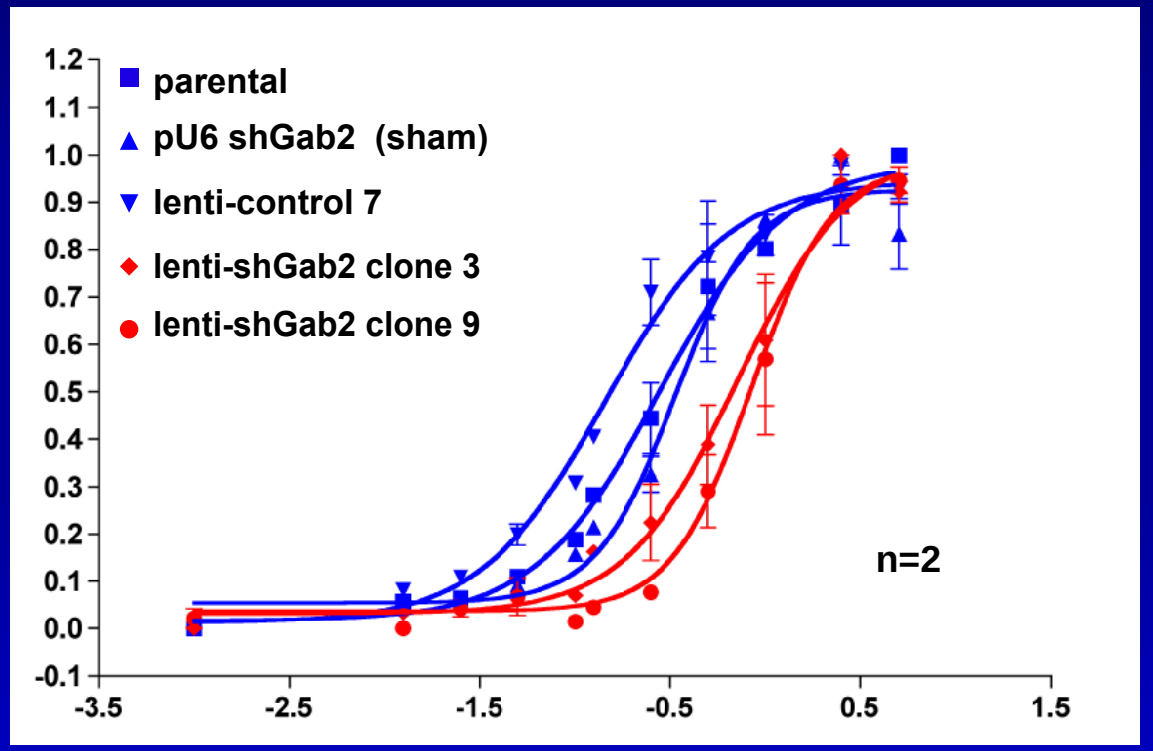
CSF-1 signals to Gab2 & Gab3 in 32D.R

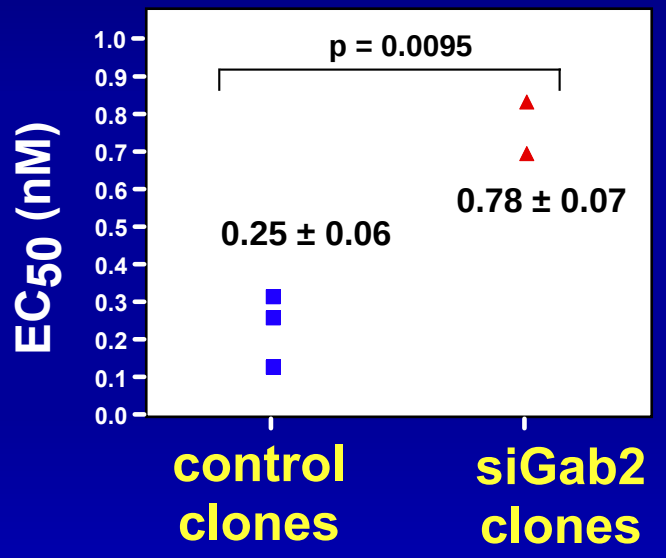
Do Gab2 and Gab3 play redundant roles in mediating CSF-1 dependent proliferation?



Angel Lee

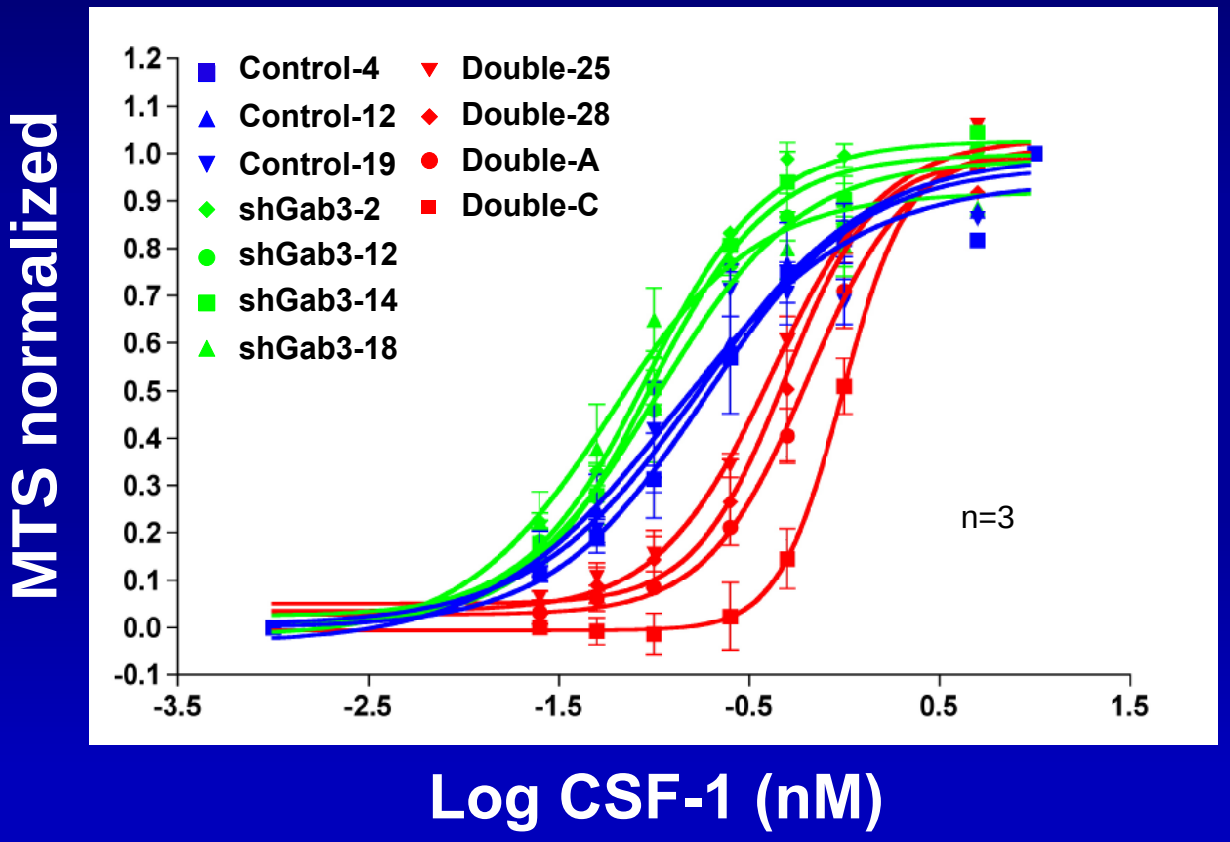
Optimal responsiveness to CSF-1-dependent proliferation in 32D.R cells requires Gab2

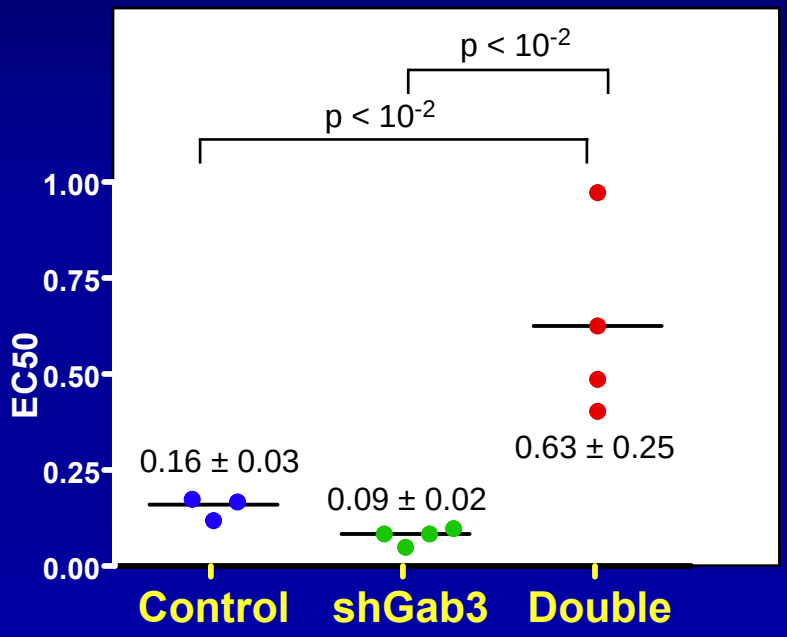




Yingwei Mao, Angel Lee

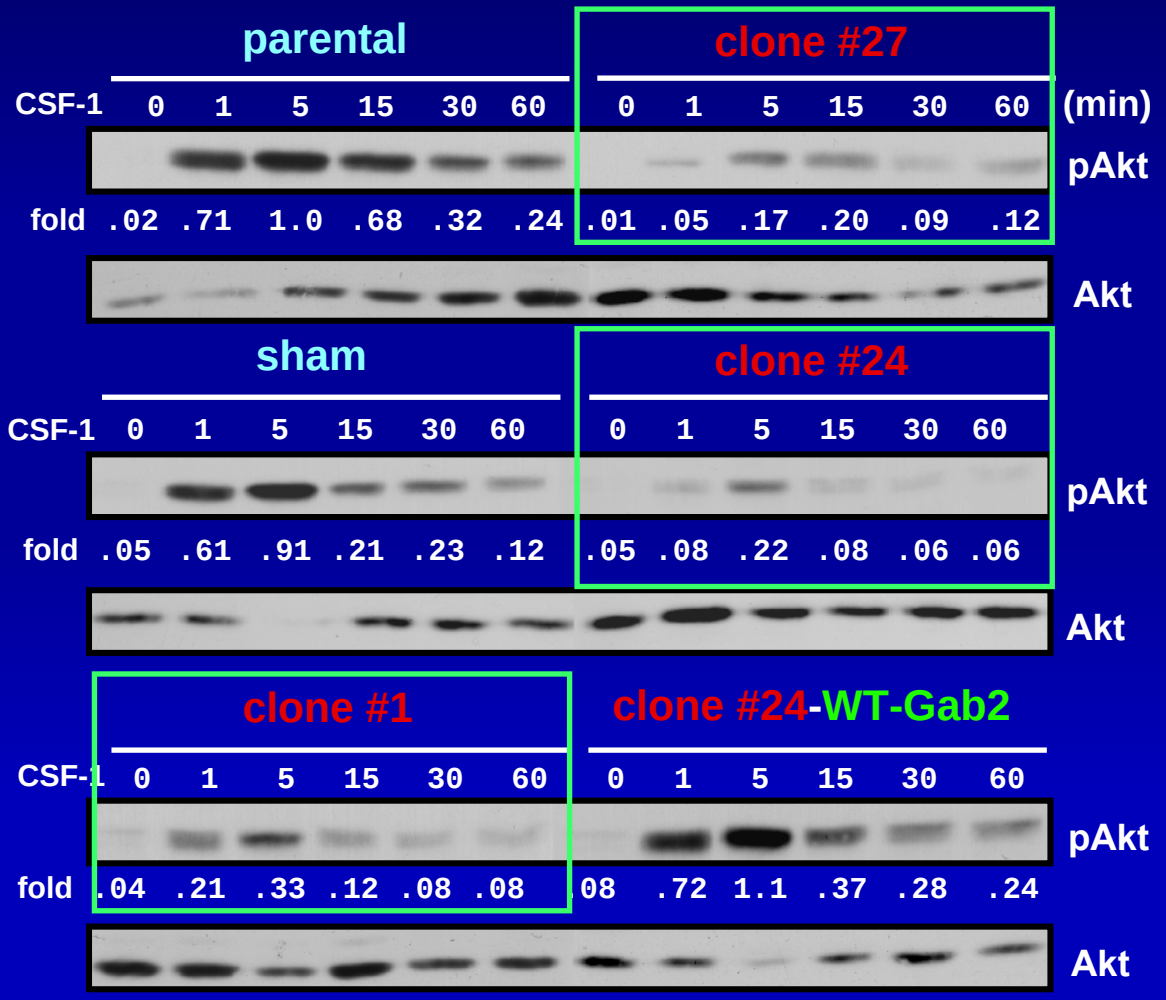
Gab3 does not appear to contribute to CSF-1 dependent proliferation





Yingwei Mao, Angel Lee

Gab2 knockdown significantly reduces CSF-1 mediated Akt phosphorylation



IB: anti-pT308-Akt

IB: anti-Akt1

IB: anti-pT308-Akt

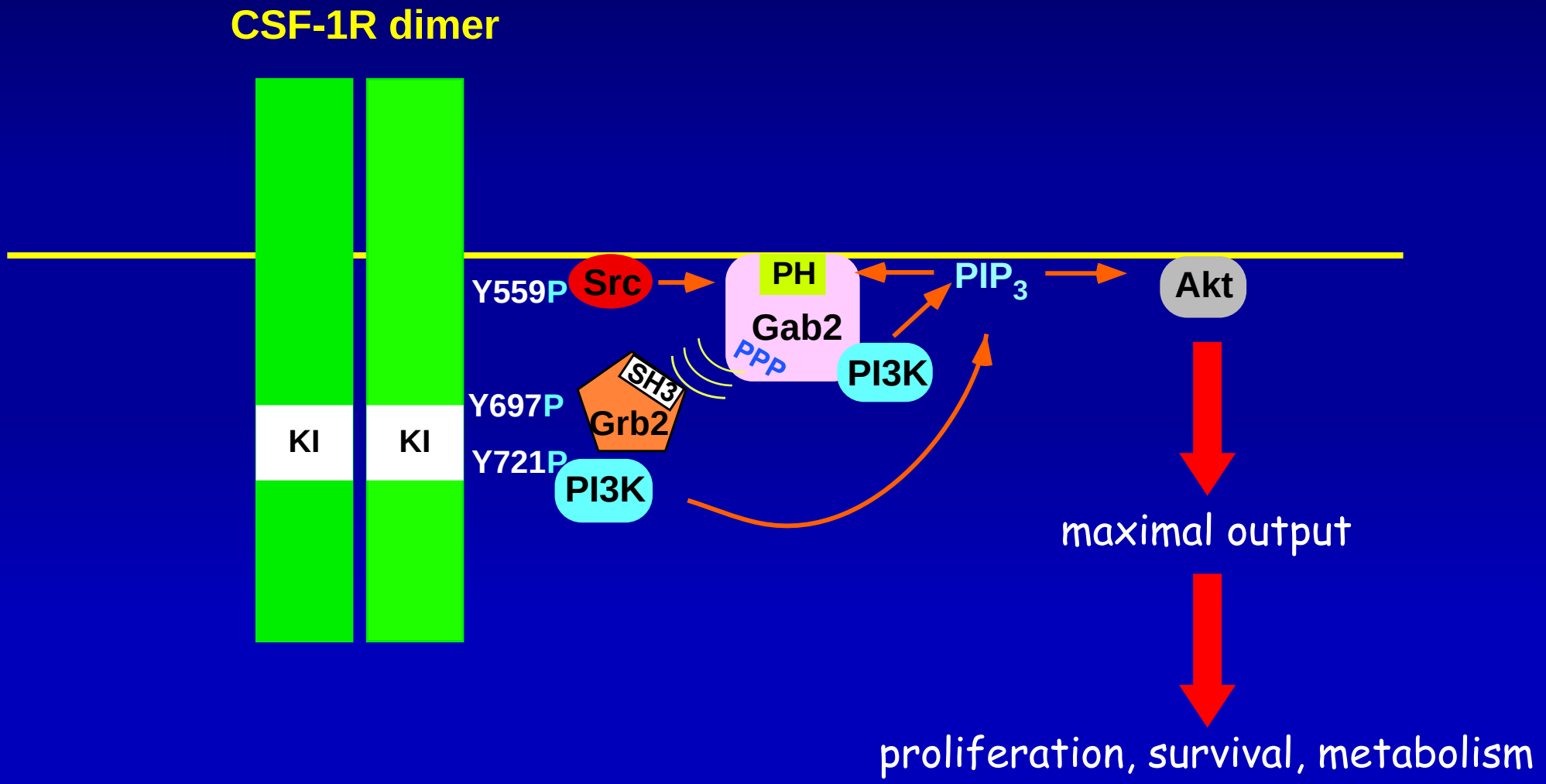
IB: anti-Akt1

IB: anti-pT308-Akt

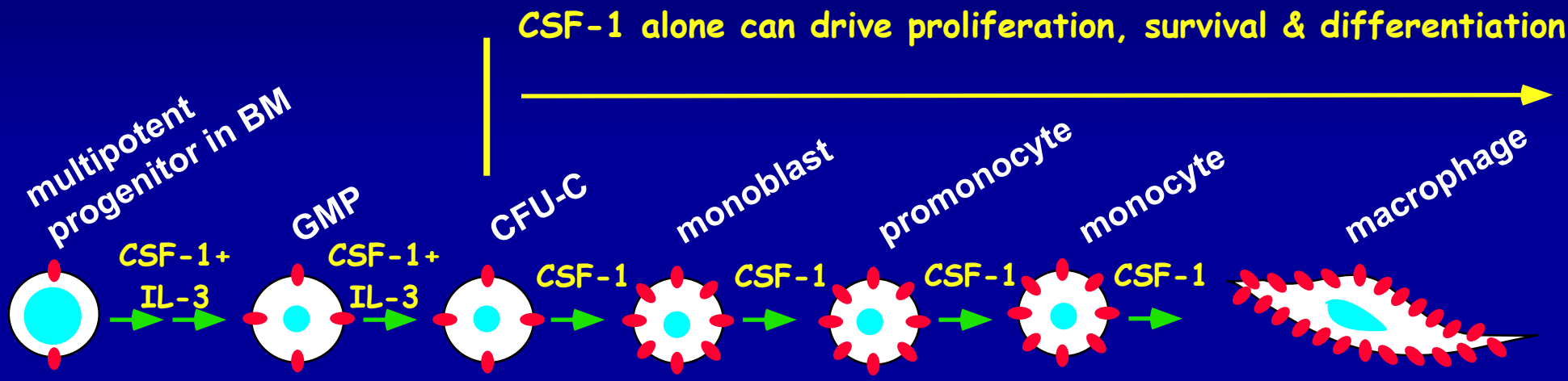
IB: anti-Akt1

Yingwei Mao

Signaling networks involved in coupling CSF-1R to the PI3K/Akt pathway



Production of macrophages from bone marrow progenitors



CSF-1R molecule

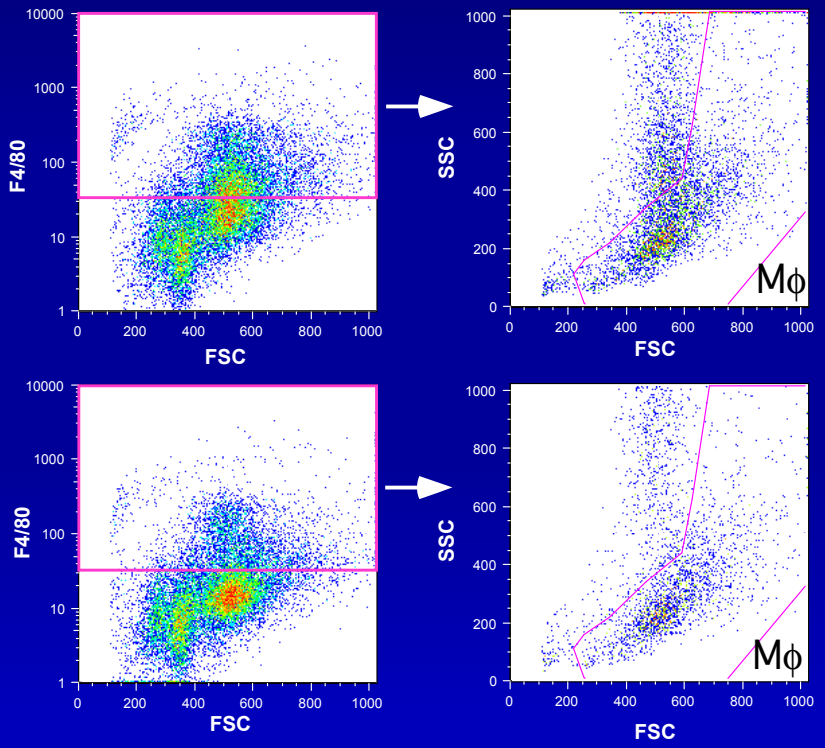
GMP: Granulocyte-macrophage progenitor

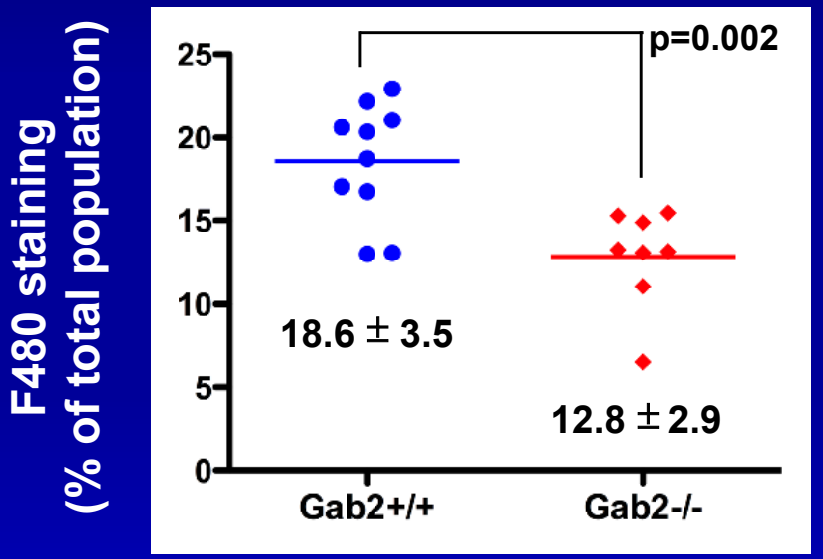
CFU-C: Colony forming unit-CSF-dependent

Day 7 BMMs will proliferate for 3 passages (5-7 logs of growth) before becoming senescent (still require CSF-1 for survival) from ER Stanley (Methods Mol. Biol. 1997)

Reduced F480 staining in Gab2 -/- BM

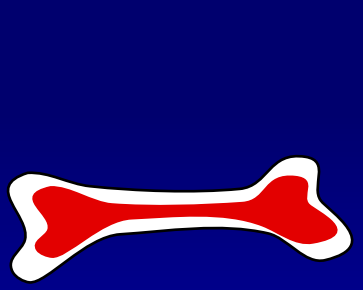
F480 staining in total bone marrow



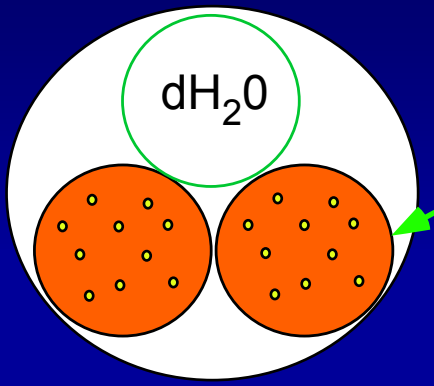


Heather Grifka

CFU-C (CSF-1 dependent) clonogenic assay



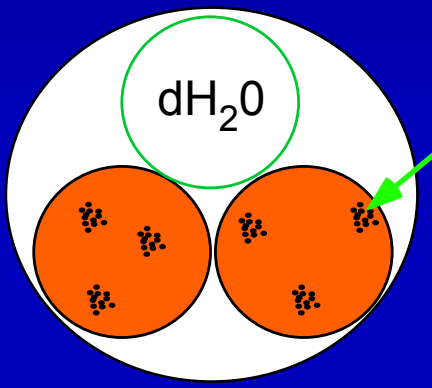
bone marrow
OR
cells at specific stages of development



35 mm dishes with methylcellulose

specific growth factors

- **frequency of progenitors**
- **proliferation capacity of progenitors**

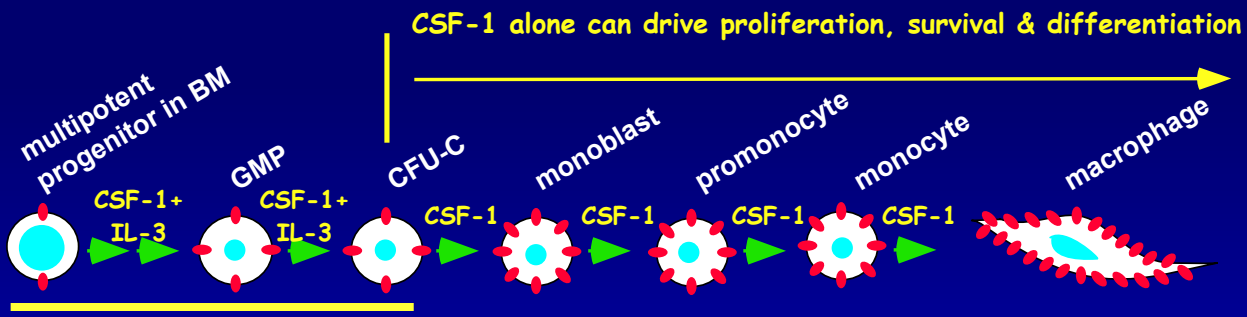


colony of cells (>50) derived from a single progenitor

7-14 days

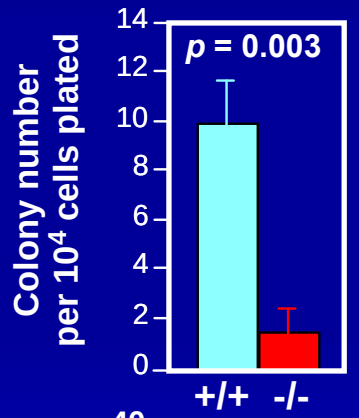
- score colonies
- isolate colonies for further studies

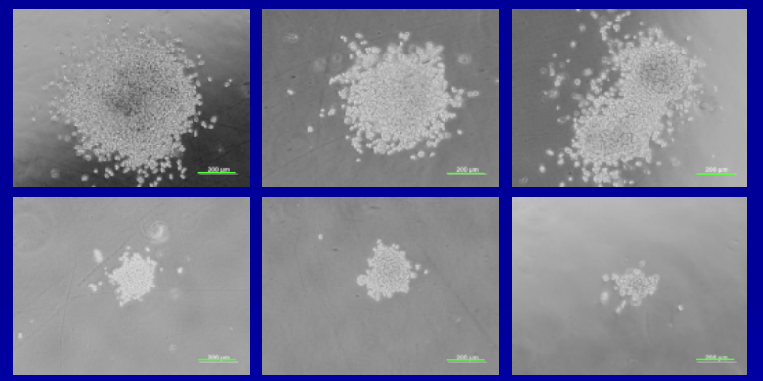
Gab2 -/- mice have (1) fewer CFU-Cs & (2) CFU-Cs have diminished proliferative capacity



whole bone marrow

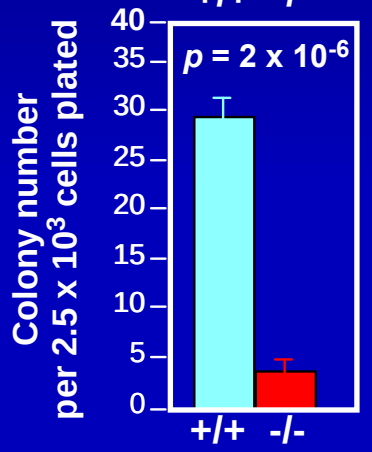
CFU-C: 1 in 1,000



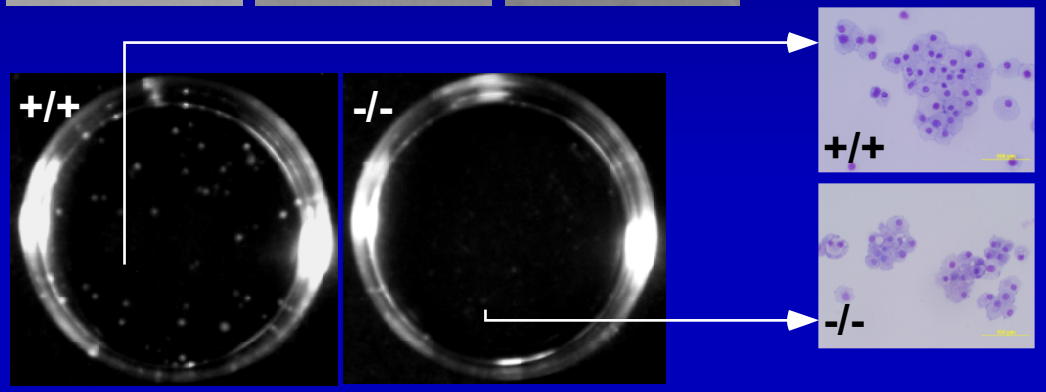


after 2 days of commitment

CFU-C: 1 in 83

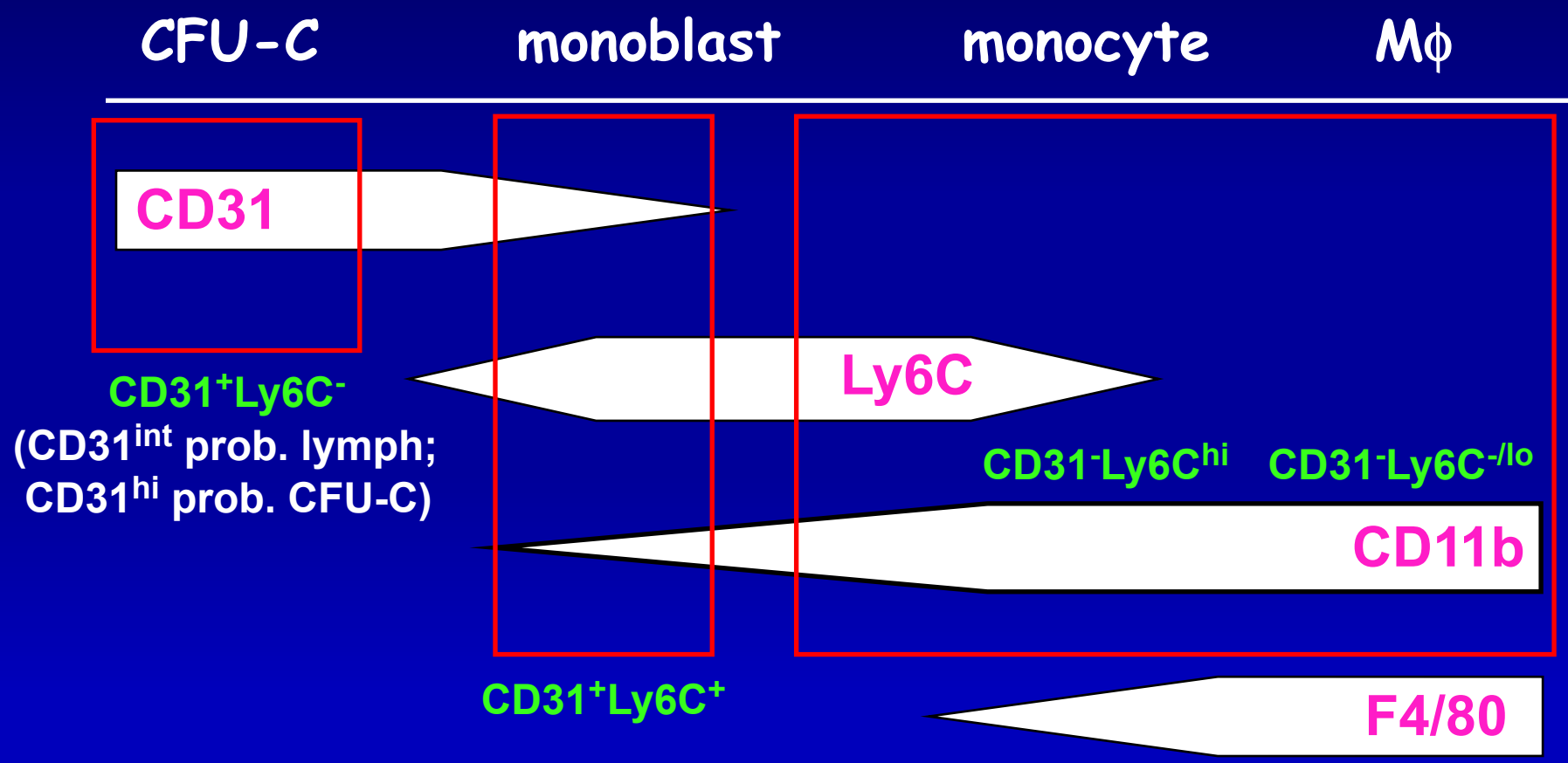


n = 6 (+/+), 7 (-/-)



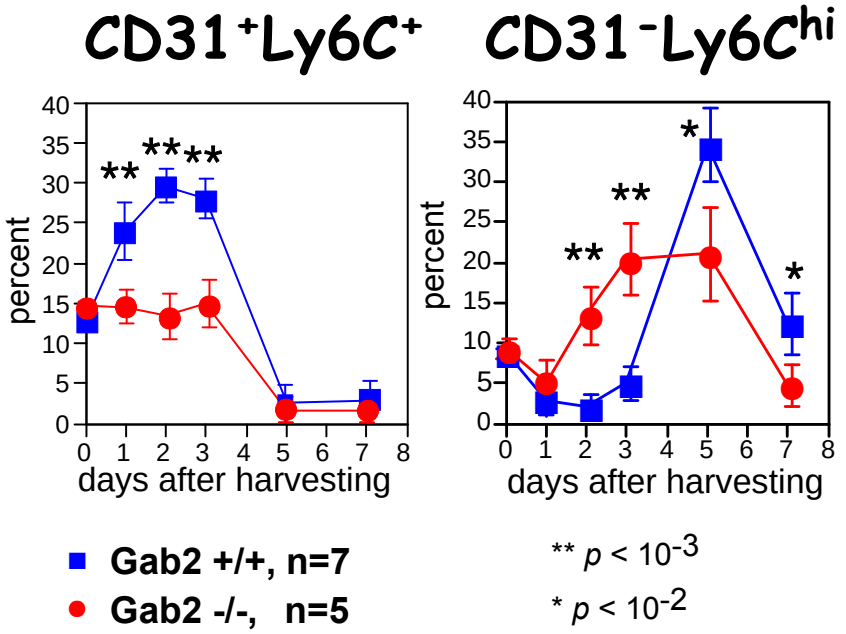
Angel Lee

Correlation of monocyte/Mϕ development with cell surface markers



Adapted from Leenen et al. Eur. J. Immunol. 1990

Phenotyping monocyte/Mϕ development in Gab2 -/- mice with CD31 and Ly6C

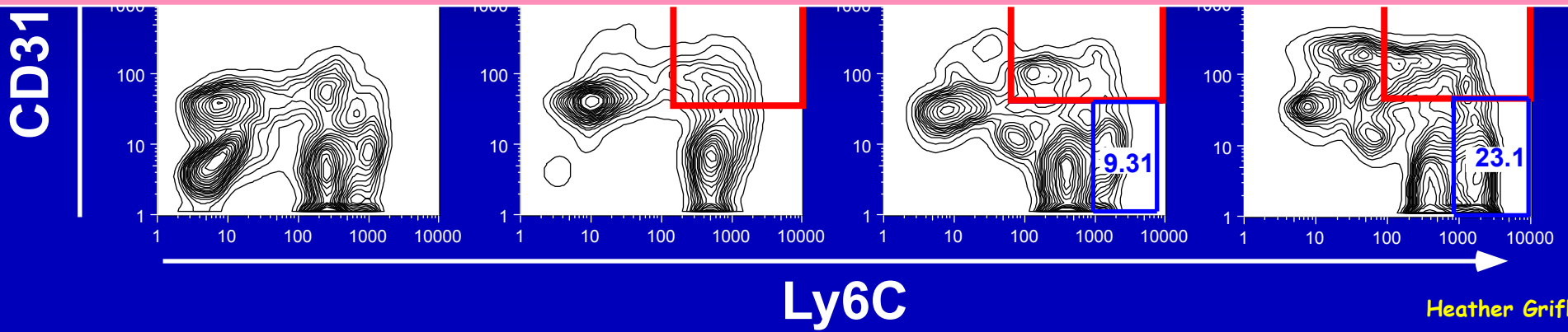


1. Gab2 +/+:

   2-fold increase in $CD31^{+}Ly6C^{+}$ cells which are a mix of monoblasts, monocytes and a few blasts

2. Gab2 -/-

   accelerated appearance of $CD31^{-}Ly6C^{hi}$ monocytes



Heather Grifka

$CD31^{hi}Ly6C^{-}$ progenitors are more abundant in Gab2 +/+ mice

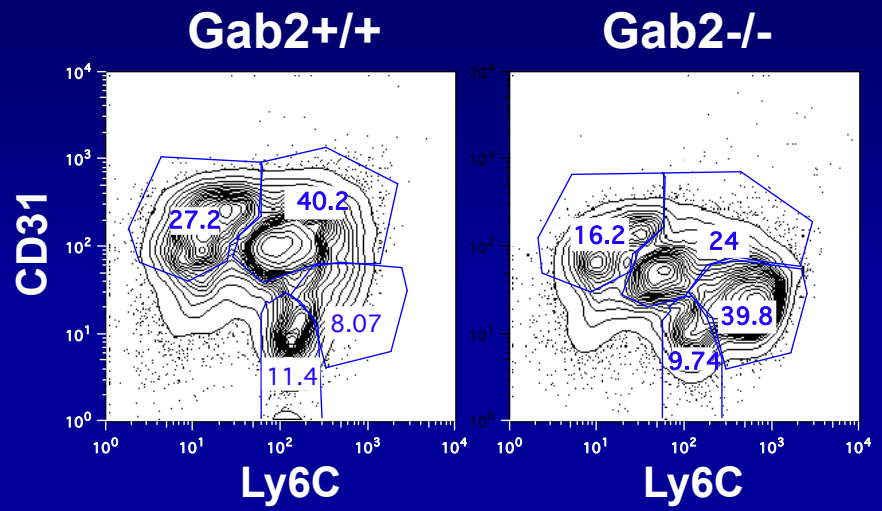
Day 2 cells

↓

MACS (Miltenyi) depletion (CD4/CD8/CD19/Ter119/Ly6G)

↓

Stain with anti-CD31, Ly6C



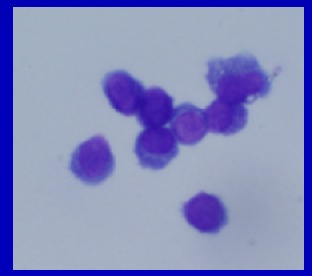
LIN*- subsets	Gab2+/+	Gab2-/-
$CD31^{high}Ly6C^{-}$	28.3 ± 6.3	18.6 ± 2.2*
$CD31^{+}Ly6C^{+}$	42.4 ± 3.7	28.6 ± 3.1**
$CD31^{-}Ly6C^{+}$	13.0 ± 5.5	10.7 ± 3.5
$CD31^{-}Ly6C^{high}$	10.7 ± 3.5	32.6 ± 6.1**

n=4, *, $p < 0.05$, **, $p < 0.005$

$CD31^{high}Ly6C^{-}$ CFU-C per 3000 cells seeded

Gab2 +/+	Gab2 -/-
85 ± 4	23 ± 0.7

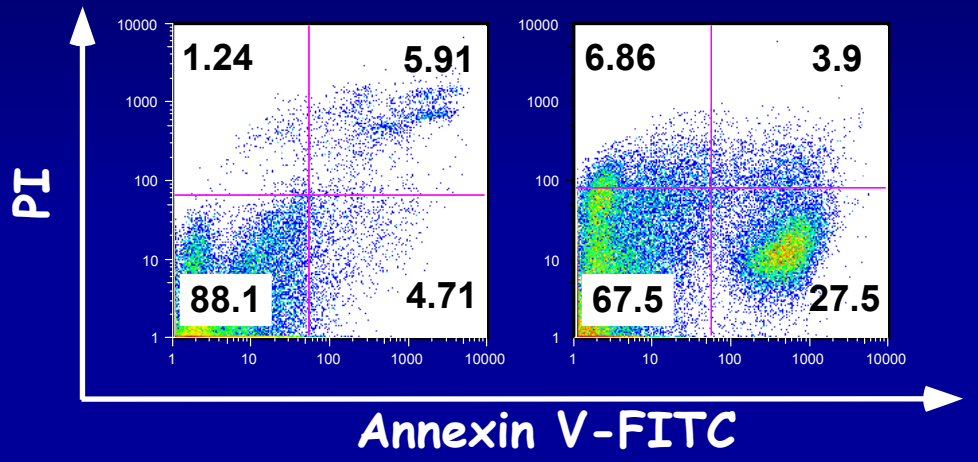
CFU-C: 1 in 35



Soojie Yu

Gab2 -/- CD31^{hi}Ly6c^{-} progenitors show reduced DNA synthesis and increased apoptosis

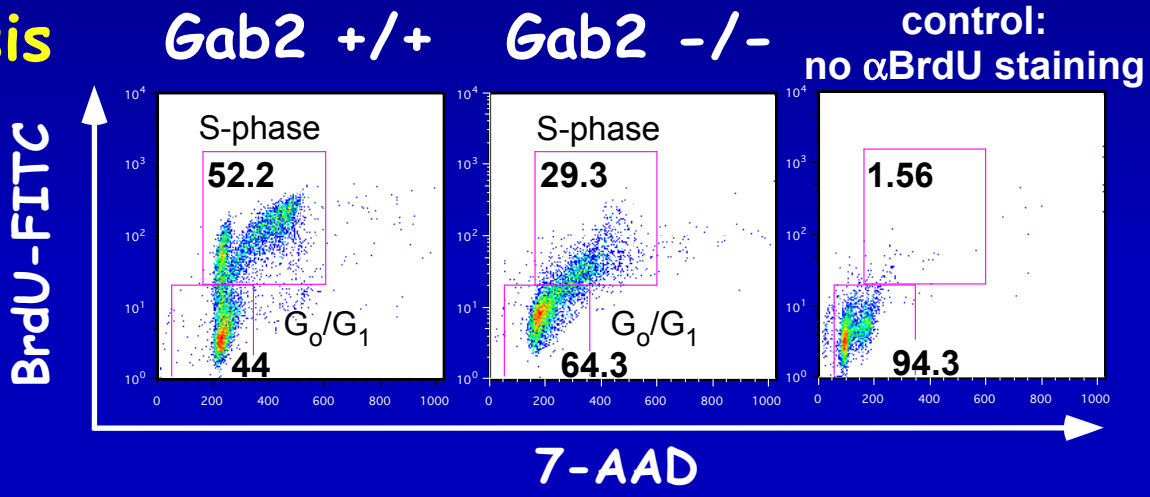
Apoptosis



day 2 depleted and sorted cells

LIN*$^{-}$CD31^{hi}Ly6C^{-}

DNA synthesis

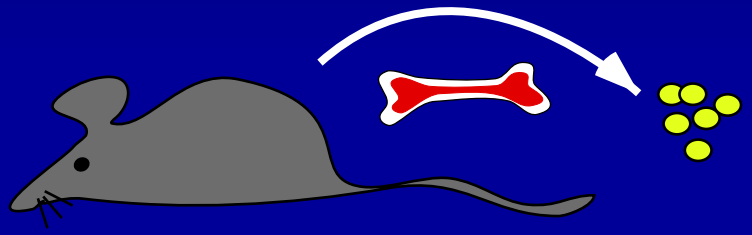


Soojie Yu

Purification of early BM progenitors that contain CSF-1 responsive precursors (LK31C)

1.

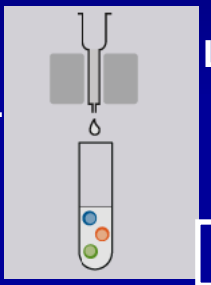
- harvest bone marrow
- treat w/ ACK lysis buffer to remove RBCs



αCD4 (T cells)
αCD8 (T cells)
αGr-1 (granulocytes)
αTer119 (erythroid)
αCD11b (late myeloid)
αF4/80 (Mϕ)
αCD19 (B cells)
(LIN- cocktail)

2.

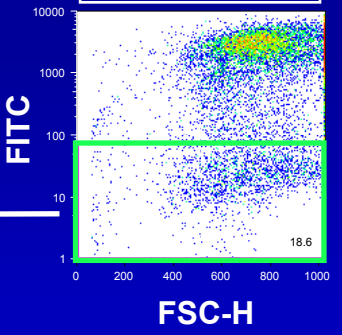
GoatαRat-IgG-microbeads (Miltenyi)

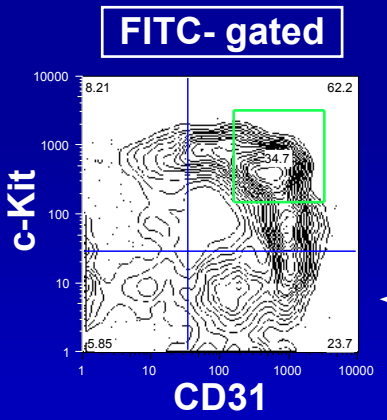


LIN+ cells

LIN- cells

αc-Kit-APC
αCD31-PE
αLy6C-FITC
(+selection)
GoatαRat-FITC
(residual lin+ cells)





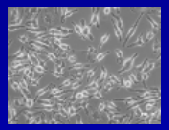
3.

FACS sort

LIN-
c-Kit+
CD31+
Ly6C-
(LK31C)

4.

culture in CSF-1

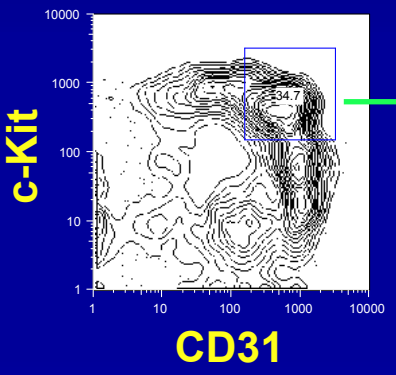


CFSE, Annexin V, signaling
Wright stain
CD31, Ly6C staining

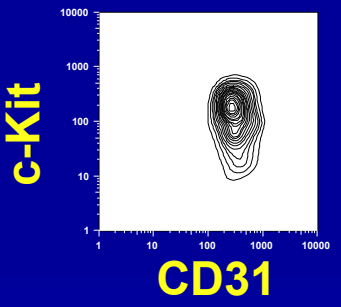
Based on Bonifer's method

LK31C cells show a 100-fold enrichment in CFU-C; Gab2 -/- mice have 5-fold fewer

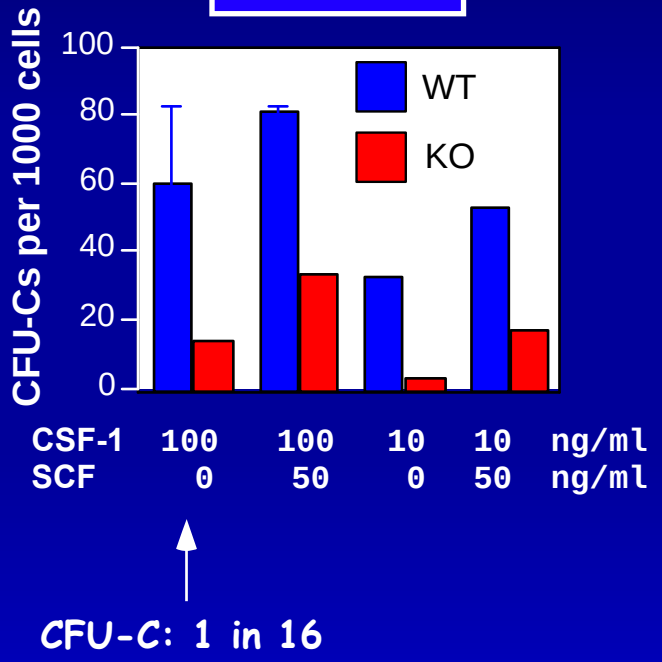
Gab2+/+ LIN$^-$ total BM cells
Ly6C$^-$ gated



LIN$^-$c-Kit$^+$CD31^{high}Ly6C$^-$
(LK31C)



CFU-Cs

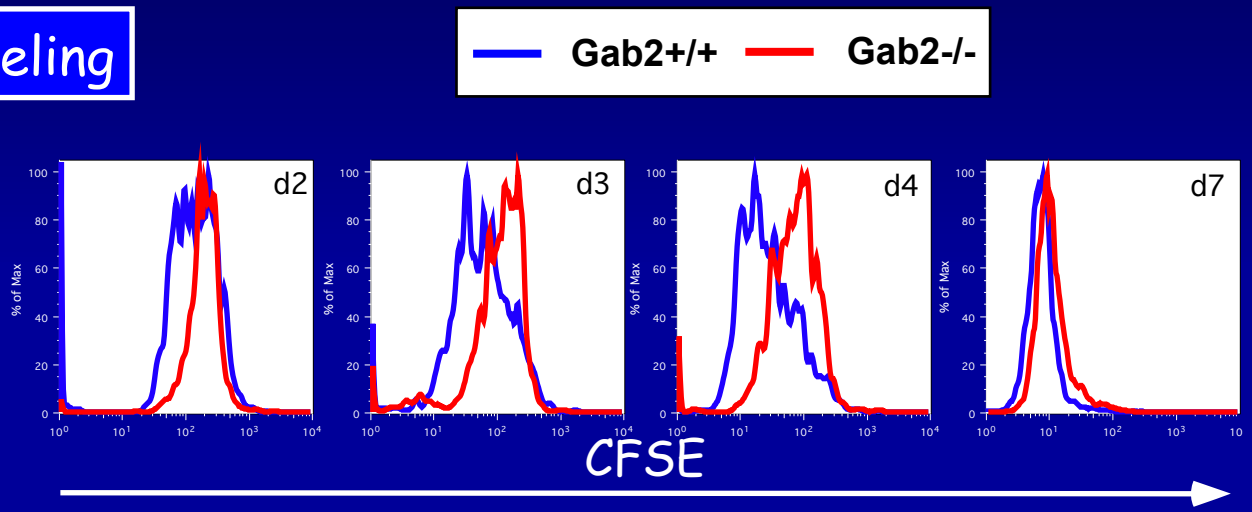


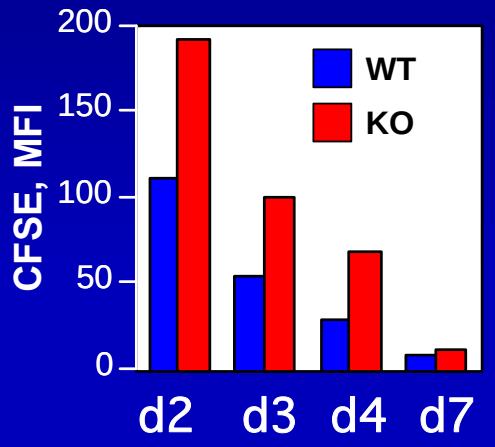
CFU-C: 1 in 16

Angel Lee

Reduced CSF-1 dependent expansion in LK31C cells from Gab2 -/- mice

d0 CFSE labeling





Heather Grifka

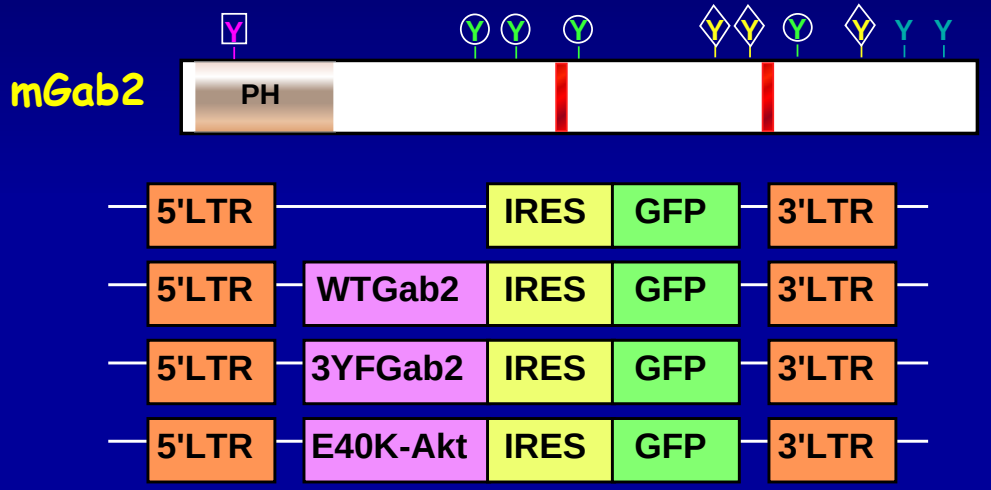
Gab2 in mouse bone marrow: summary

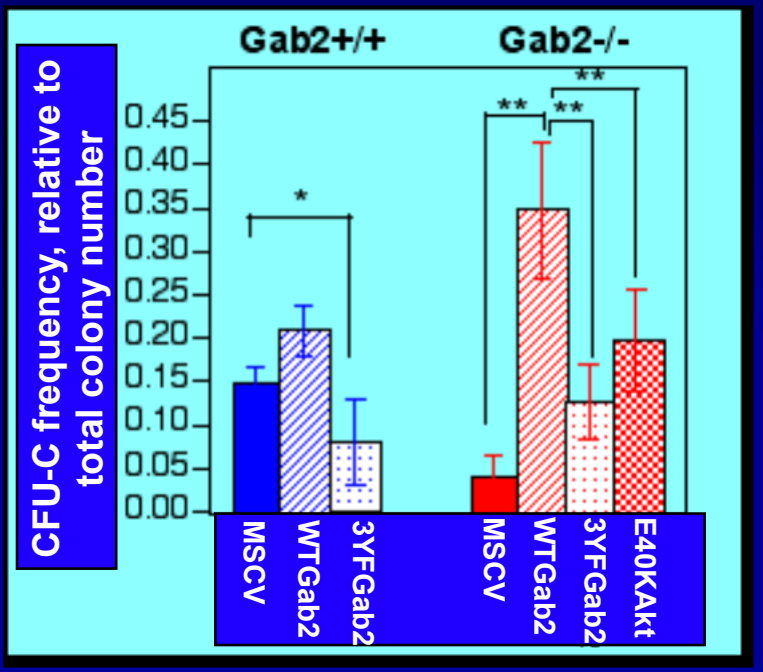
So far:

In mouse bone marrow, Gab2 is an important CSF-1R effector in the regulation of MNP development at the early progenitor stage, by promoting optimal expansion of progenitors (LIN*$^{-}$CD31^{hi}Ly6C^{-} subset or LK31C cells) and by ensuring timely differentiation along the MNP lineage.

What are the signaling pathways acting downstream of Gab2 that are required for CSF-1 mediated expansion of early stage MNP cells ?

WTGab2 but not 3YFGab2 can completely restore CFU-C numbers and expansion





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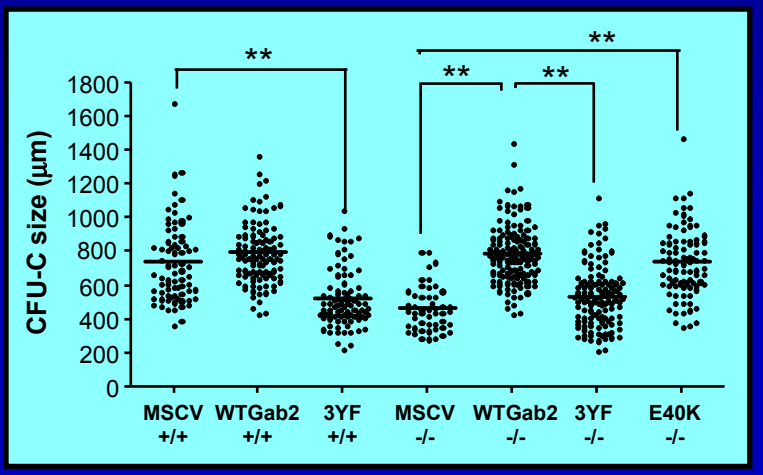
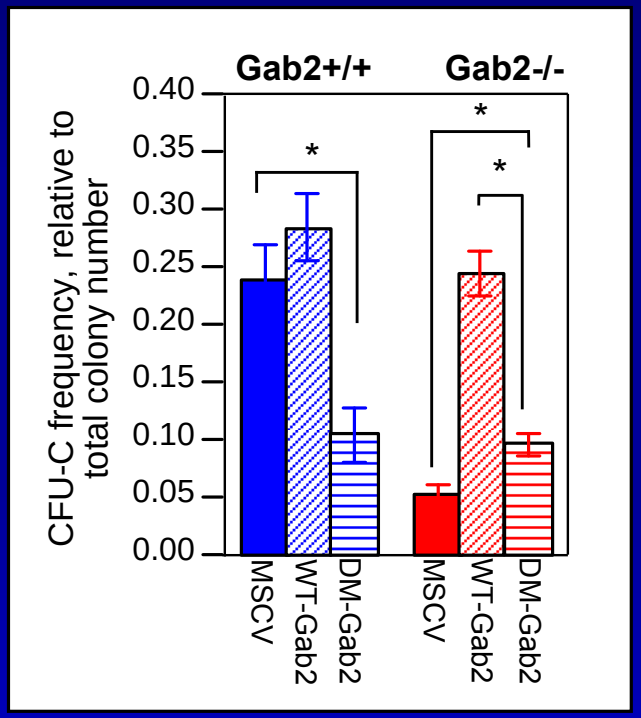


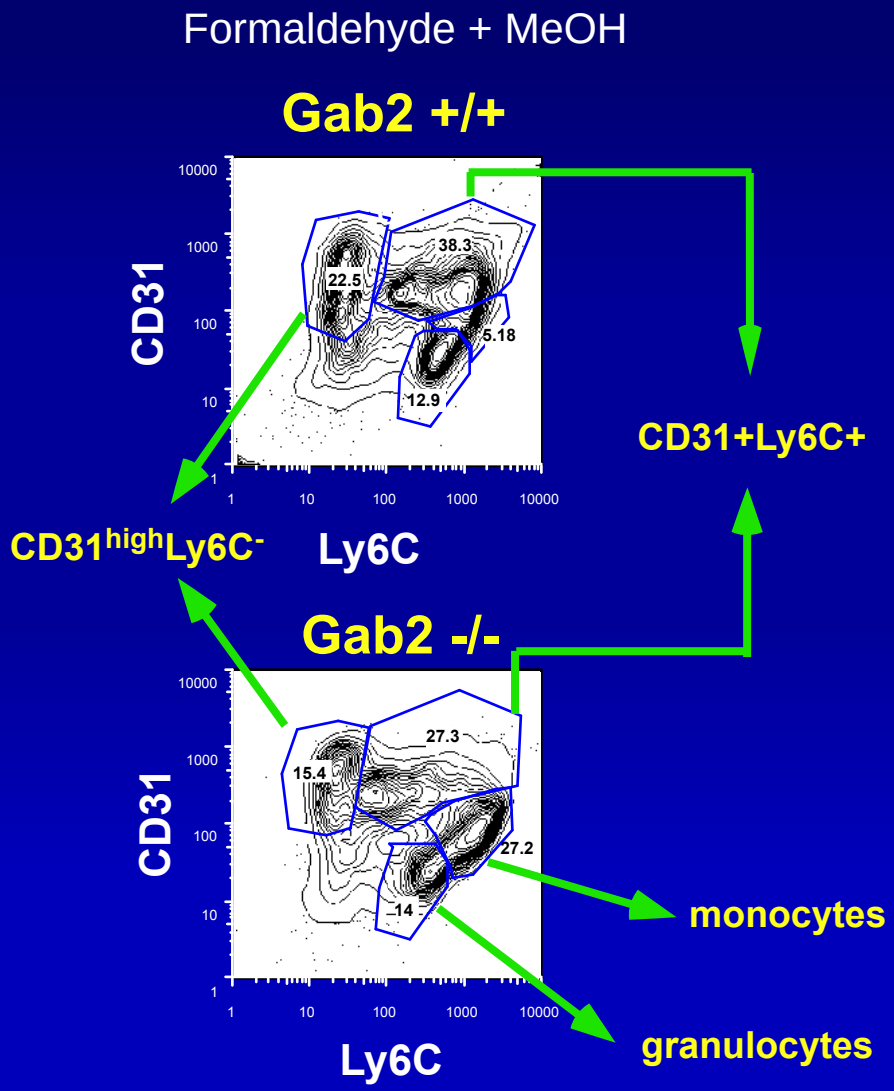
Heather Grifka
Angel Lee

Gab2 recruitment of Shp2 is also required for maximal CFU-C numbers and expansion



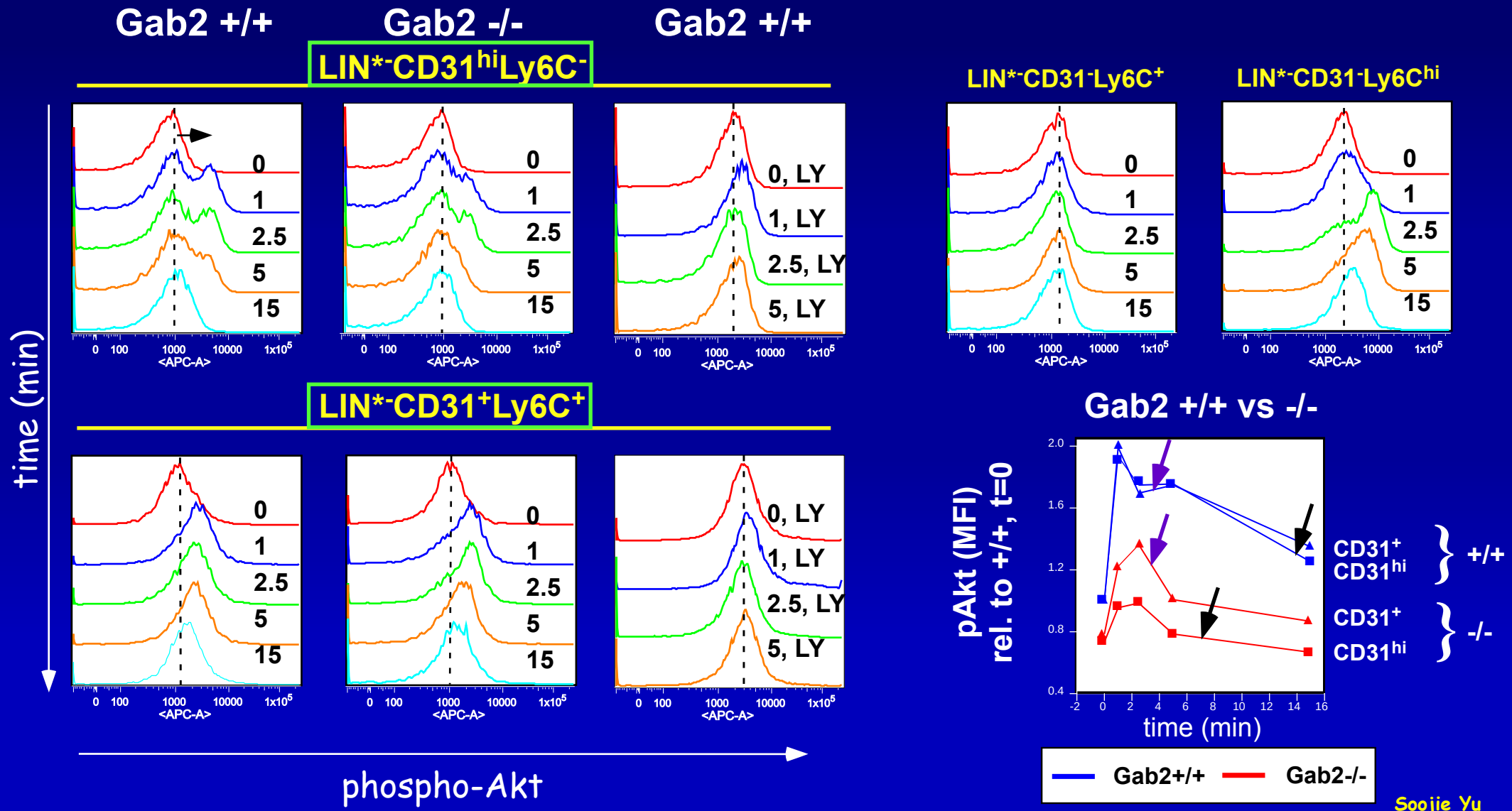
Soojie Yu
Angel Lee

Intracellular signaling in MNP progenitors analyzed by flow cytometry



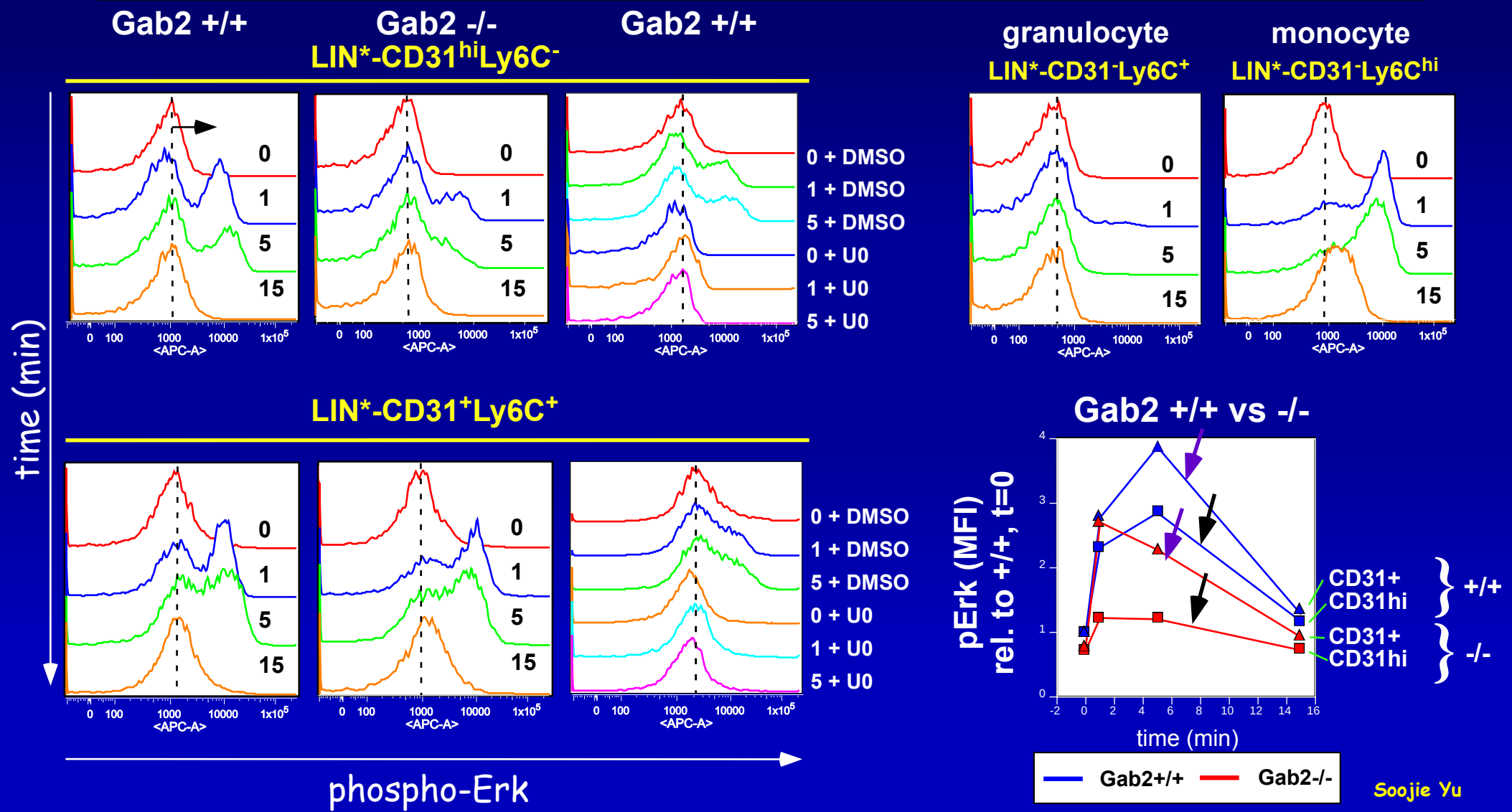
1. MACS depletion of day 2 cells (CD4/CD8/CD19/Ter119/Ly6G)
2. Starve in serum free medium
3. CSF-1 stimulation
4. Fix in formaldehyde
5. Stain with αLy6C-FITC
6. Methanol permeabilization
7. Stain with αCD31-PE, αphospho-Ab, GoatαRat-APC
8. Flow cytometry

Gab2 is required for maximal phosphorylation of Akt in CSF-1 responsive progenitors



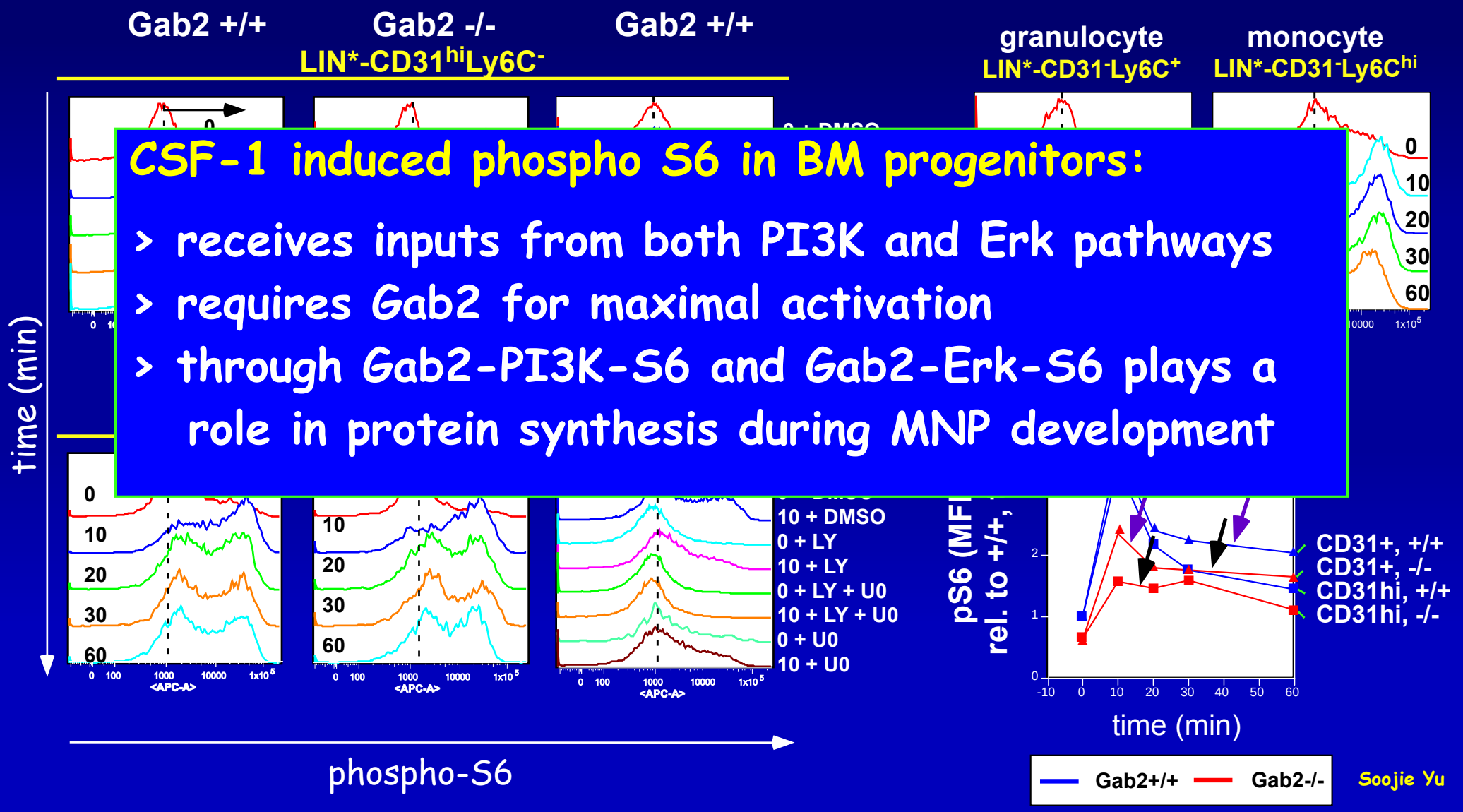
Soojie Yu

Gab2 is also required for full activation of the Erk pathway in CSF-1 responsive progenitors



Soojie Yu

Gab2 is required for maximal phosphorylation of S6 in CSF-1 responsive progenitors



CSF-1 induced phospho S6 in BM progenitors:

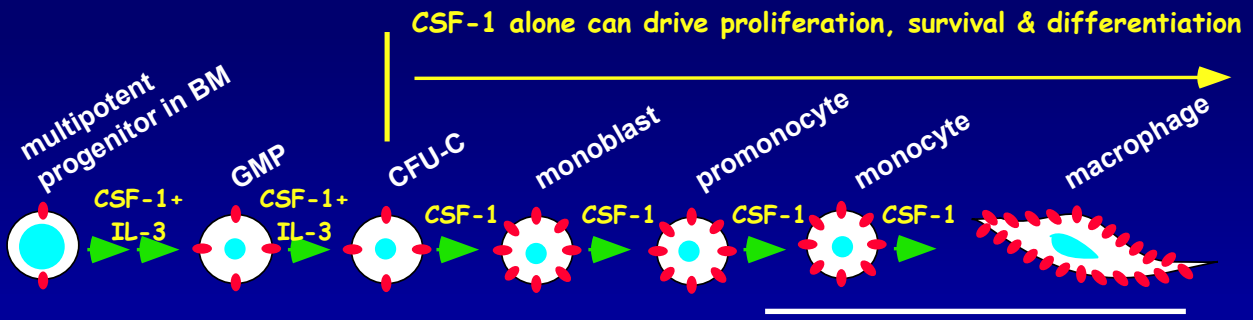
- > receives inputs from both PI3K and Erk pathways
- > requires Gab2 for maximal activation
- > through Gab2-PI3K-S6 and Gab2-Erk-S6 plays a role in protein synthesis during MNP development

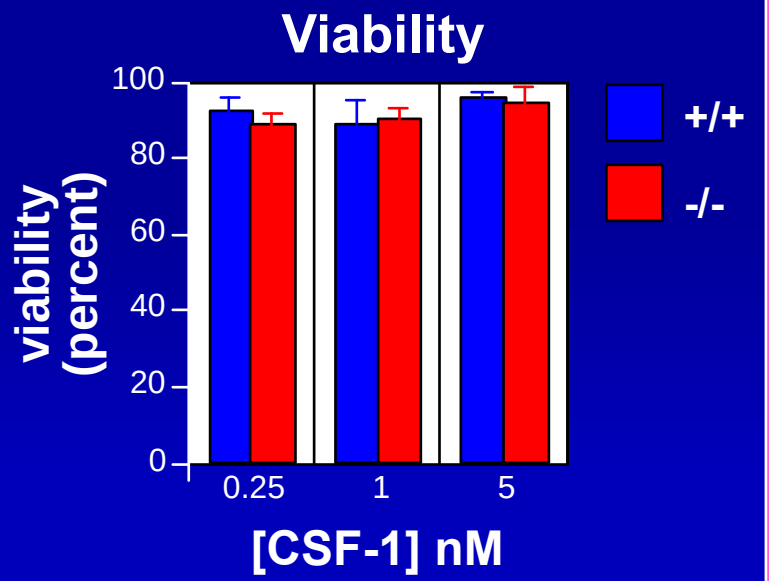
Soojie Yu

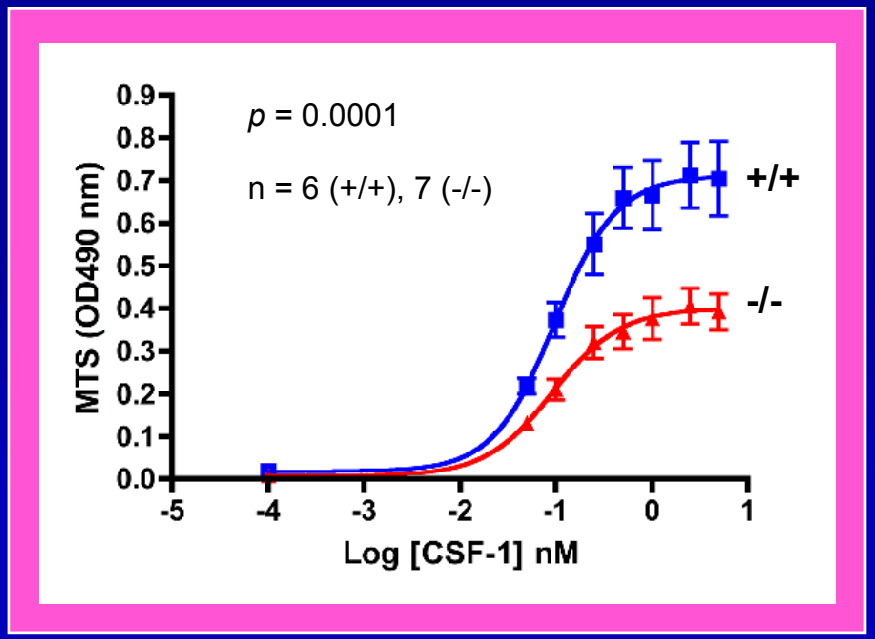
Gab2 regulated signaling in MNP development: summary

- Gab2-PI3K-Akt is important in mediating CSF-1 dependent MNP development in the bone marrow
- Gab2-PI3K-Akt synergizes with a second Gab2 dependent pathway, Gab2-Shp2, to promote MNP development
- Using a novel phospho-flow approach, we showed that Gab2 is needed for optimal activation of Akt & Erk in MNP progenitors. Gab2-Shp2 most likely promotes activation of Erk

Gab2 -/- bone marrow Mϕs show diminished CSF-1 dependent proliferation



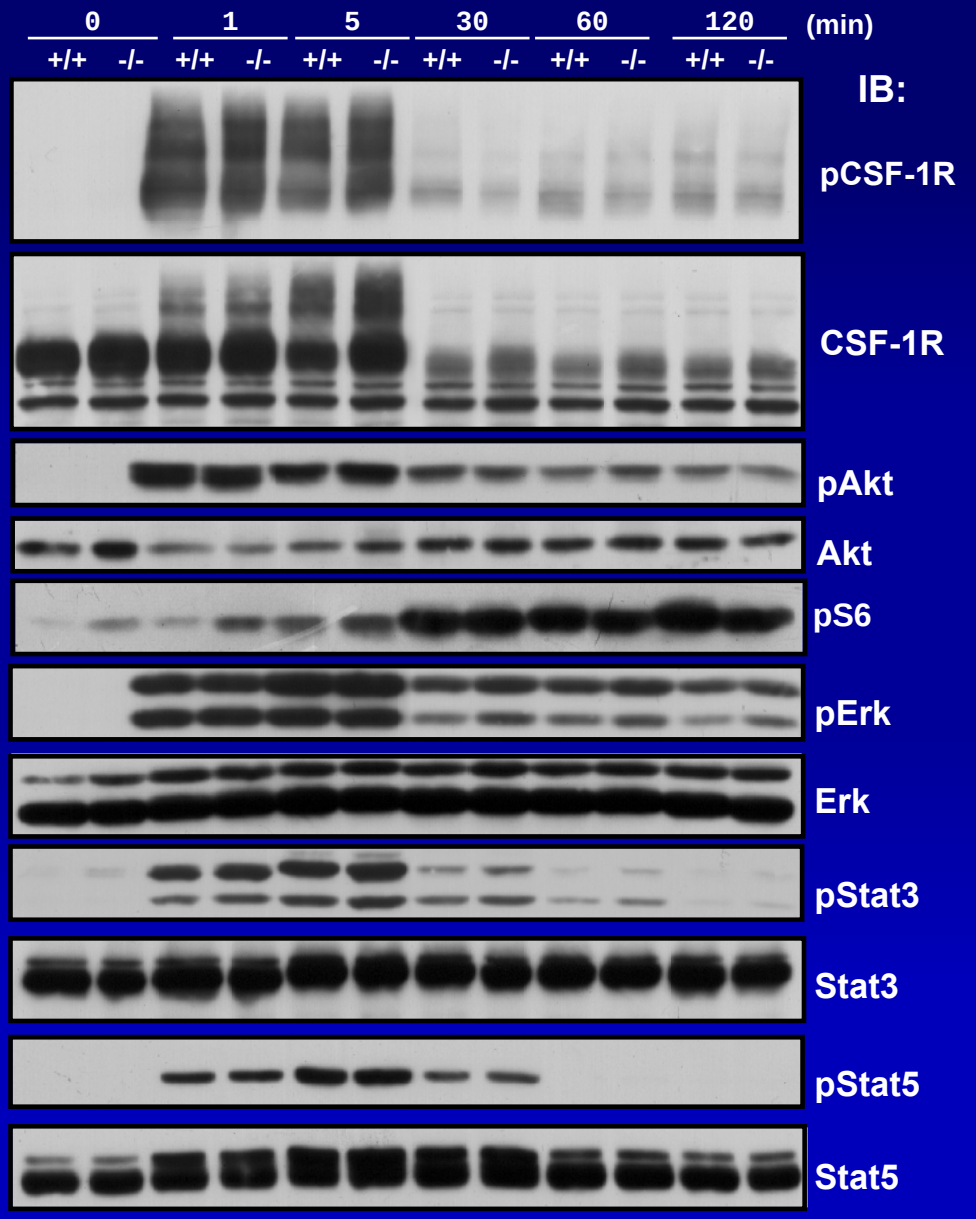


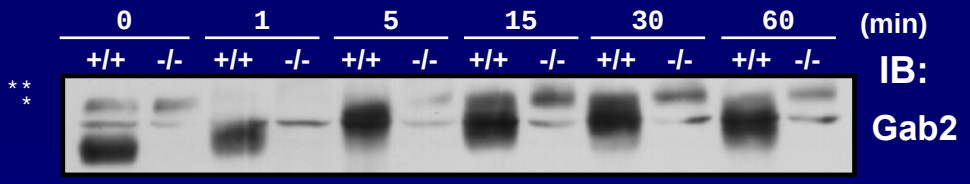


MTS assay (d5 - d7)

Angel Lee

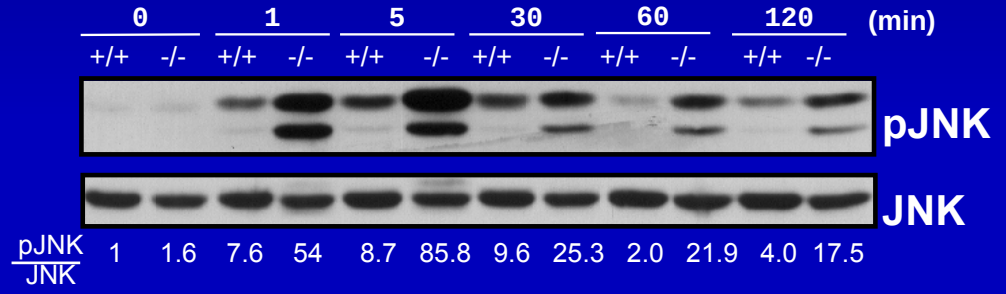
Signaling in Mϕs from Gab2 -/- mice





Constitutive Gab2 deletion

- does not affect CSF-1R phosphorylation or degradation
- does not affect CSF-1 induced Akt, Erk, S6 and Stat3/5 phosphorylation



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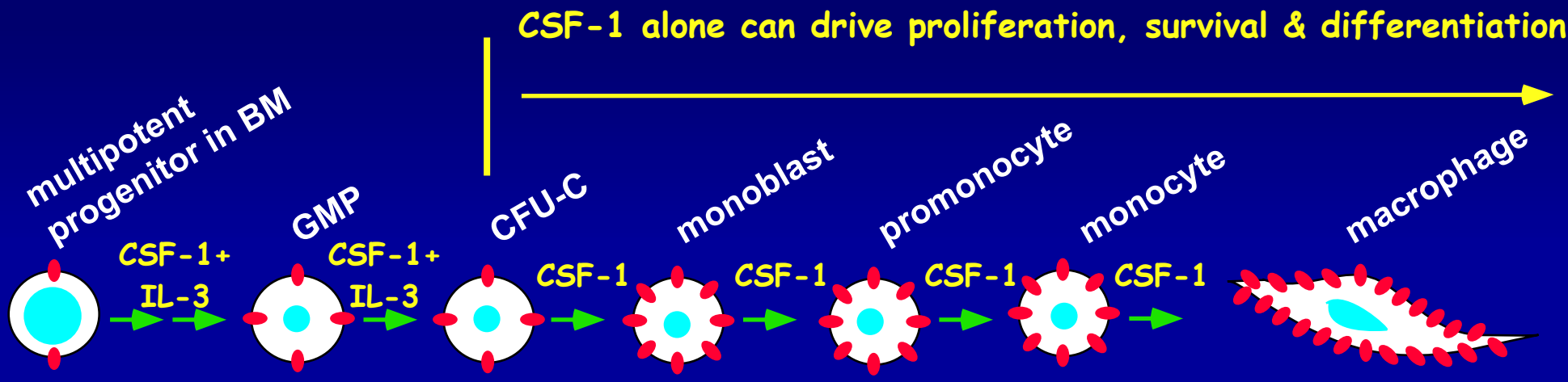
Gab2 as a CSF-1R effector in MNPs



commitment to MNP lineage
expansion of MNP progenitors

Akt, Erk, S6

proliferation of MNPs

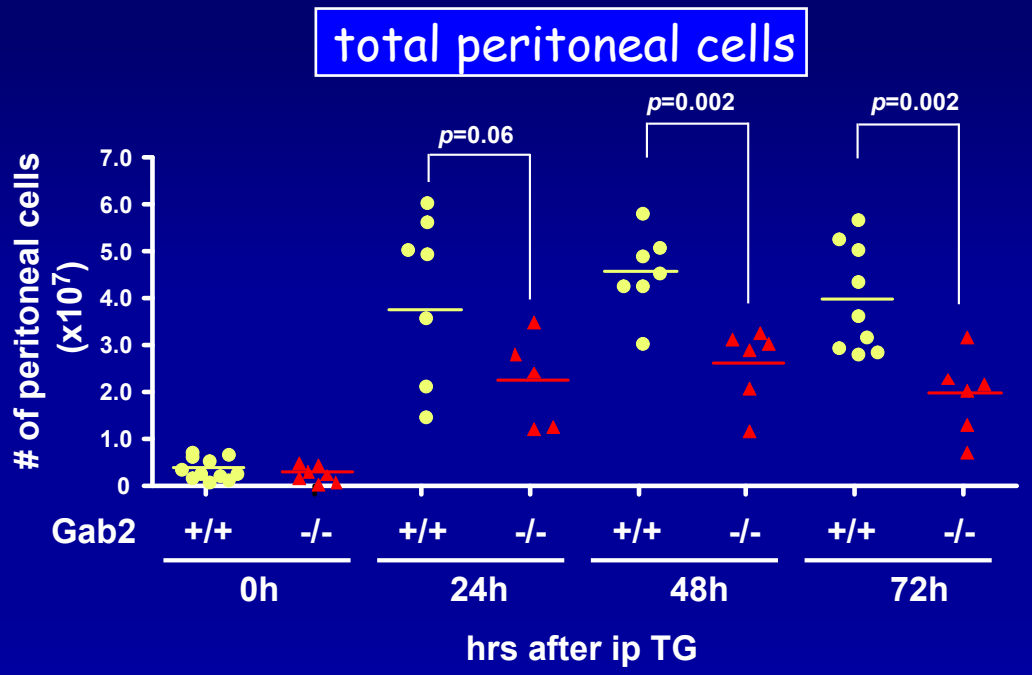
?JNK?

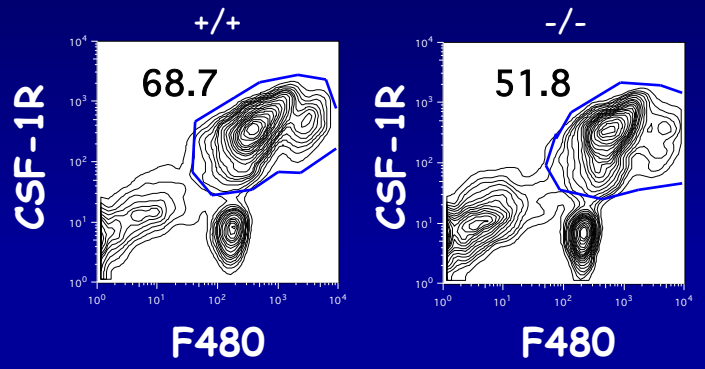
Gab2

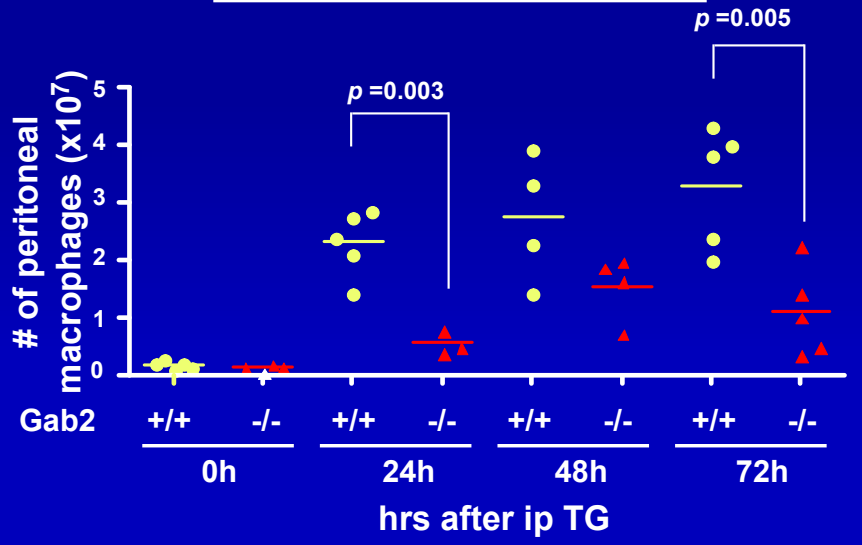
target in inflammation?

Are there in vivo consequences to Gab2 deficiency in cases of increased MNP demands?

Gab2 -/- mice responds submaximally to increased demands for MNPs







Sterile peritonitis model
(ip injection with thioglycollate)

Heather Grifka, Soojie Yu

Gab2-/- BM shows a minimal response in CFU-Cs to thioglycollate challenge

Gab2 deficiency results in a diminished BM response in situations with an increased demand for monocytes and Mϕs.

We predict that in infections or other inflammatory conditions with a high Mϕ infiltrate (e.g. breast cancer), that Gab2 deficiency could have a more serious consequence.

Heather Grifka, Soojie Yu

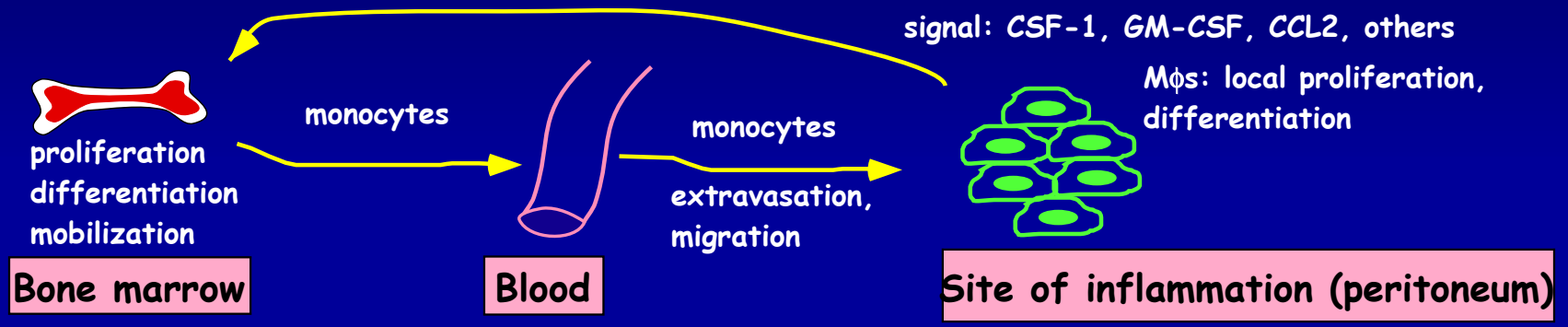
Future directions -1

Macrophage development in the bone marrow

- Use the LK31C ($Lin^-cKit^+CD31^+Ly6C^-$) bone marrow progenitor model to study signal transduction mechanisms that regulate the commitment and expansion of early bone marrow progenitors along the MNP lineage

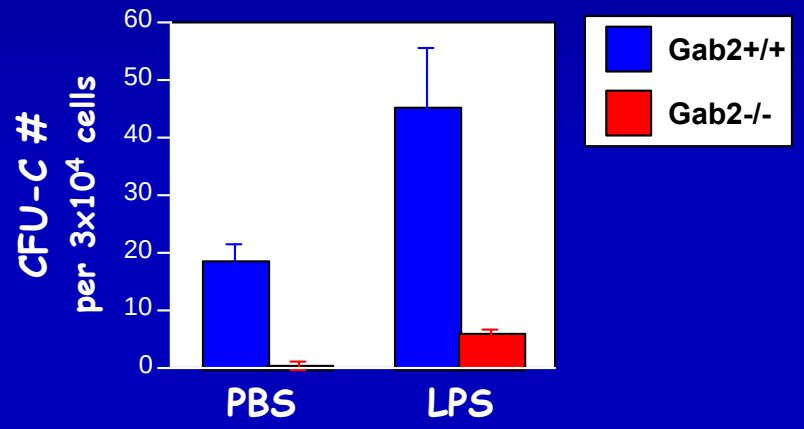
Future directions -1, cont'd

› Use the LK31C model (via bone marrow transplant) to study the mechanism underlying the diminished Mϕ recruitment to the peritoneum in the thioglycollate sterile peritonitis model

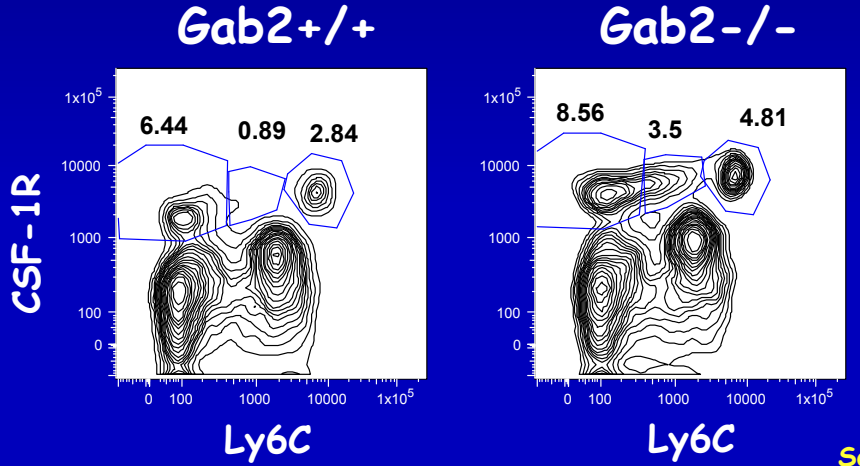


LPS Sepsis Model

Bone Marrow



Blood monocytes

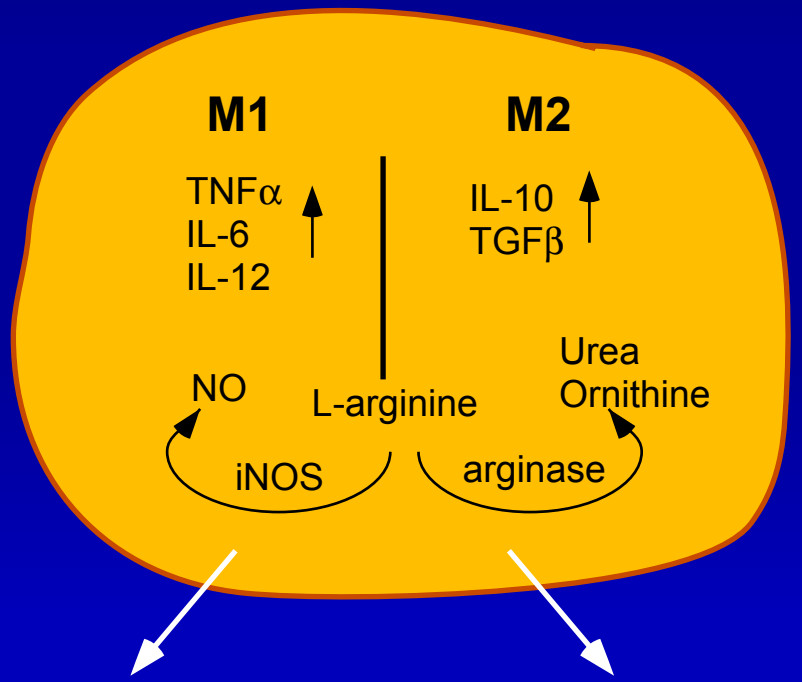


Soojie Yu

Future directions -2

Macrophage polarization & role in human diseases

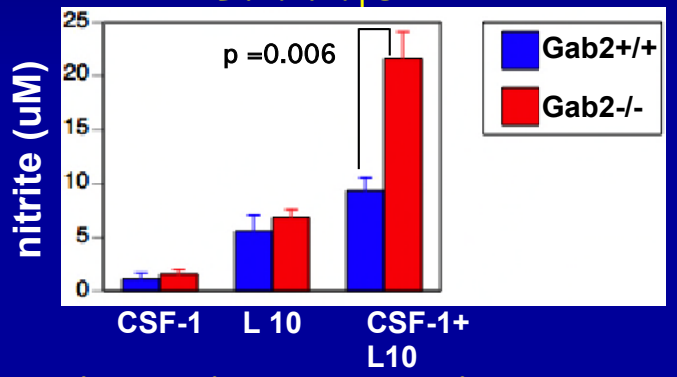
- short term: understand how Gab2 polarizes Mϕs
- long term: understand how signaling molecules impact Mϕ's role in in cancer (TAMs in tumor microenvironment)



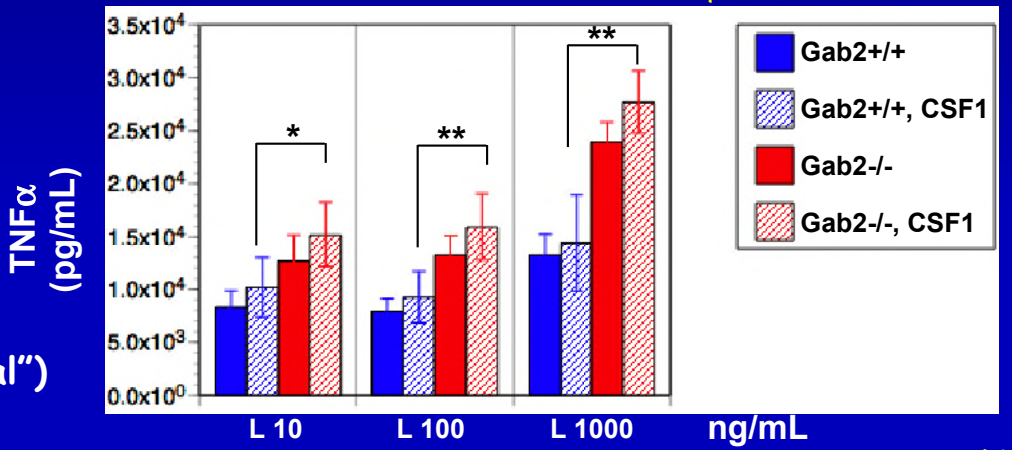
"killer macrophages"
(microbial infections)

"healer macrophages"
1. CSF-1 exposed Mϕs ("normal")
2. TAMs

BM Mϕs



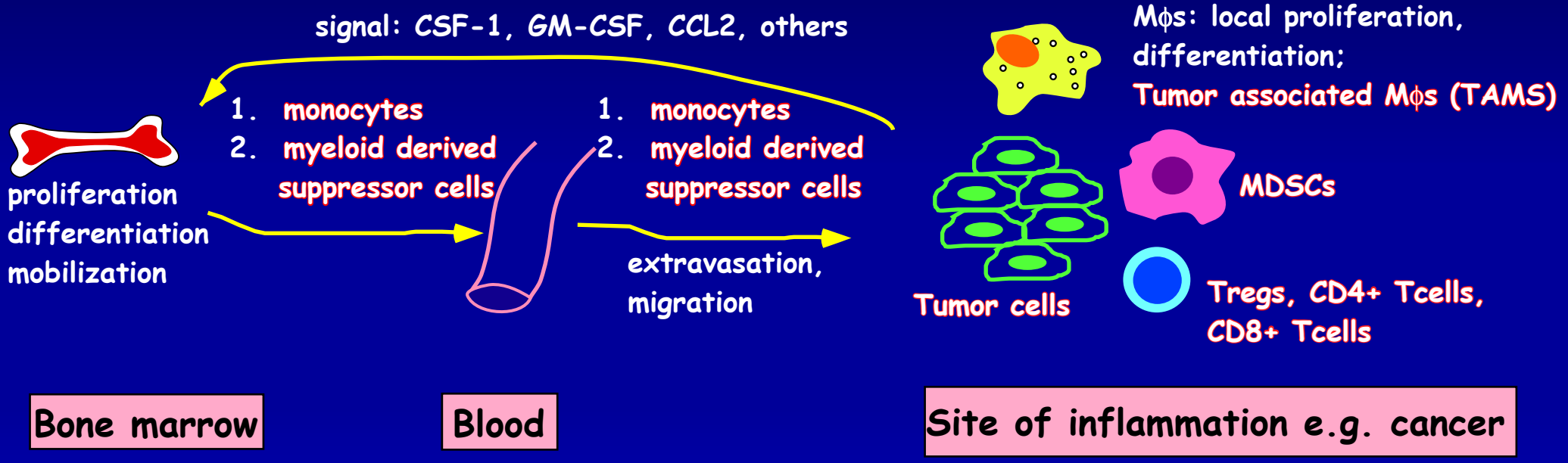
Elicited Peritoneal Mϕs



Angel Lee

Future directions -3

Bone marrow response to increased MNP demands



Myeloid derived suppressor cells (MDSCs)

- heterogeneous population of bone marrow derived myeloid cells found in pathological conditions e.g. cancer (both mice and humans)
- found in tumors and lympoid tissues
  - in mouse tumor models, may be as many as 20-40% of nucleated splenocytes
- defined as $Gr1^{+}CD11b^{+}$ (mouse) and $CD11b^{+}CD14^{-}CD33^{+}$ (human)
- many MDSCs in tumor-bearing mice co-express CD115 (CSF-1R) and CD124 (IL-4Rα)
- tumor secreted factors induce myeloid expansion and inhibit differentiation:
  - factors implicated in expansion: COX2, PG, SCF, CSF-1, IL-6, GMCSF, VEGF
  - postulated to go through Stat3
- increased circulating MDSCs in cancer patients at all stages disease
- highest numbers of MDSCs in patients with extensive tumor mets

Myeloid derived suppressor cells (MDSCs) - cont'd

- considered one of the major effectors of tumor induced immune deficiencies
- upregulated expression of iNOS (NO production) and Arginase I (depletion of arginine):
  - NO inhibits T cell function via different mechanisms (inhibition of Jak3-Stat5 function; inhibition of MHC class II expression, induction of T cell apoptosis)
  - low arginine levels inhibits T cell proliferation via different mechanisms (decreased CD3ζ chain expression, decreased CycD3 an Cdk4 expression); inhibitory effects require actively proliferation T cells
  - peroxynitrite: $O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$
    - potent oxidant: induces the nitration and nitrosylation of cysteine, methionine, tryptophan, tyrosine;
    - increased production at sites where MDSCs accumulate
    - nitration of tyrosines in the TCR-CD8 complex → $CD8^+$ T cell tolerance
- promote the de novo development of $FoxoP3^+$ regulatory T cells

Tumor associated macrophages (TAMs)

- all solid tumors recruit macrophages into their microenvironment
- preferentially localize to hypoxic regions in tumors
  - hypoxia induces HIF-1-dependent upregulation of CXCR4 on TAMs, tumor and stromal cells
  - hypoxia induces HIF-1-dependent upregulation of VEGF (angiogenic switch)
- derived from circulating monocytes recruited to tumors by CCL2, VEGF, CSF-1; may also arise from MDSCs
- $CD11b^{+}CD68^{+}F4/80^{+}CD14^{+}CD31^{-}CSF1R^{+}VEGFR1^{+}Arg1^{+}MR^{+}SR^{+}$
  - CD68 is expressed on monocytes/Mϕs; MR is the mannose receptor, SR is Scavenger Receptor-A
- M2 phenotype: $IL\text{-}10^{high}TGF\text{-}\beta^{+}Arg1^{+}$ (low expression of IL-12, TNFα, IL-6)
- defective NF-κB activation in TAMs from advanced tumors
  - p50 homodimers are responsible for the sluggish activation of TAMs
  - correlates with impaired expression of NF-κB-dependent inflammatory functions e.g. expression of TNF-α, IL-1, IL-12

Tumor associated macrophages (TAMs) cont'd

- correlation between TAM abundance and poor prognosis: esp. breast, prostate,ovarian and cervical cancers
- pro-tumoral functions:
  - pro-angiogenesis through expression of many factors (VEGF, FGF2, CCL2,..)
  - production of growth factors (e.g. VEGF, PDGF, EGF)
  - induction of matrix remodeling through production of TGF-β, CCL2, MMPs
  - immune suppression through cytokines (TGF-β, IL-10), recruitment of Tregs
  - skewing of adaptive immunity to a Th2 response through CCL17, CCL18, CCL22

TAMs: biomarker for cancer?

TAM content is a strong predictor of tumor aggressiveness in head & neck squamous cell CA Marcus et al. Cancer 101:2779, 2004

TABLE 2
Correlations with Elevated Macrophage Counts at the Primary Tumor Site

Elevated macrophage count correlated with	*P* value
Presence of metastatic lymph nodes	< 0.0001
Presence of extracapsular spread	0.0001
Advanced clinical stage	0.002
Alcohol use	0.003
Lymph node metastasis in small primary tumors (T1, T2)	0.001
Extracapsular spread in small primary tumors (T1, T2)	0.03

1. Why is there variation in TAM content for the "same" disease in different patients?
2. What is the role of TAMs?
3. Do TAMs in different tumors have the same function?

TAM content may be useful for stratifying treatment in follicular lymphoma Canioni et al. J. Clin. Oncol. 24:400, 2008