A Novel Regulator In Tumor Microenvironment: Ca^{2+}/Calmodulin Kinase Kinase 2 (CaMKK2)

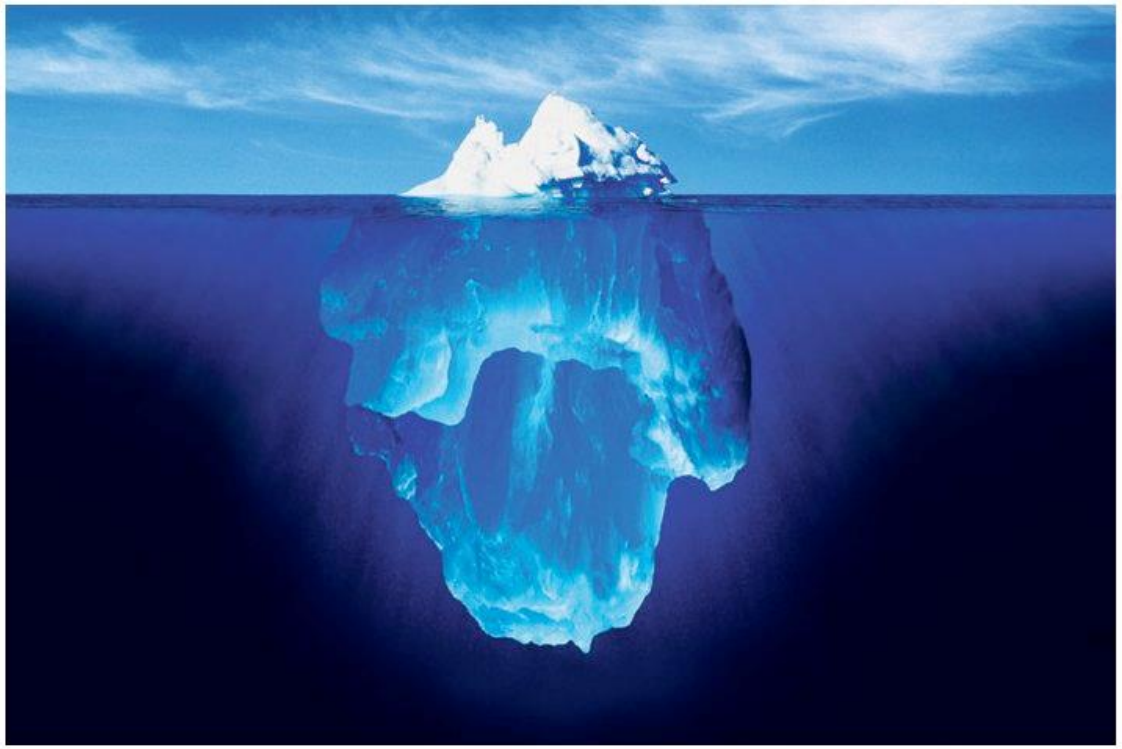
Nelson Chao, MD, MBA

Duke University

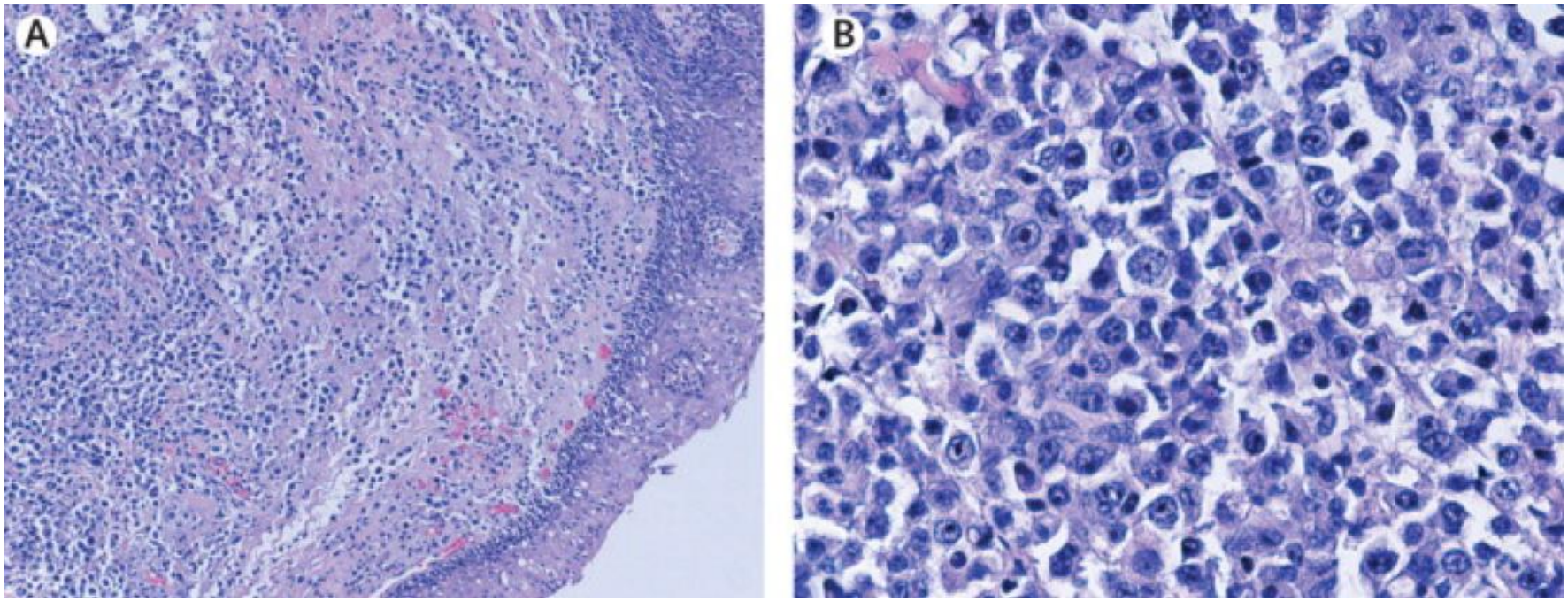
History

- 38 year old, previously healthy presents with one month history of sweats, fatigue and fever. Seen by PCP, nothing remarkable on exam, given a course of antibiotics without improvement.
- Exam not remarkable, CXR demonstrates a large mediastinal mass.
- Repeat exam shows supraclavicular lymphadenopathy.
- Biopsy of lung mass and lymph node performed.

This is not cancer…



What happens in a lymphoma?



Journal of Cell Science
jcs.biologists.org

The Tumor Microenvironment at a Glance

Frances R. Balkwill, Melania Capasso and Thorsten Hagemann

Lymphatic endothelial cells

- Tumor cells can invade existing lymphatics or stimulate lymphatic vessel sprouting with the production of factors, such as VEGFC or VEGFD.
- Lymphatic vessels are important in the dissemination of malignant cells, but they might also promote tumor development by mechanomodulation of the TME and altering the host immune response to the tumor.

T lymphocytes

- Abundant in the majority of human and experimental cancers (up to 10% of all cells in the tumor).
- Found within and surrounding the tumor mass.
- Phenotypes of pro- and anti-tumor T cells can vary with disease type and stage. $CD8^+$ cytotoxic T cells, $CD4^+$ Th1 helper T cells and $\gamma\delta$ T cells are usually associated with a good prognosis.
- $FOXP3^+$ T regulatory cells, $CD4^+$ Th2 helper T cells and TH17 cells are usually associated with a poor prognosis.

B lymphocytes

- Sometimes found at the invasive margin of some tumors, but more often in secondary and tertiary structures adjacent to the TME.
- B cell infiltration is associated with good prognosis in some human cancers. However, deposition of B cells and immunoglobulin is tumor-promoting in some mouse cancer models.
- Immunosuppressive IL-10 producing subtypes of B cells, B10 or Breg cells also have tumor-promoting activity in mouse models.

Myeloid cells

Consist of several subtypes; probably the most abundant cell lineage in the TME.

Tumor-associated macrophages (TAMs)
- Typically tumor-promoting.
- $IL\text{-}10^{hi}$, $IL\text{-}12^{low}$ phenotype and mannose-receptor-positive.
- TAMs also produce angiogenic factors and accumulate in hypoxic or nectrotic areas of the TME.

Myeloid-derived suppressor cells (MDSCs)
- Inhibitory immune cells producing large amounts of IL-10.
- Inhibit cytotoxic T cells and polarize TAMs to a tumor-promoting phenotype.

Tumor-associated neutrophils (TANs)
- Can have both pro- and anti- tumor activity.

Terminally-differentiated myeloid dendritic cells
- Might be defective in the TME and cannot adequately stimulate an immune response to tumor-associated antigens.

NK and NKT cells

- Innate cytotoxic lymphocytes, NK cells and NKT cells are usually found outside the tumor area.
- For some cancers they can predict a good prognosis.

Cancer-associated fibroblasts

- Found in many human and experimental cancers, especially at the invasive margins.
- Produce tumor-promoting growth factors, chemokines, cytokines, ECM components and ECM remodeling enzymes.
- Can also have important immunosuppressive activity.

Vascular endothelial cells

- Angiogenic factors produced by malignant cells, myeloid cells or CAFs in the TME stimulate sprouting of endothelial cells.
- The new blood vessels have chaotic branching and uneven vessel lumina. The vessels are also leaky, raising interstitial pressure, with uneven blood flow, oxygenation, nutrient and drug delivery in the TME.

Key

- Malignant cells
- Malignant cells in necrotic or hypoxic areas
- NK and NKT cells
- Macrophage
- T lymphocyte
- Adipocyte
- B lymphocyte
- Lymphatic endothelial cell
- Pericyte
- Endothelial cell
- CAF
- Dendritic cell
- Mesenchymal stem cell
- Red blood cell
- Extracellular matrix

Mesenchymal stem cells

Mesenchymal stems cells can be recruited from the bone marrow and give rise to CAFs, pericytes, adipocytes and smooth muscle cells in the TME.

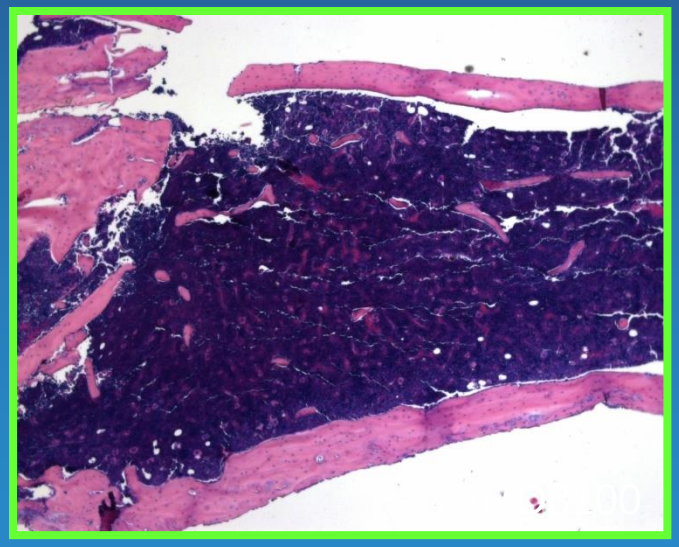
Adipocytes

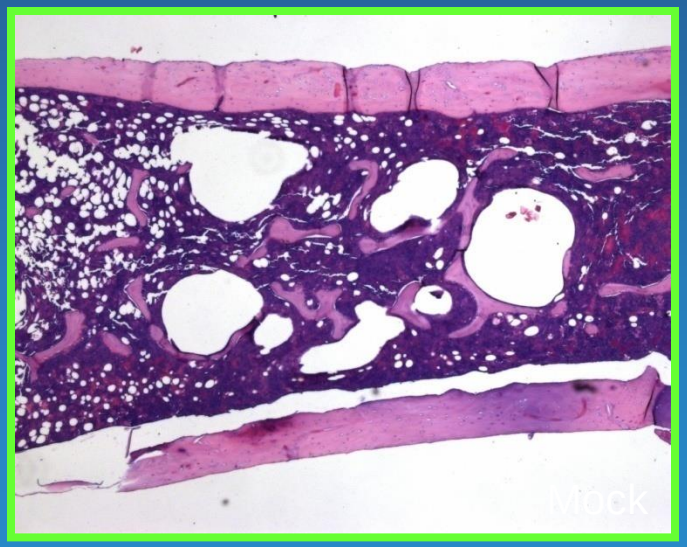
- In some cancers, adipocytes actively aid recruitment of malignant cells through the secretion of adipokines.
- They also promote malignant cell growth by providing fatty acids as fuel for cancer cells.

Pericytes

- Perivascular stromal cells, pericytes, provide structural support for blood vessels in the TME.
- Low pericyte coverage of TME vessels correlates with poor prognosis and increased metastases.

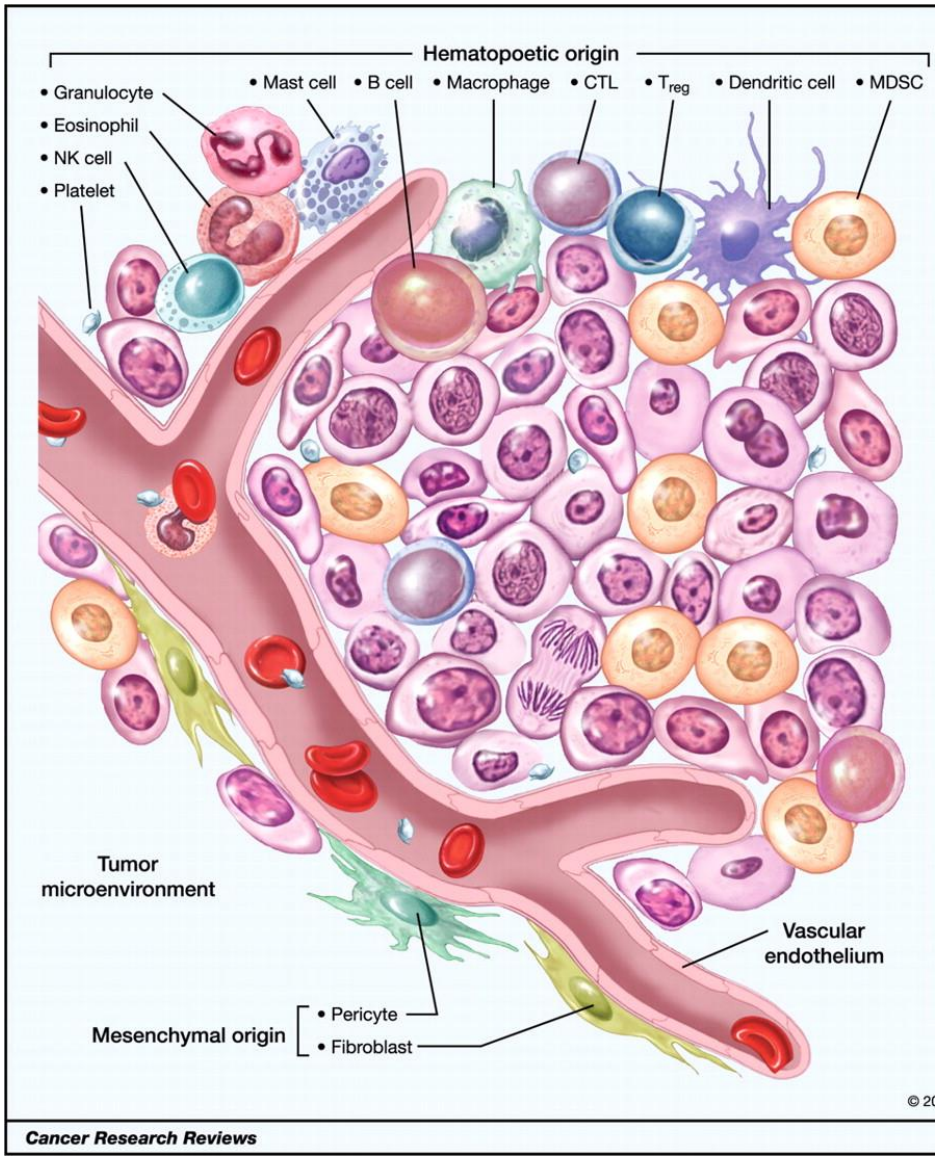
AMD3100 enhances marrow engraftment

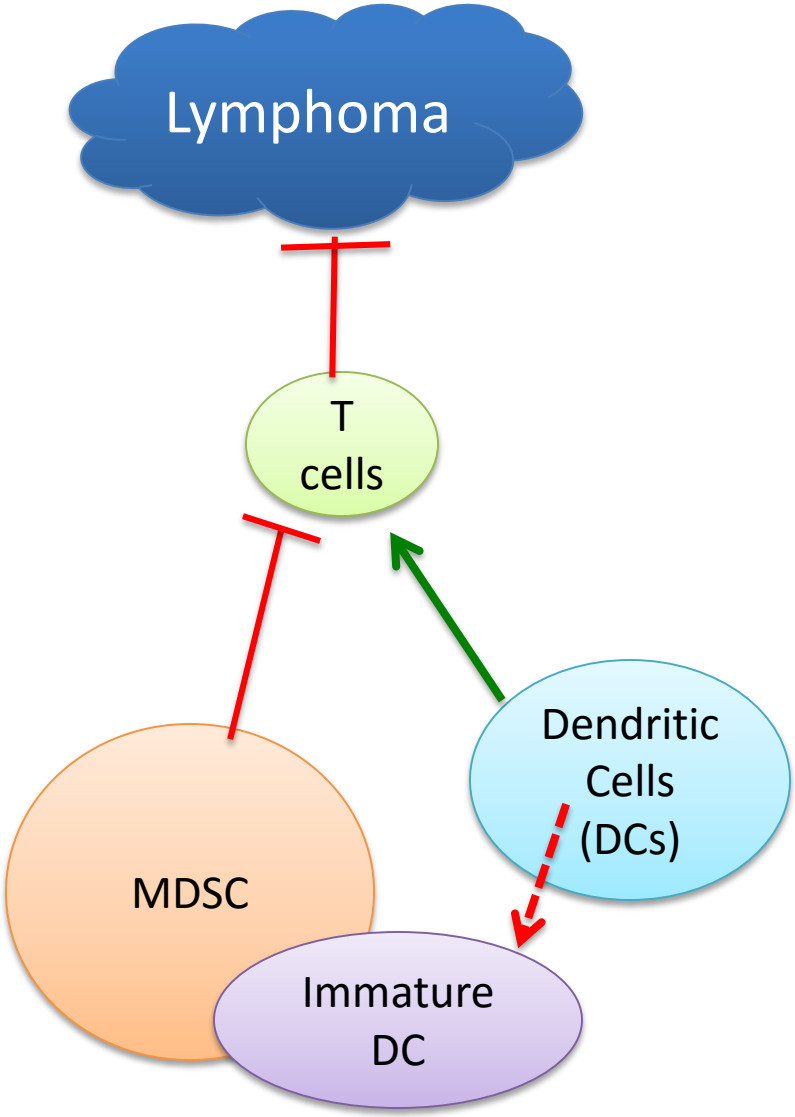




Histology (day +65)

Tumor microenvironment





Kerkar, Restiso. *Cancer Res. 2012*

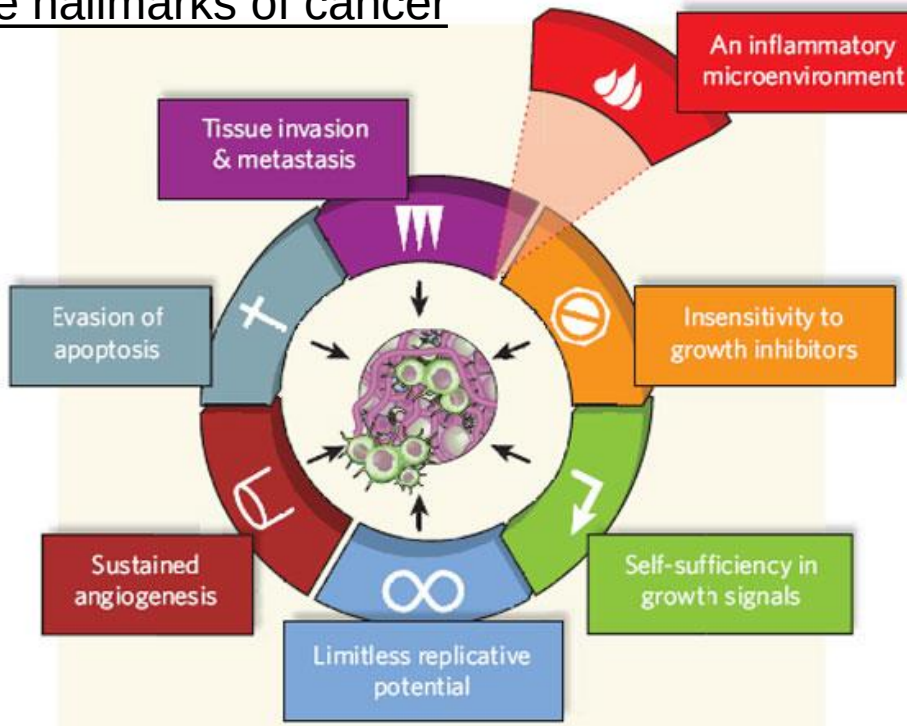
Controlling inflammation changes tumor microenvironment

Calcium/Calmodulin-dependent Protein Kinase Kinase 2 Regulates Macrophage-mediated Inflammatory Responses*[S]

Received for publication, December 20, 2011, and in revised form, February 11, 2012 Published, JBC Papers in Press, February 14, 2012, DOI 10.1074/jbc.M111.336032

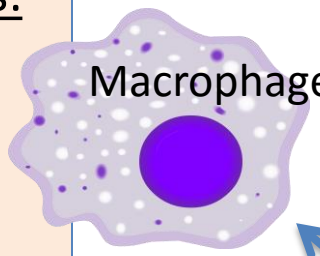
Luigi Racioppi[‡§¶1], Pamela K. Noeldner[‡], Fumin Lin[‡], Stephanie Arvai[‡§], and Anthony R. Means[‡§¶]

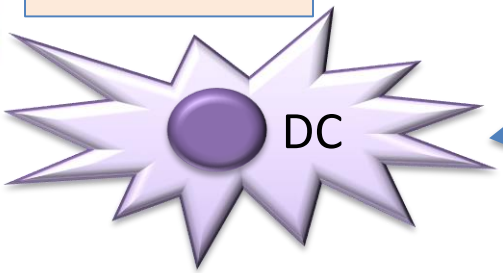
The hallmarks of cancer



Hanahan, Weinberg. *Cell. (2000)*
Mantovani. *Nature. (2009)*

cytokines:
IL-1
IL-6
TNF
RANKL





CaMKK2

?

CaMKK2 Regulates Inflammatory Response

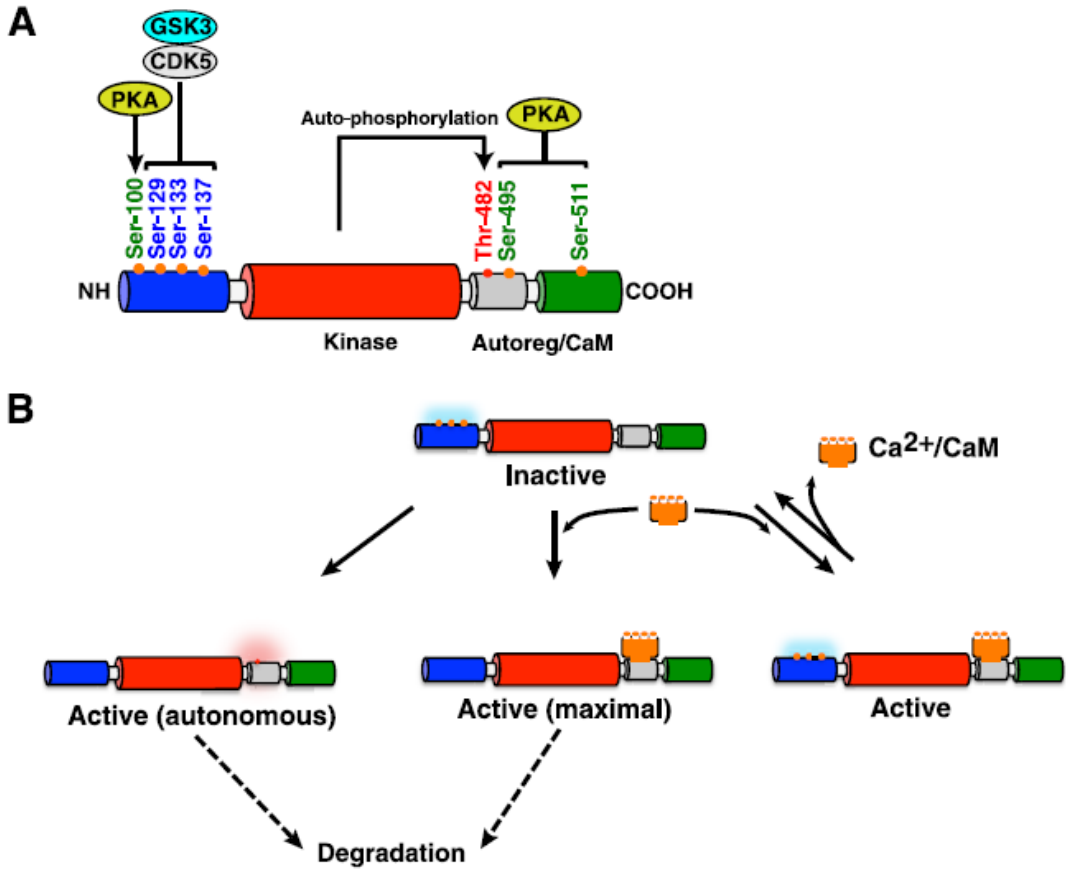
Genetic ablation of CaMKK2:

1. Protects adipose from high-fat diet induced inflammation
2. Protects from endotoxin shock
3. Protects from fulminant hepatitis
4. Impairs macrophage activation
    a. Spreading
    a. Migration
    b. Phagocytosis
    c. cytokines & chemokines release

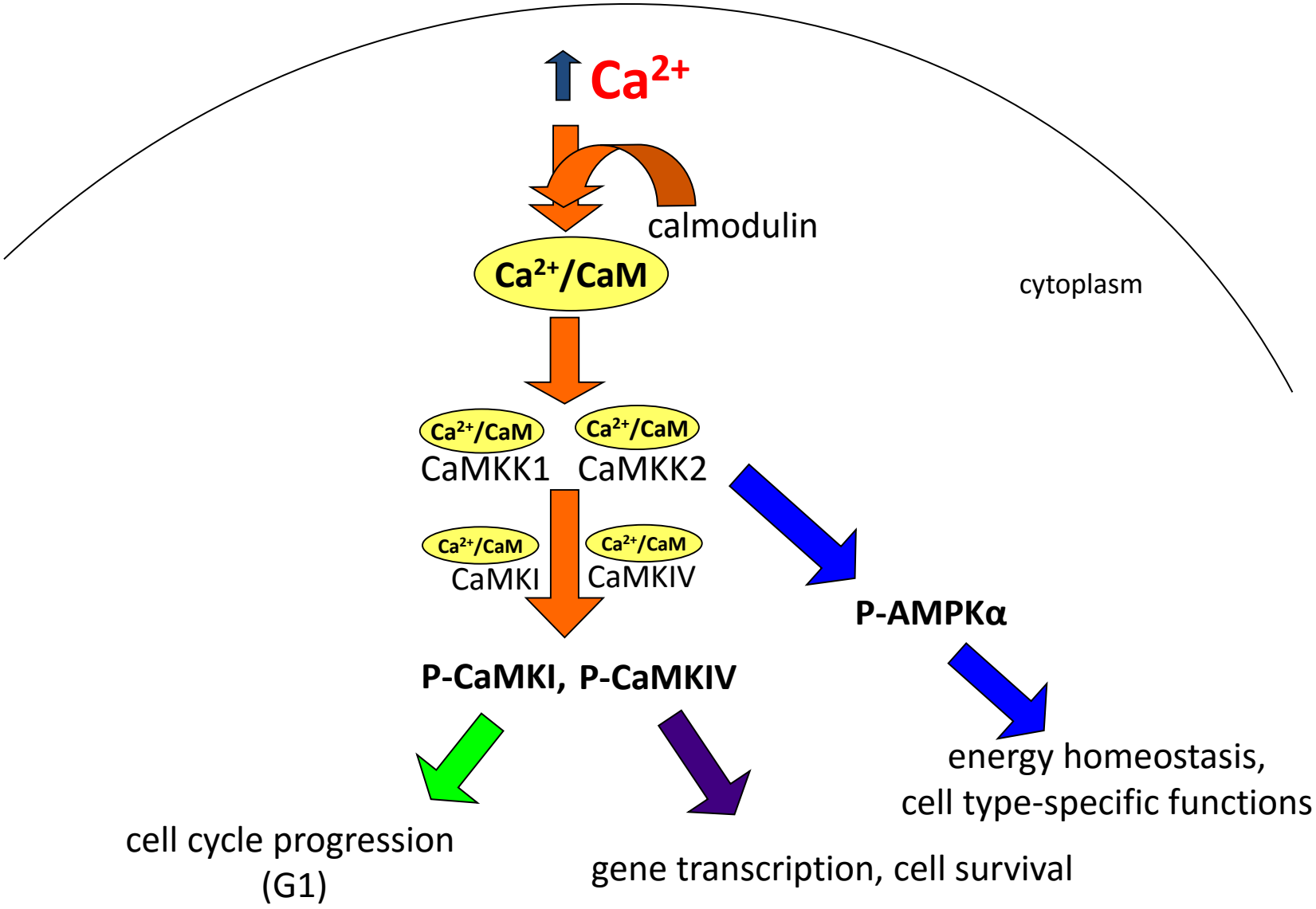
Luigi Racioppi et al. (2012) J Biol Chem

CAMKK2

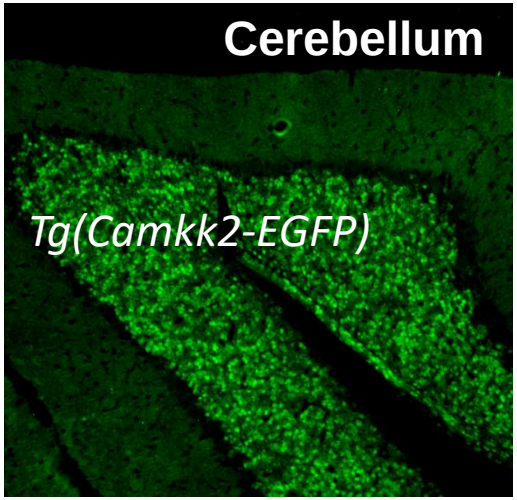
- Calcium/calmodulin-dependent kinase kinase II
- Structure: complex oligomeric structure

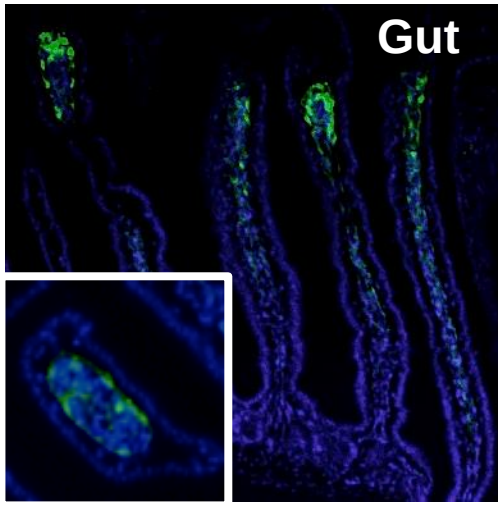


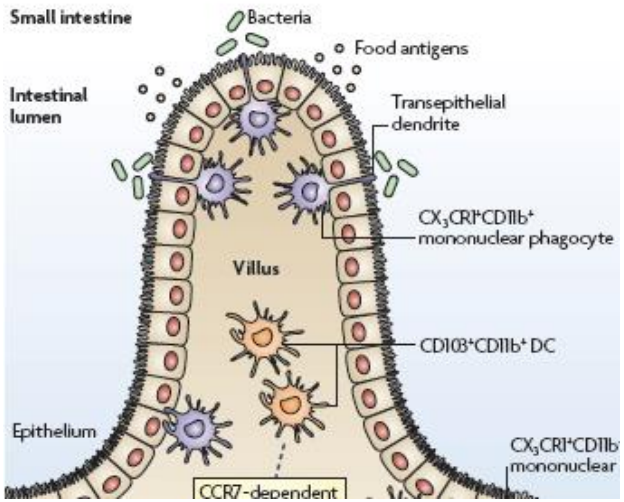
Calmodulin Kinase Cascades and Cell Function



Macrophage and monocyte express CaMKK2



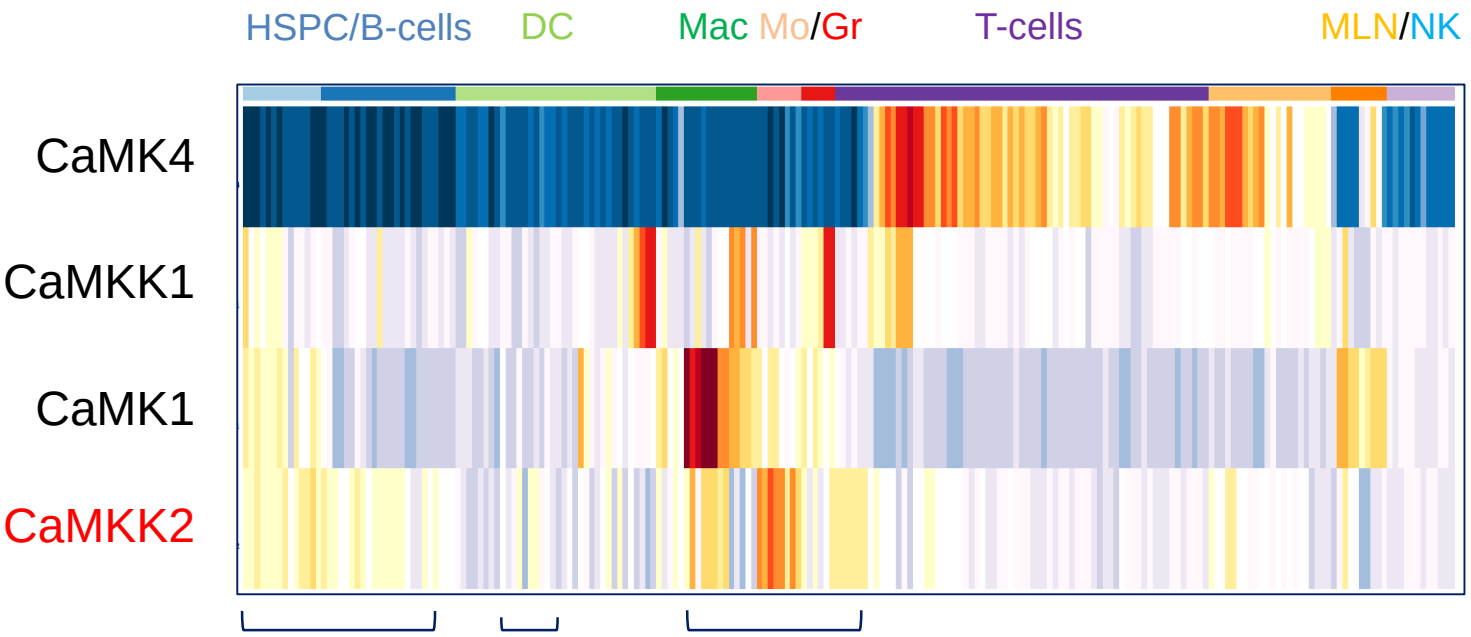




Luigi Racioppi et al. (2012) J Biol Chem

Hypothesis: CaMKK2 Controls Myeloid Differentiation and Immunity

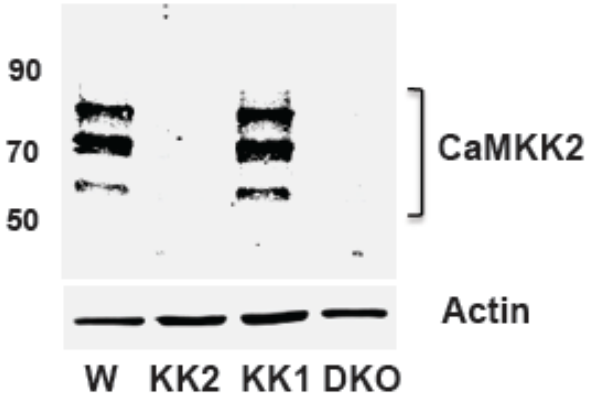
The CaMKs family in Blood Cells



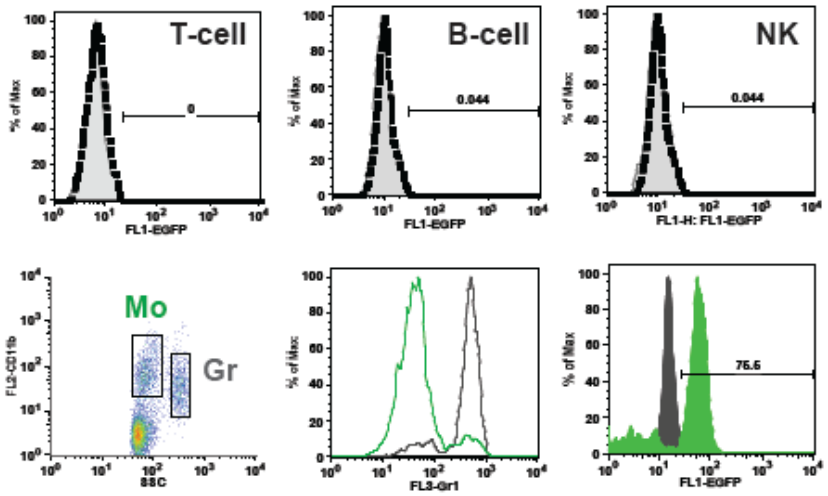
http://rstats.immgen.org/MyGeneSet/

KK2 Knock out data

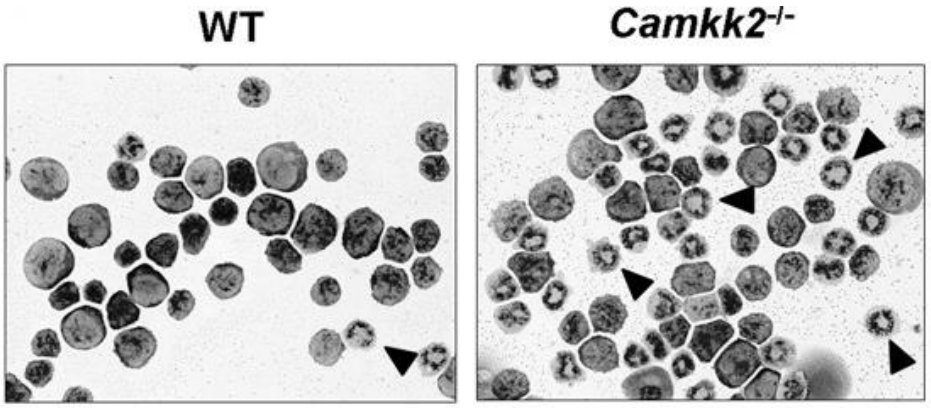
Macrophage

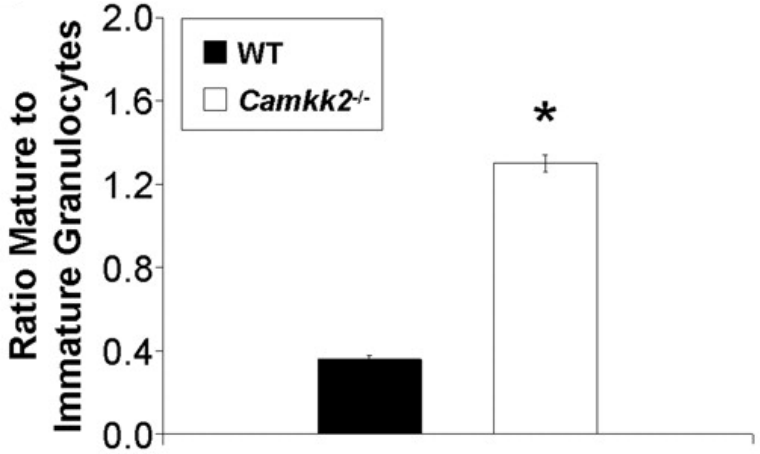


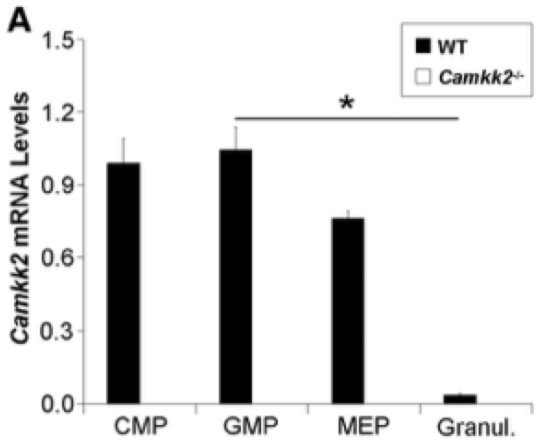
Blood



CaMKK2 regulates Granulopoiesis







Ellen C. Teng et al. J Leukoc Biol 2011;90:897-909

JLB JOURNAL OF LEUKOCYTE BIOLOGY

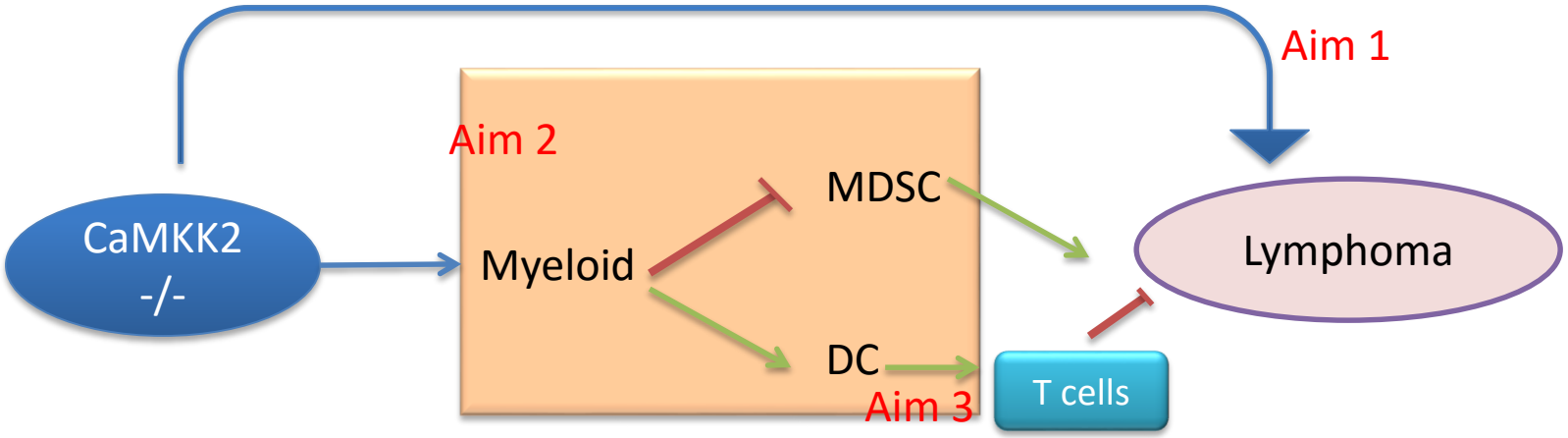
Myeloid-Derived Suppressor Cells (MDSC)

- Myeloid origin, immature state, suppressing T-cell responses
- Contribute to the negative regulation of immune responses during cancer and other diseases
- Regulate innate immune responses by modulating the cytokine production of macrophages
- Expanded in pathological conditions
- Mouse: CD11b+ Gr1+
  - 20-30% in BM, 2-4% in spleen, 0% in LN
  - Expand in cancer, chronic infection/sepsis, trauma, transplantation
  - **Monocytic morphology: CD11b+ Ly6G** low **Ly6C** high (differentiation)
  - **Granulocytic morphology: CD11b+ Ly6G** high **Ly6C** low (expansion)

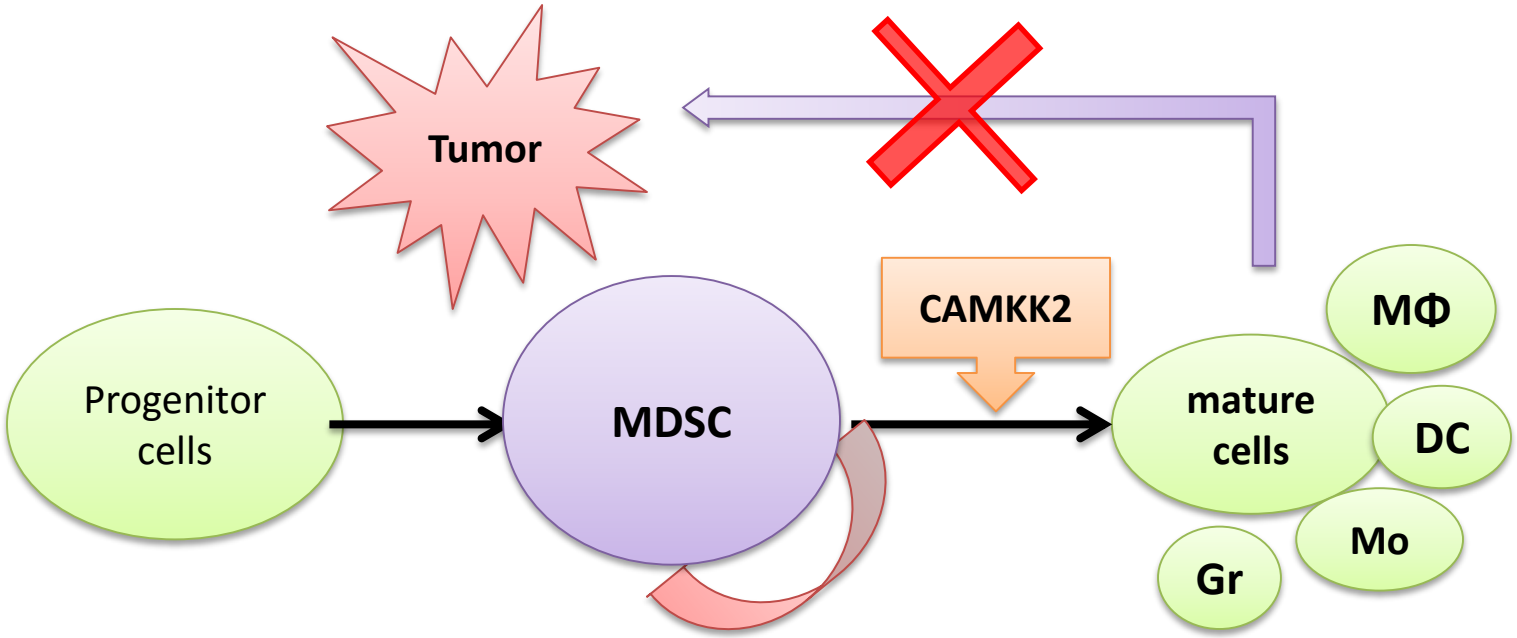
Gabrilovich, Nagaraj. *Nature Reviews Immunology*. 2009.

Hypothesis

- CaMKK2-/- environment impairs lymphoma progression by regulating myeloid cells maturation and function
  - Does CaMKK2-/- microenvironment suppress lymphoma progression?
  - Does CaMKK2 regulates myeloid cells maturation and function in tumor environment?
    - Myeloid derived suppressor cells (MDSC)
    - Dendritic cells (DC)
  - Does CaMKK2-/- DC stimulates T-cell proliferation better?



CAMKK2 Controls Myeloid Development?



Models: E.G7 Injection

- E.G7:
  - derived from the C57BL/6 mouse lymphoma cell line EL4;
  - OVA expression for antigen-specific response
  - transfected with GFP-LUC.

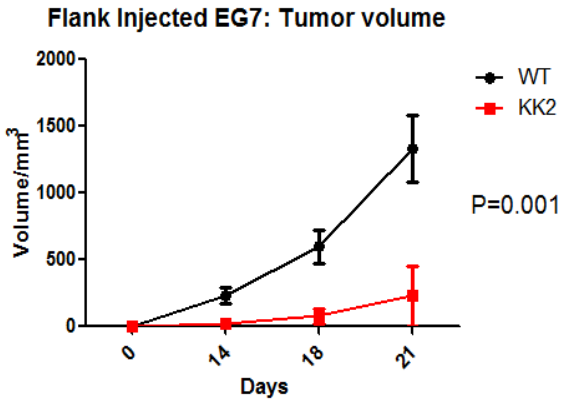
- Model:
  - Intracranial injection
  - Flank injection
  - CaMKK2 inhibitor: STO609
  - LysM-Cre;fl/fl lineage knockout mice

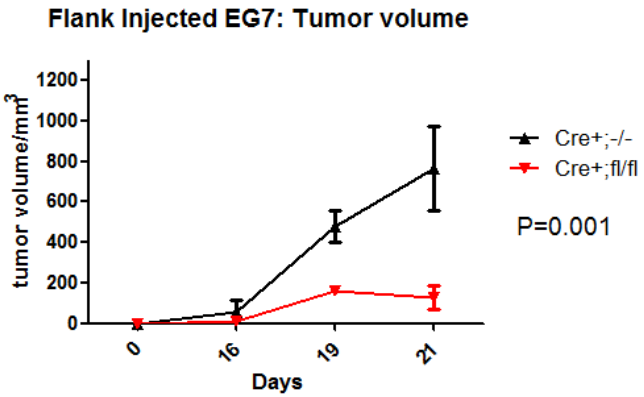
CaMKK2 -/- mice have impaired lymphoma growth *in vivo*

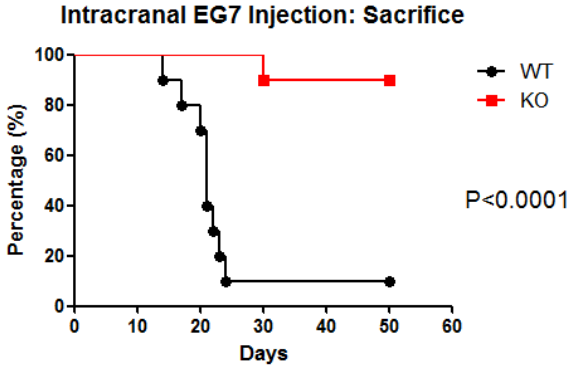
Model 1.
Tumor: EG7
Location: Flank
Strain: Global CaMKK2 -/-

Model 2.
Tumor: EG7
Location: Flank
Strain: LysM CaMKK2 -/-

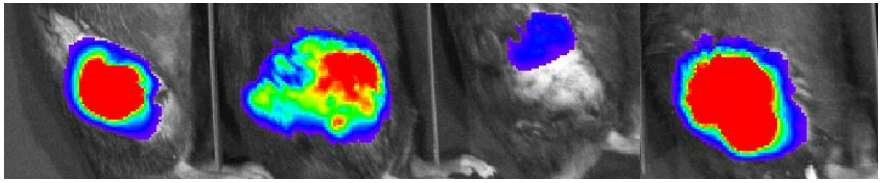
Model 3.
Tumor: EG7
Location: caudate nucleus
Strain: Global CaMKK2 -/-



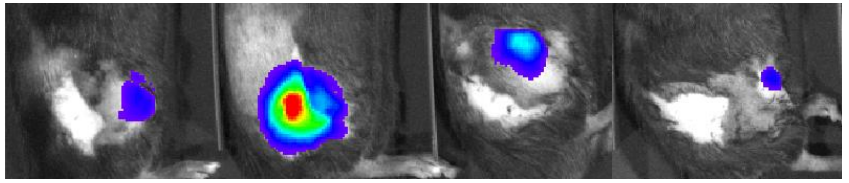




Control Luciferase

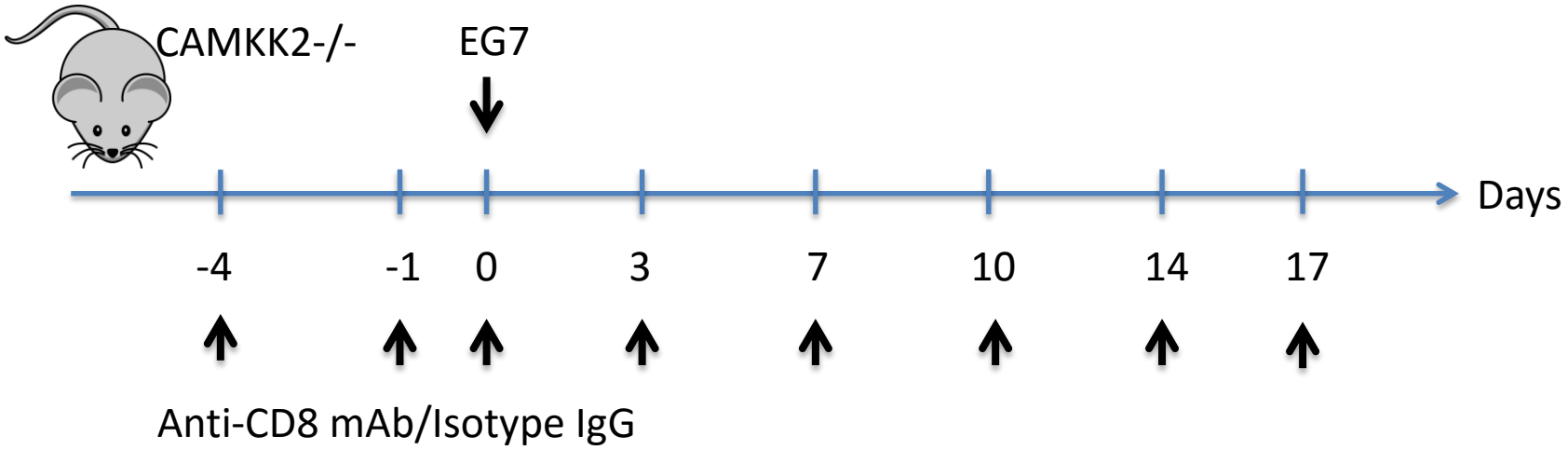


CaMKK2 -/- Luciferase



CAMKK2-/- mediated tumor suppression is CD8 T-cell dependent

- Deplete CD8 cells to verify the role of CD8 cells in anti-tumor effect in CAMKK2-/- mice with EG7
- Anti-CD8 mAb injection, 100ug, i.p. Controlled by isotype IgG, and untreated mice
- Inject EG7 s.c. 1e5 to CAMKK2 KO mice



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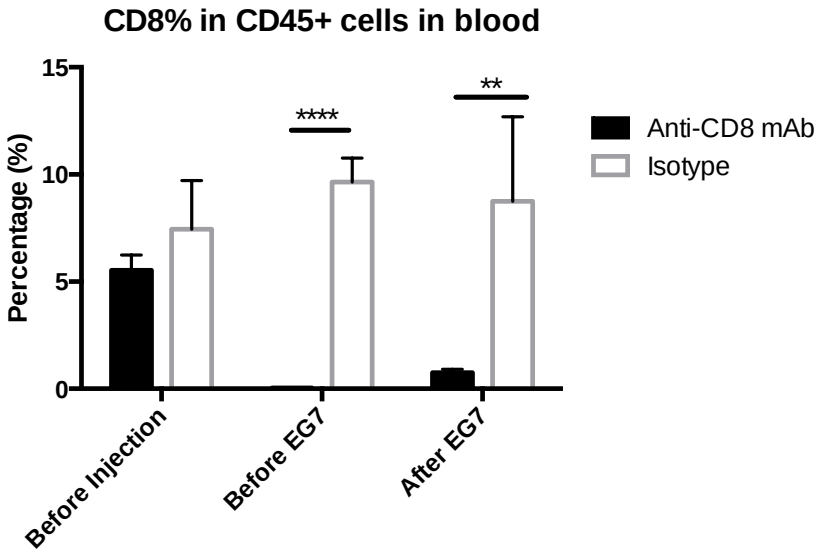
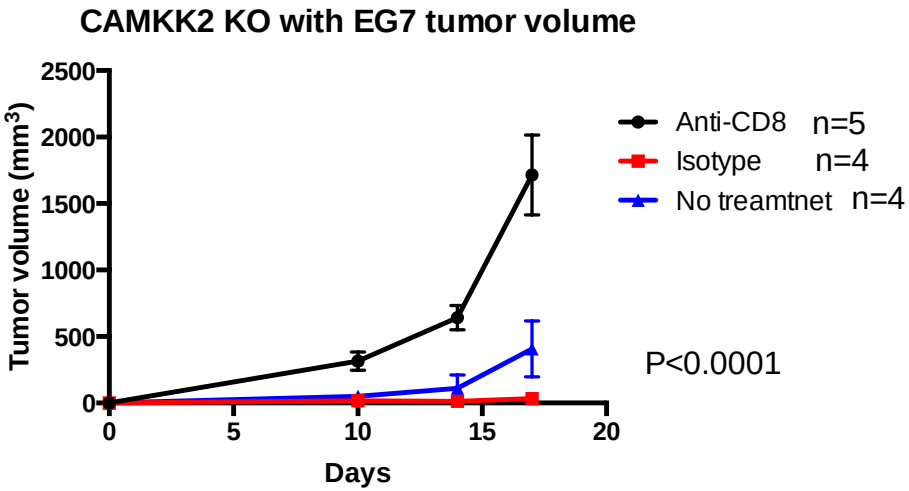
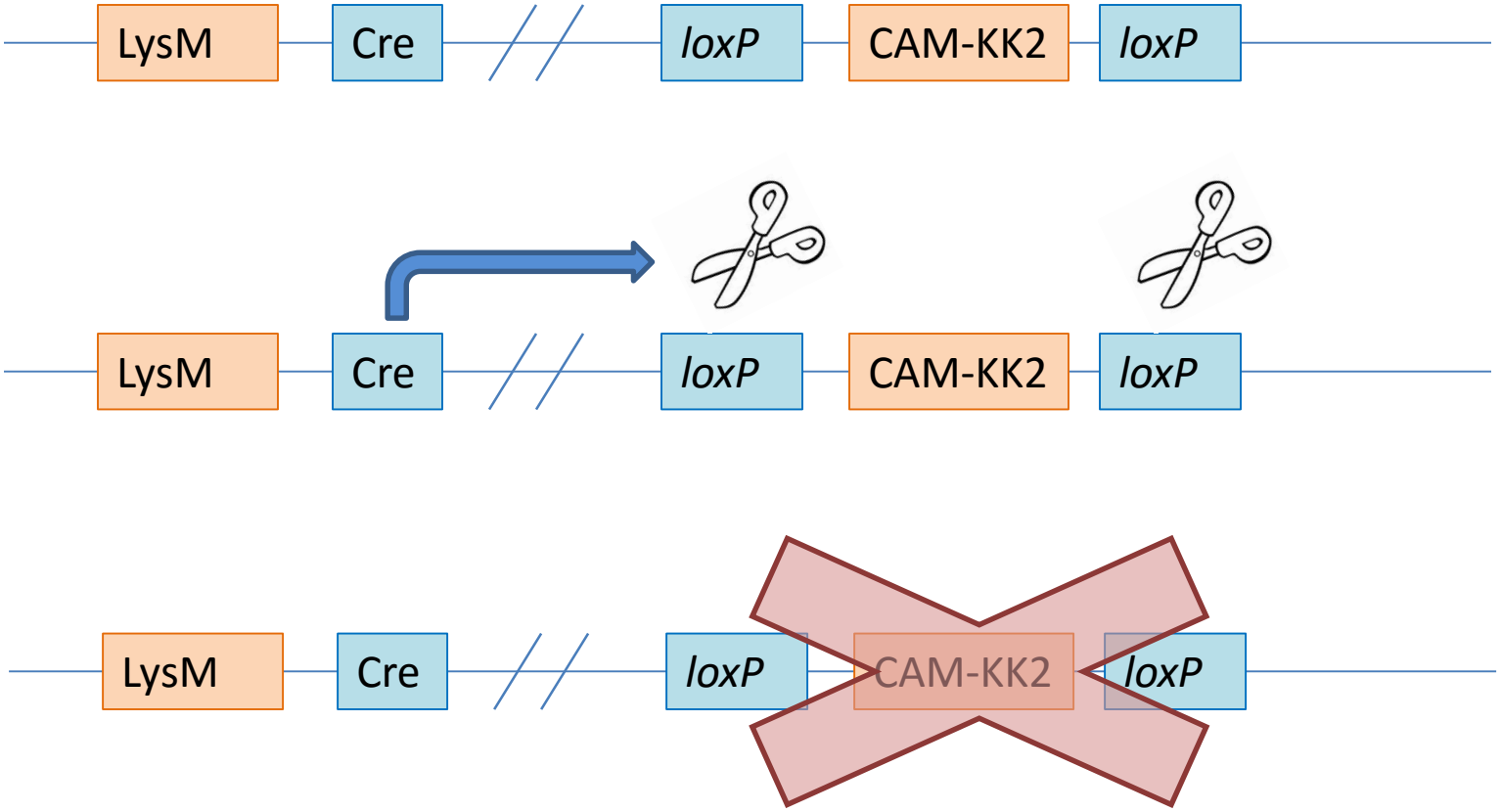




LysM Cre+/-;*lox-CAMKK2-lox* mice

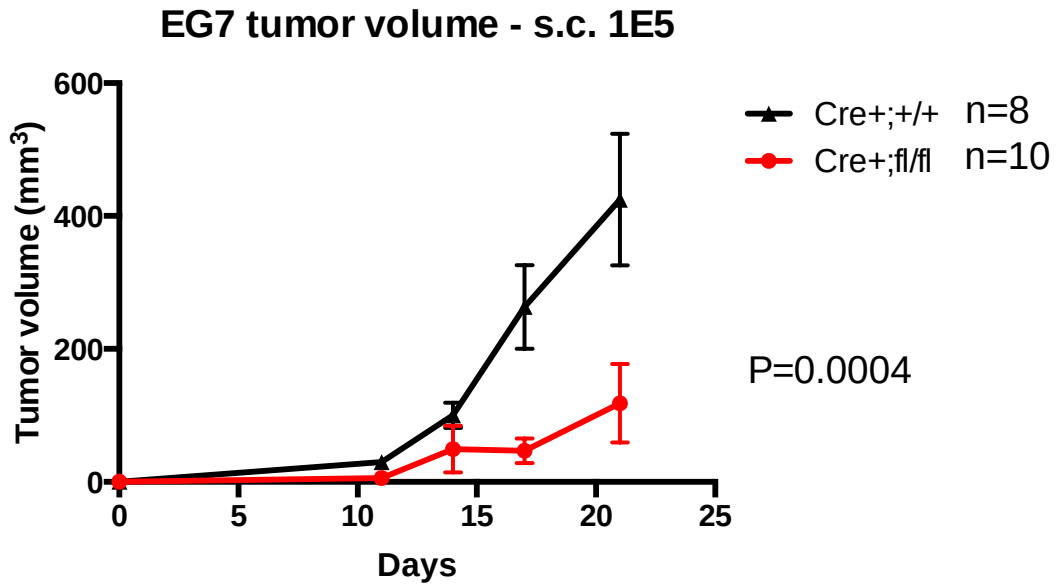


Lysozyme M gene: conditional gene targeting in myeloid cells

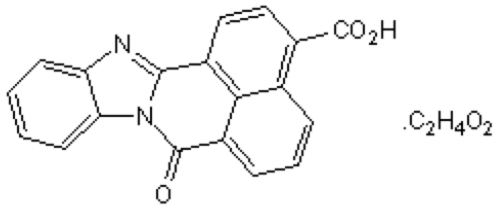
Clausen, Förster, et al. *Transgenic Res.* **8**, 265–277 (1999).

E.G7 flank injection: LysM-Cre mice

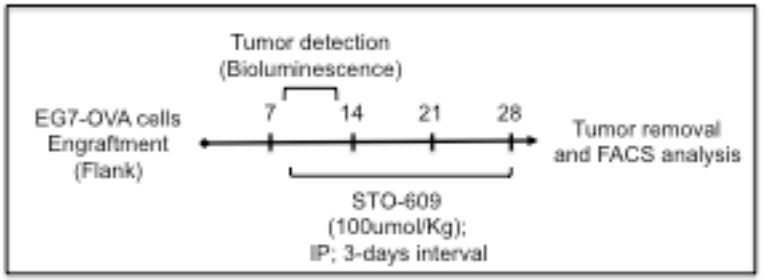
Model 3.
Tumor: E.G7
Location: Flank
Strain: LysM CaMKK2 -/-

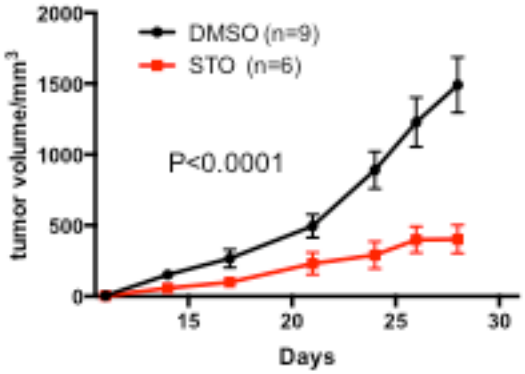


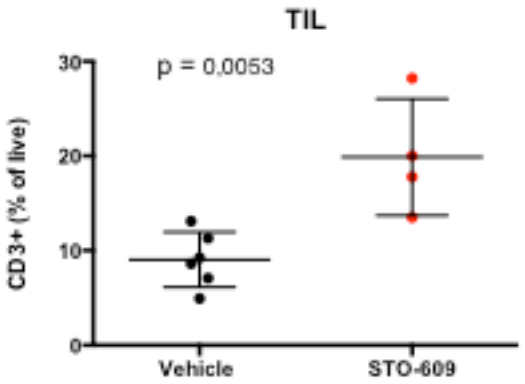
Effects of STO-609 on EG7-OVA growth and tumor microenvironment



- B6 mice with EG7 injection, s.c.
- STO609 0.1M i.p. injection every other day, started from tumor detectable by bioluminescence





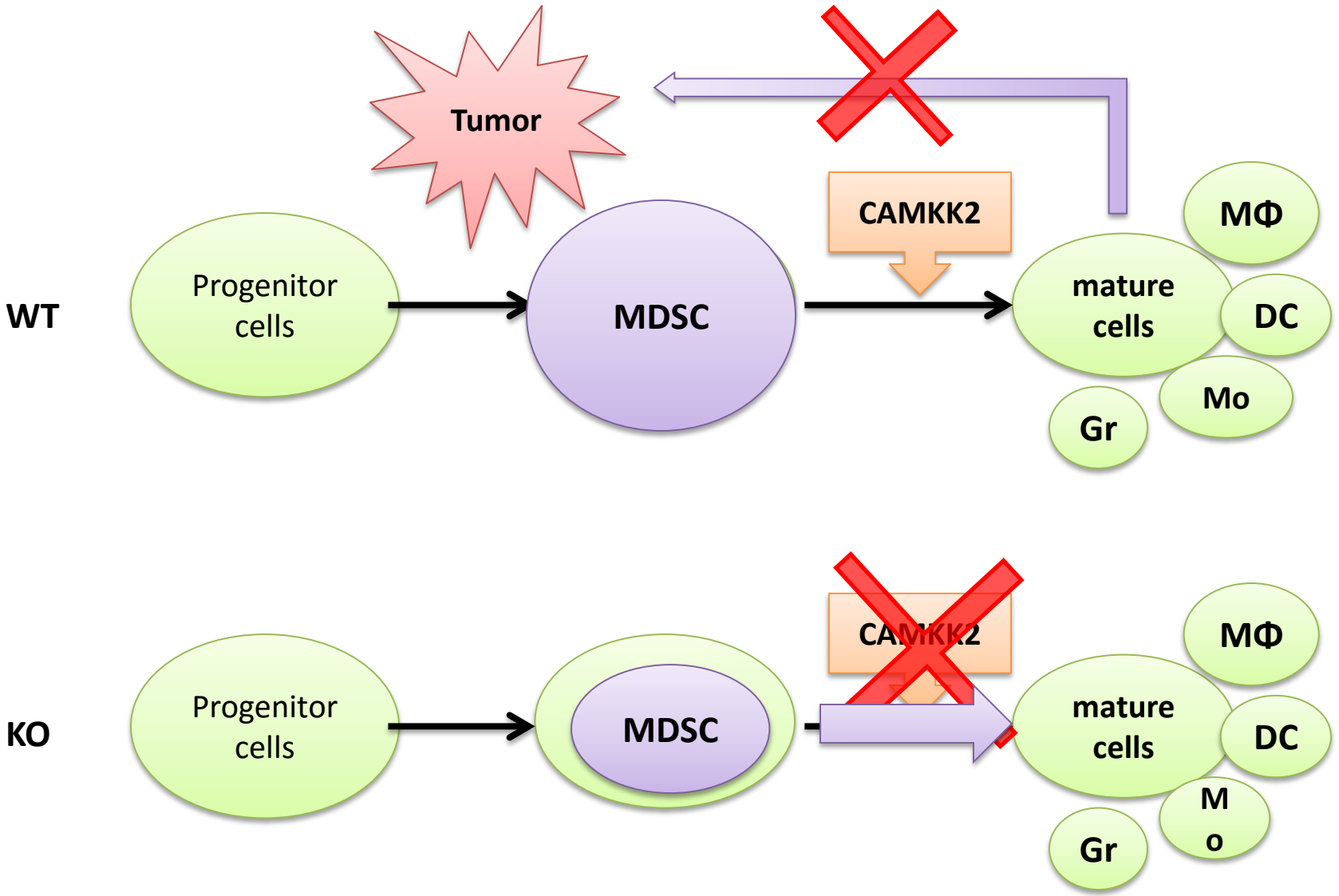


Summary 1

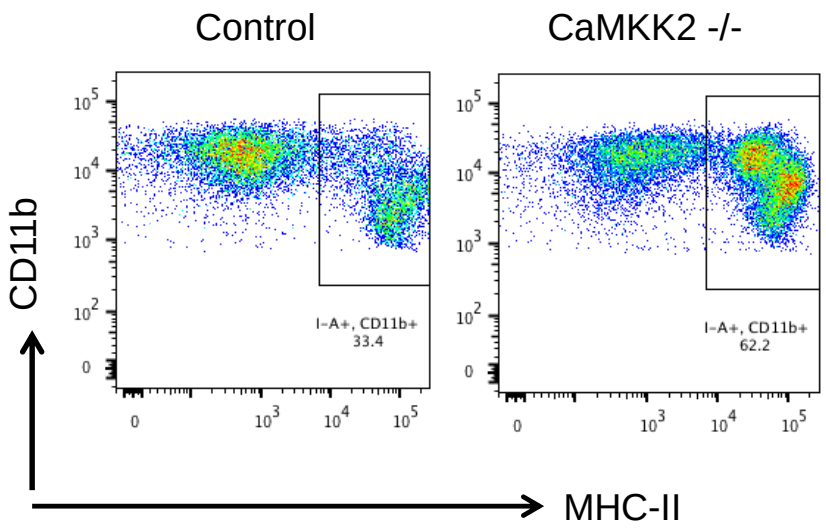
- CaMKK2 global ablation inhibits lymphoma progression.
- CD8+ cells suppress E.G7 growth in CaMKK2 deficient mice.
- CaMKK2 ablation in myeloid cells are sufficient for lymphoma suppression confirmed by LysM-Cre model.
- STO609 is a small molecule that is able to inhibit CaMKK2 and reproduce similar findings.

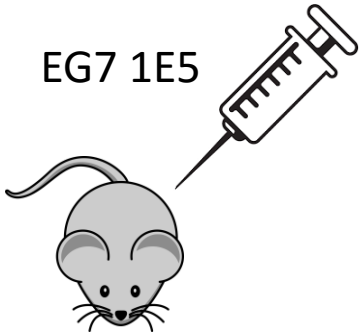
- What is the role of CaMKK2 in myeloid cells in tumor model
  - Myeloid derived suppressor cells
  - Dendritic cells

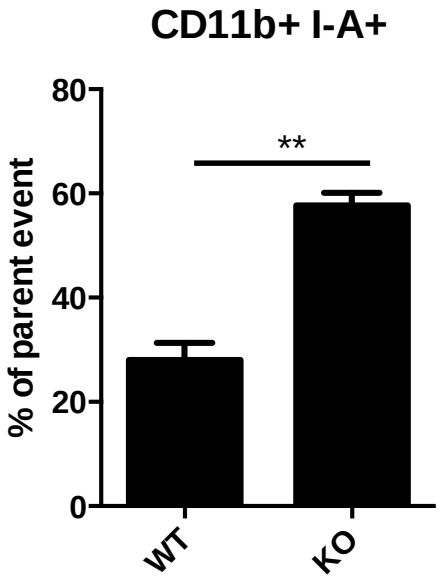
CAMKK2 Controls Myeloid Development?



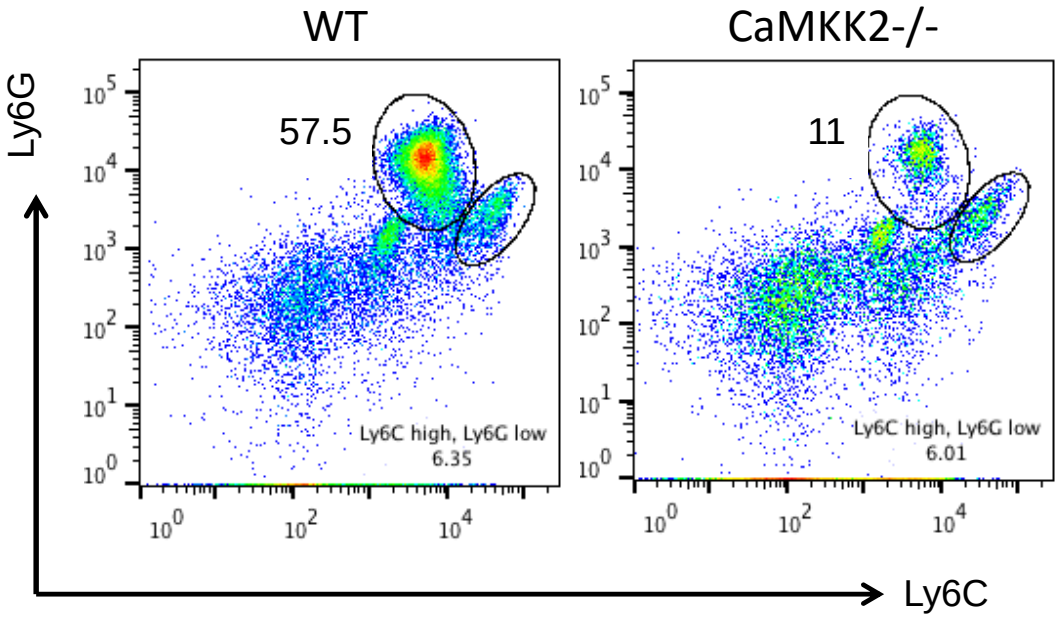
CaMKK2 Ly6G cells in tumor bearing mice have more MHCII expression

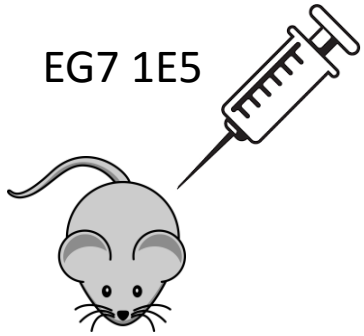


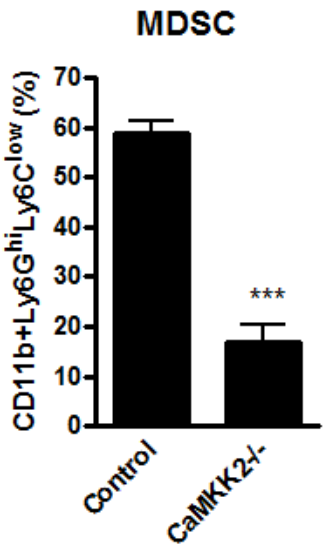




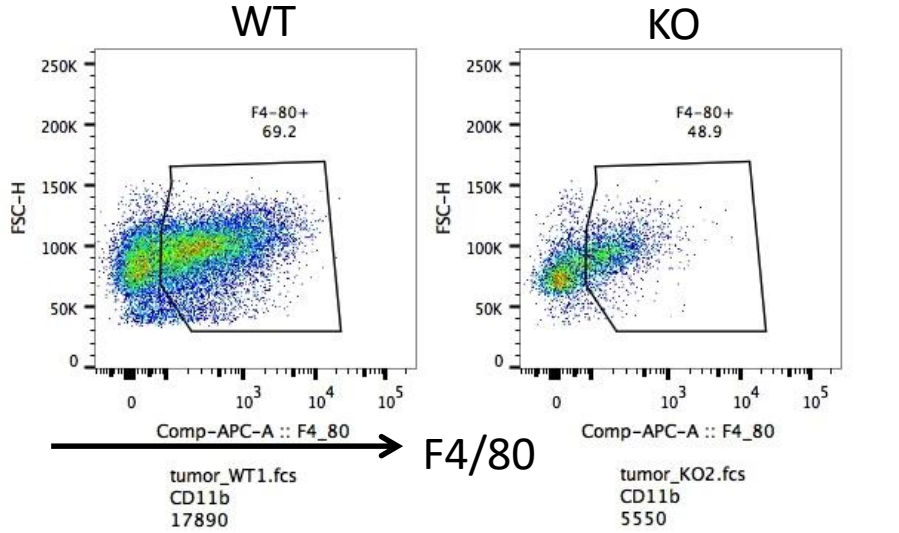
CaMKK2 is required for granulocytic MDSC accumulation in vivo

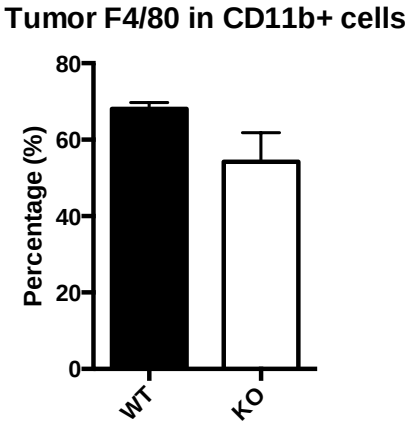


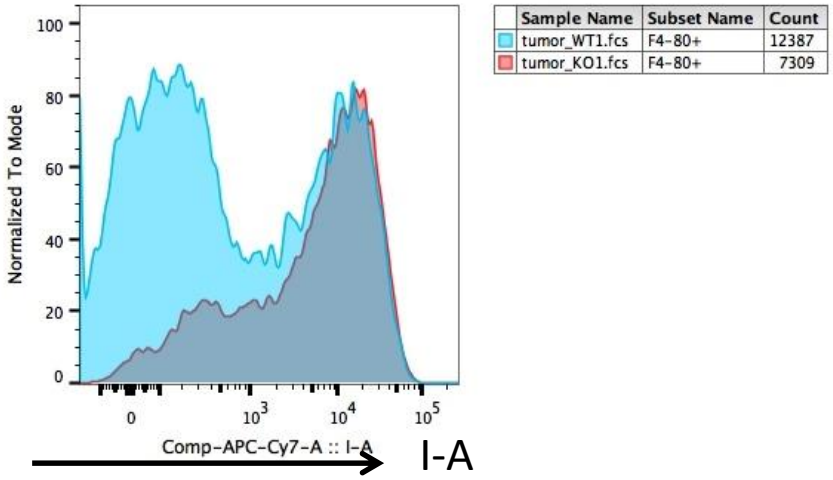




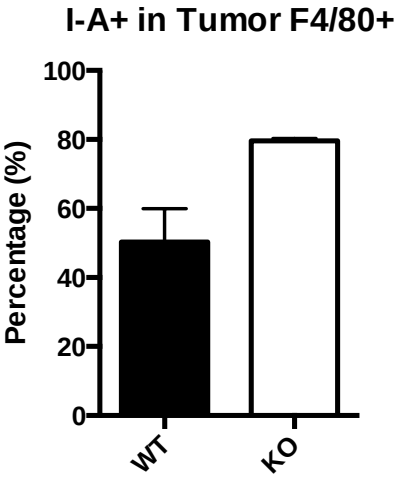
CAMKK2 KO tumor bearing mice have less F4/80 but higher I-A expression



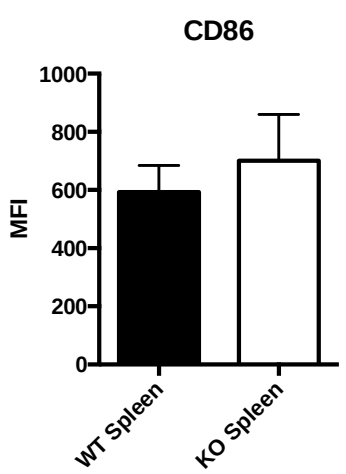


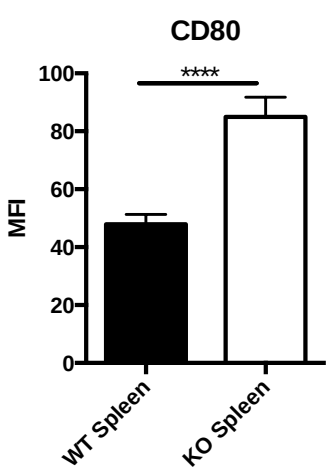


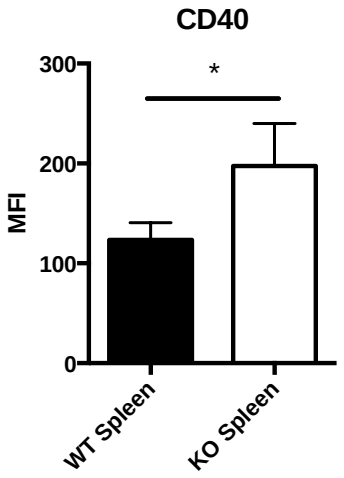
Sample Name	Subset Name	Count
tumor_WT1.fcs	F4-80+	12387
tumor_KO1.fcs	F4-80+	7309

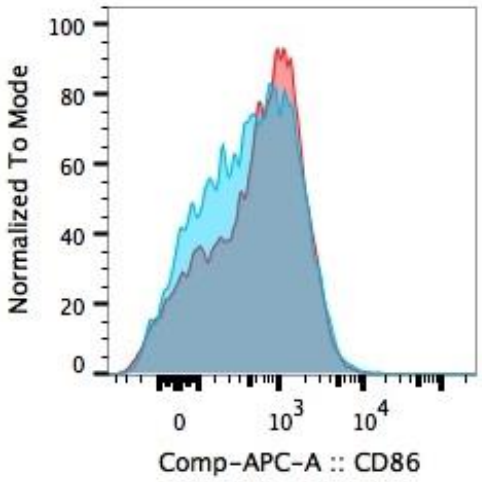


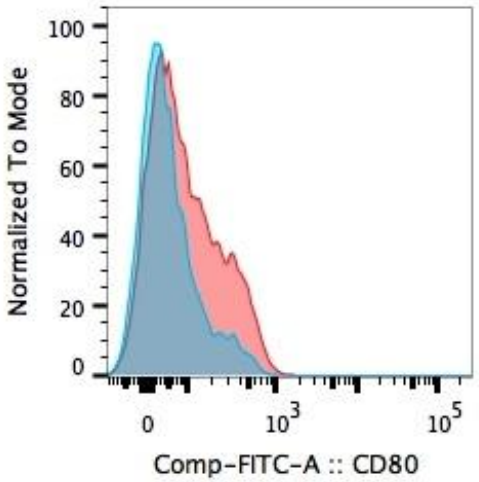
CaMKK2 KO splenic DCs have higher costimulatory molecules expression

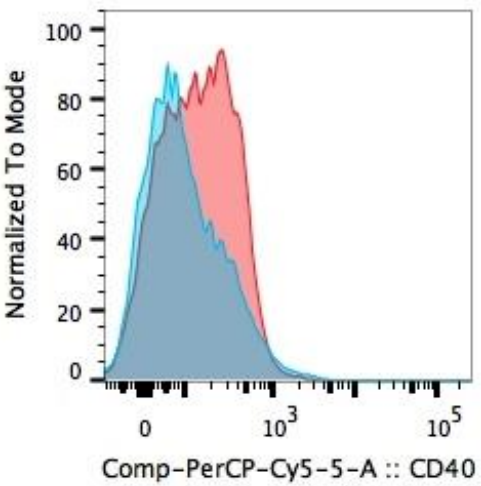






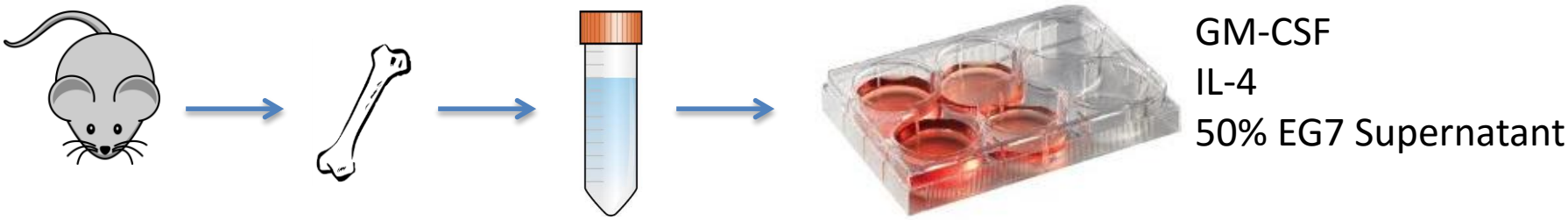






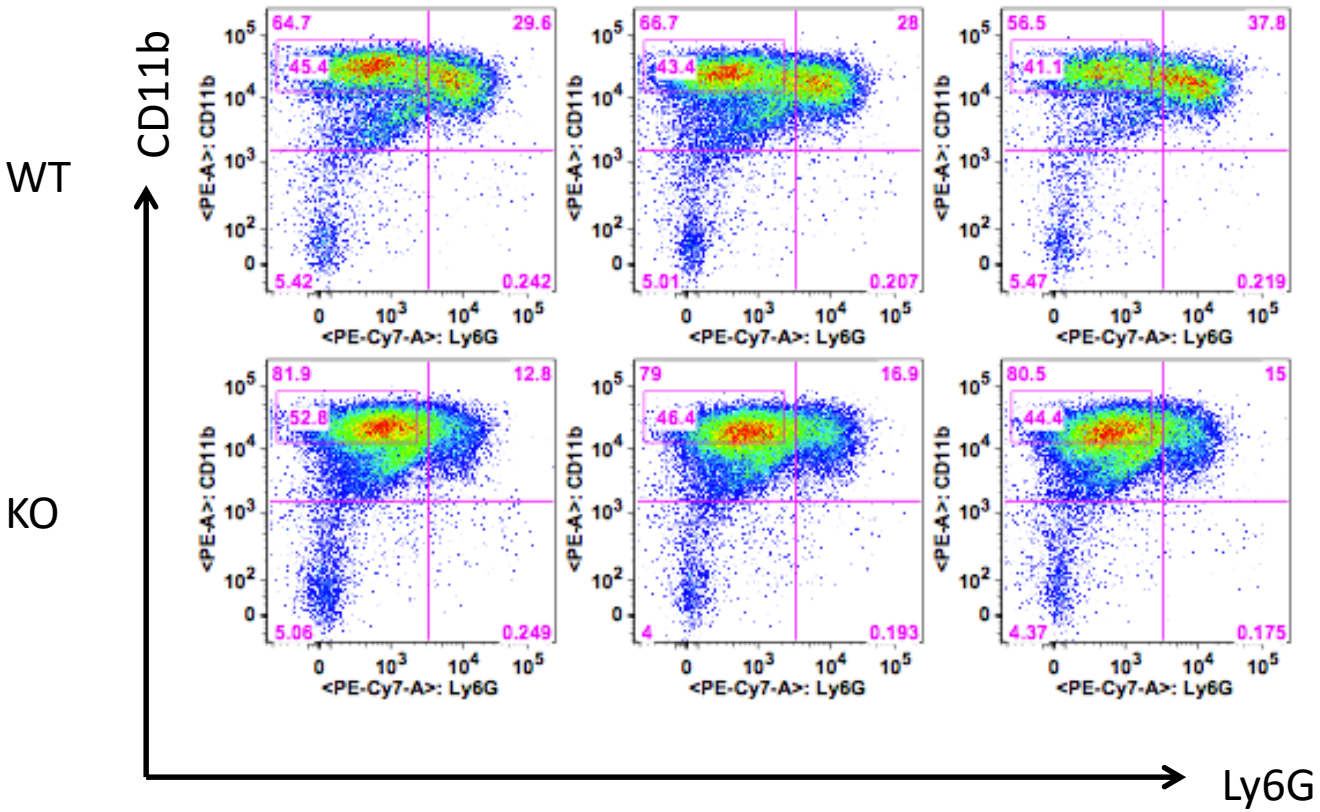
in vitro MDSC generation

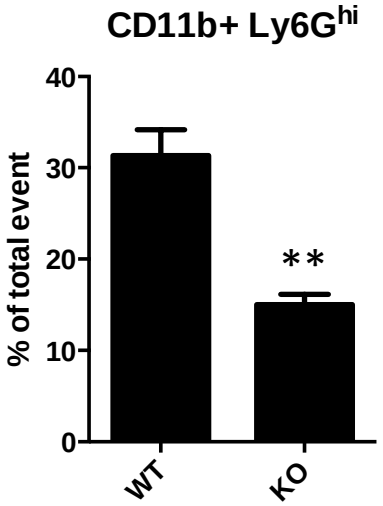
- BM cells from femurs and tibias
- 1e6 cells in DMEM + 10% FBS, 40 ng/ml GM-CSF, 40 ng/ml IL-4, 50% EG7 supernatant.
- Change half of the medium on Day3
- Collect on Day5



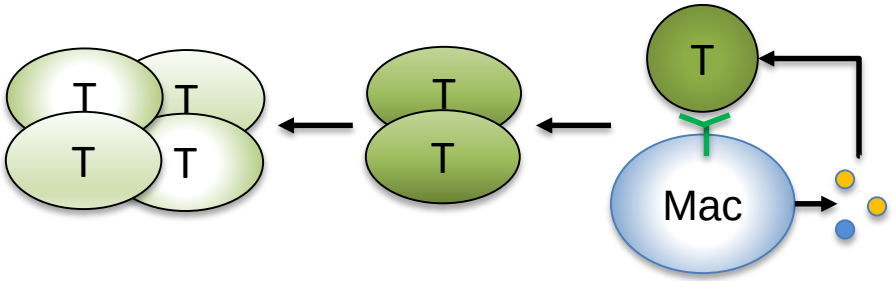
GM-CSF
IL-4
50% EG7 Supernatant

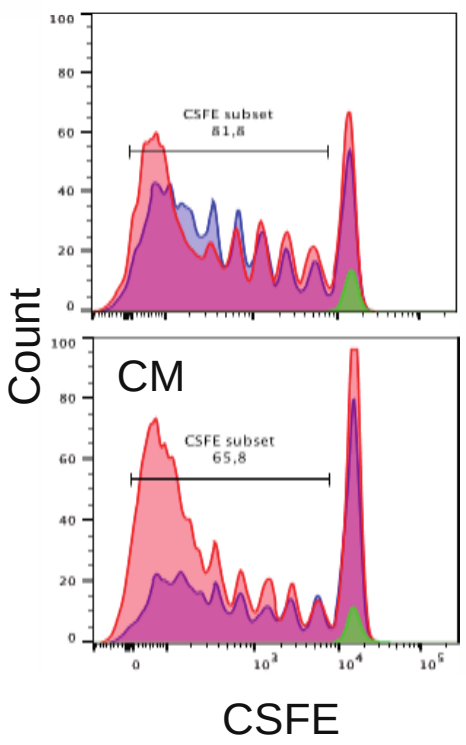
CaMKK2 is required for granulocytic MDSC induction in vitro

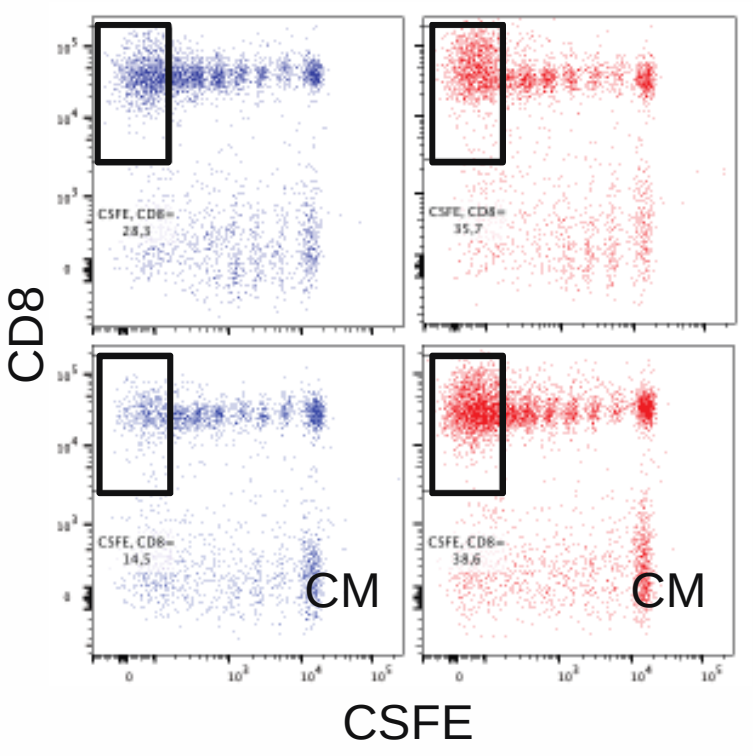


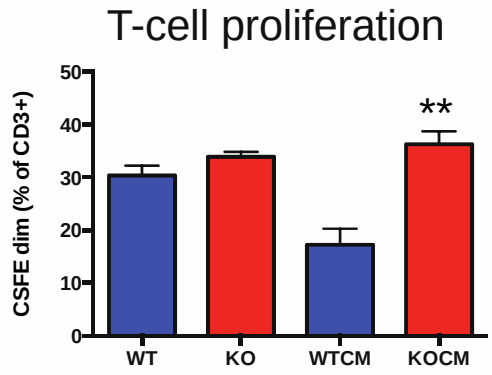


CaMKK2 controls the macrophages responsiveness to tumor-derived factors







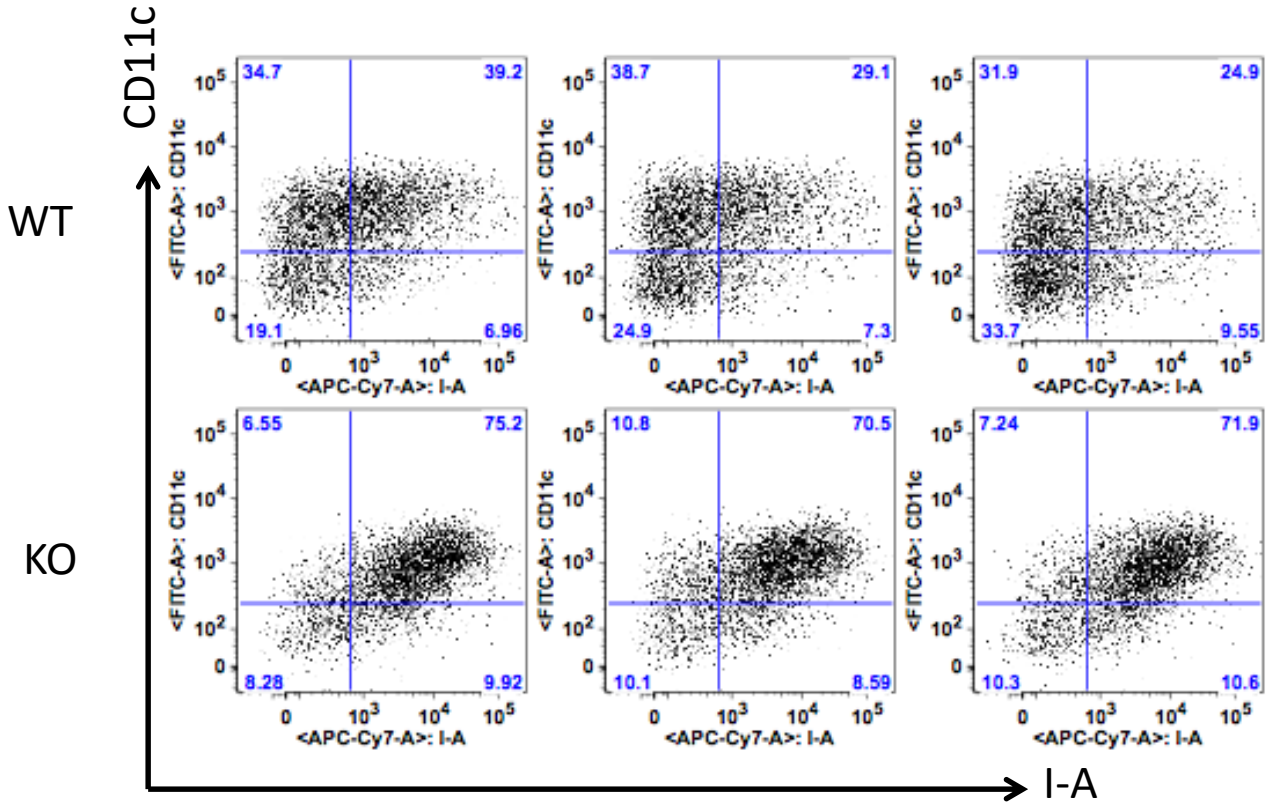


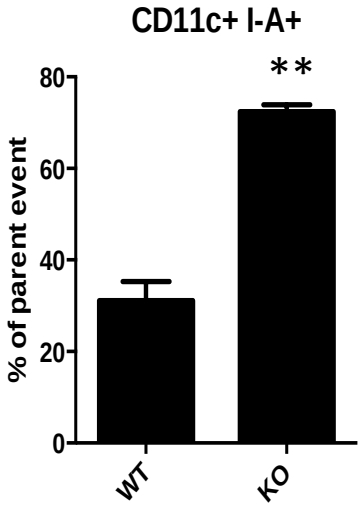
Summary 2

- CaMKK2 ablation promotes myeloid cell maturation in tumor bearing mice
  - Limited MDSC accumulation
  - Higher expression of MHC II on macrophages
  - Higher expression of MHC II and costimulatory molecules on DCs
- Same phenomenon observed in *in vitro* system

- Does CaMKK2-/- DCs stimulate T-cell proliferation more robustly?

CaMKK2 ablation facilitates dendritic cells maturation





in vitro DC generation

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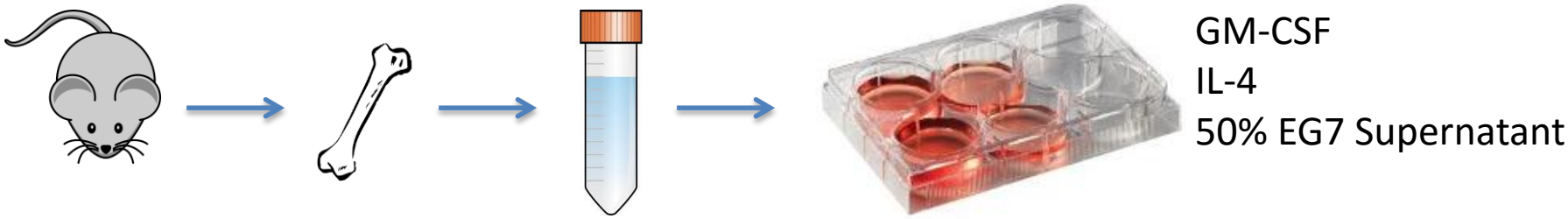
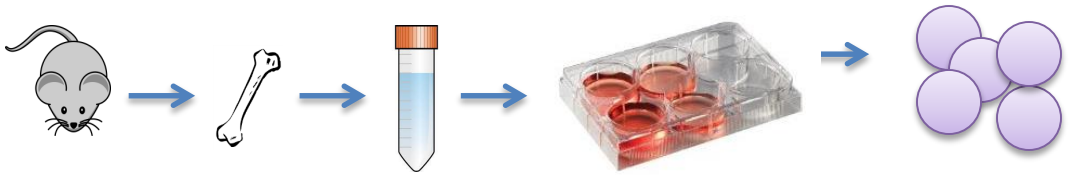


DC-mediated T-cell proliferation

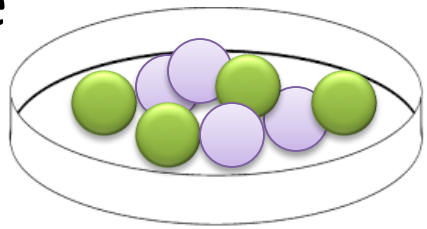
- Bone marrow derived dendritic cells



CD11b+CD11c+

- T cells

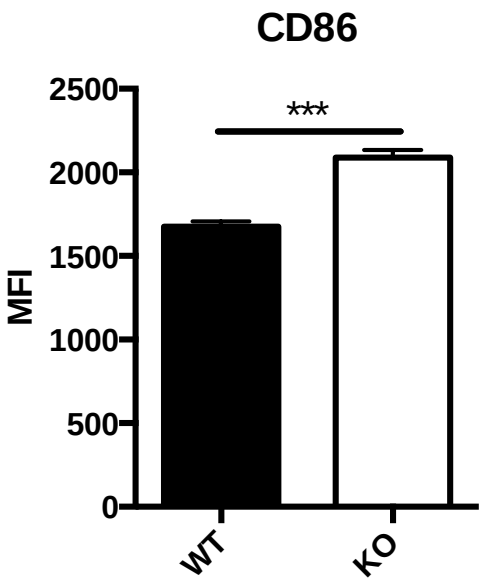
- Coculture

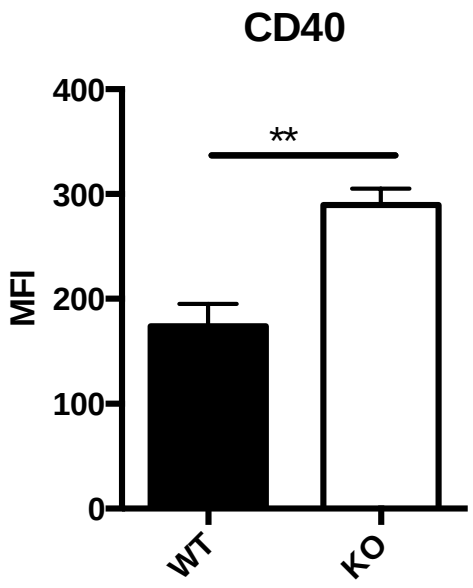


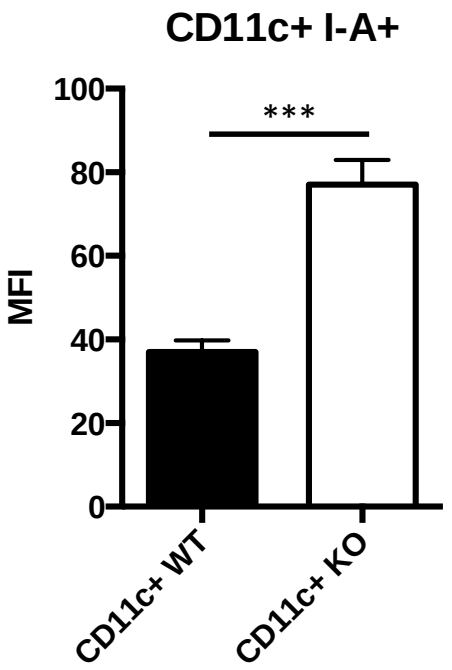
CD11b+CD11c+ 1E4

T cells 1E5

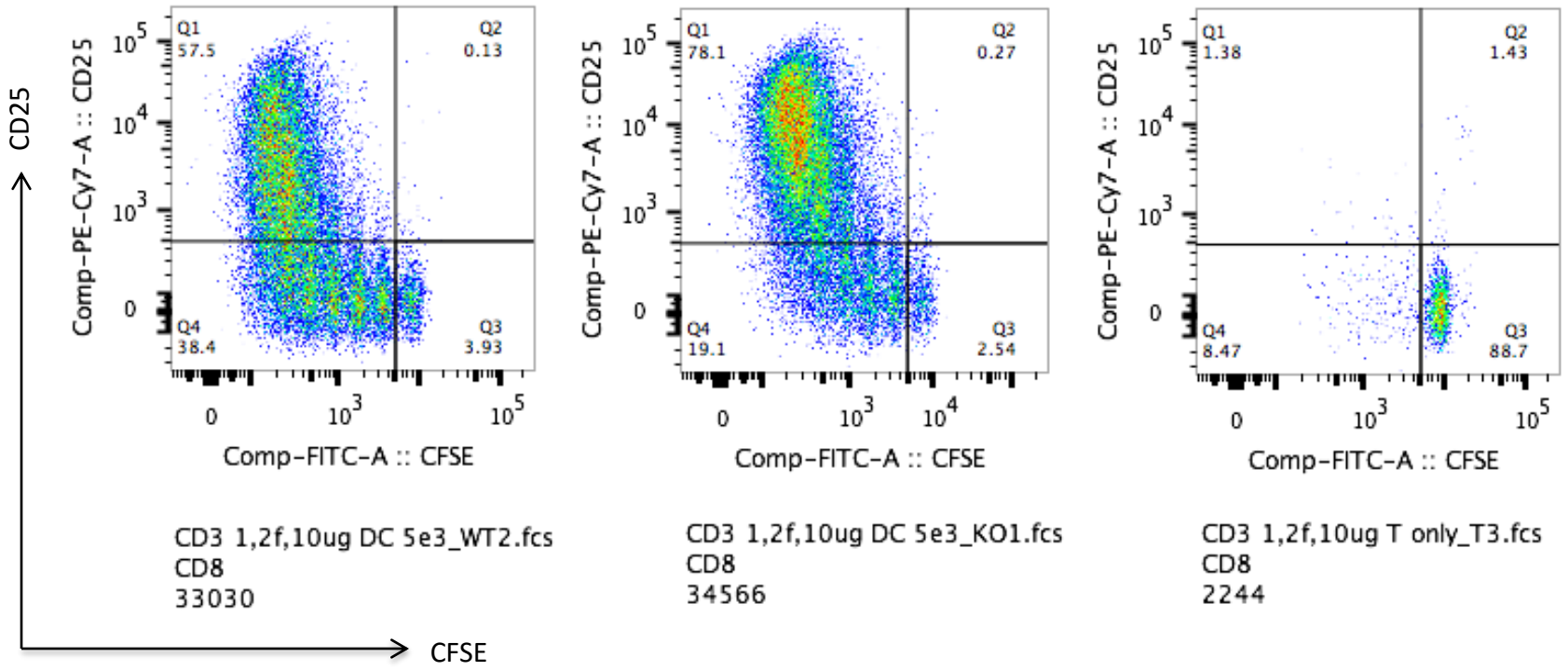
Higher costimulatory molecules expression is induced on CaMKK2 DCs cocultured with T cells







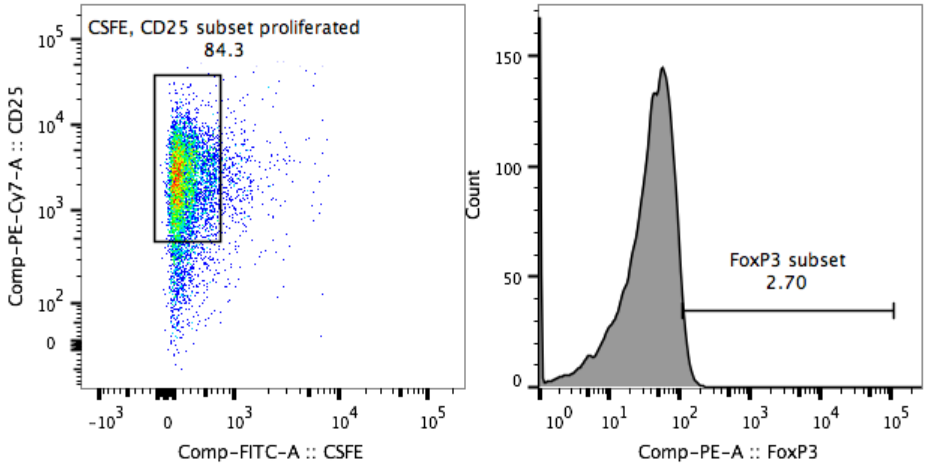
CAMKK2 KO DCs stimulates more T cell proliferation – CD8 (and similarly for CD4)



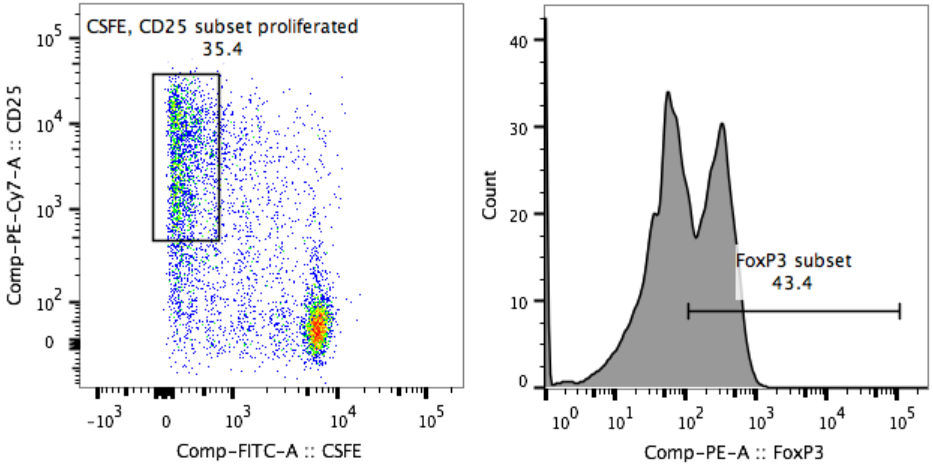
WT KO No DCs

Proliferating CD25+ T cells are not Treg

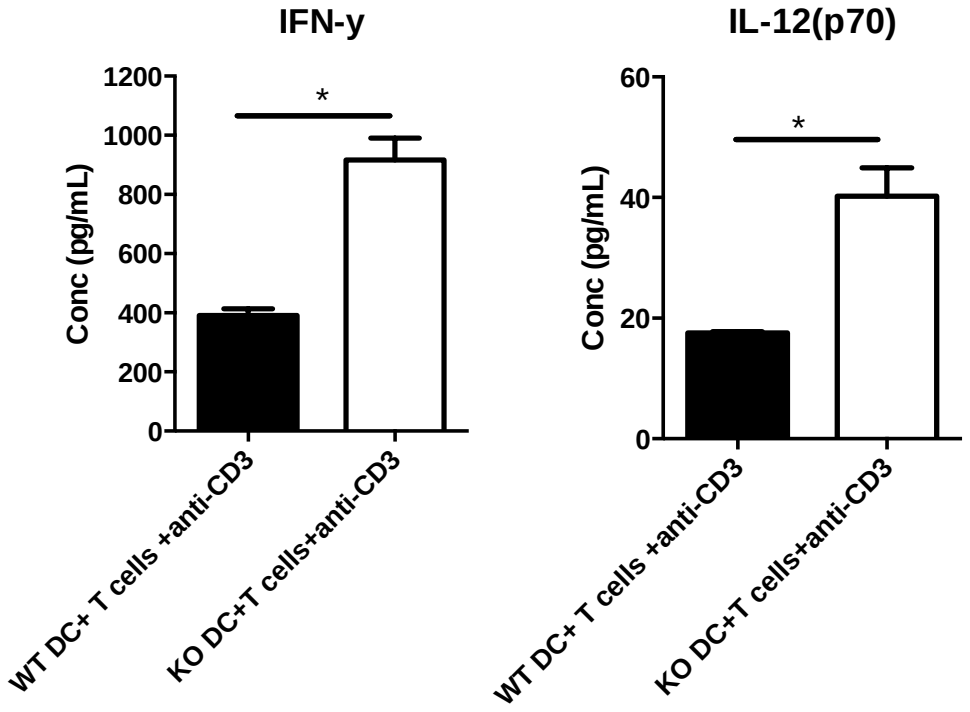
KO DCs
Anti-CD3 mAb

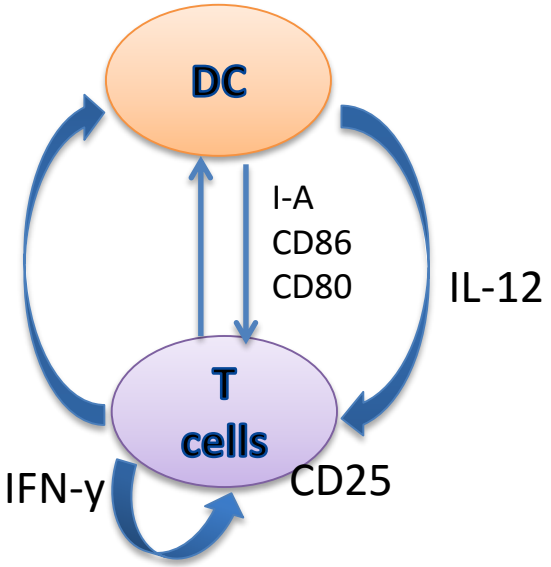


KO DCs
no Anti-CD3 mAb



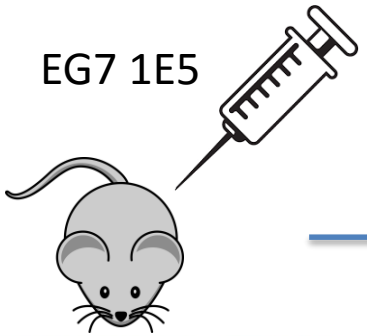
Cytokine Secretion In DC And T cells Coculture





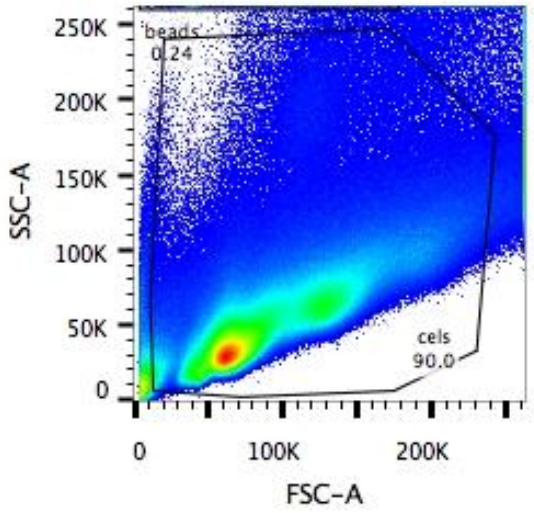
OT2 cells have more proliferation in EG7-bearing CaMKK2 KO mice

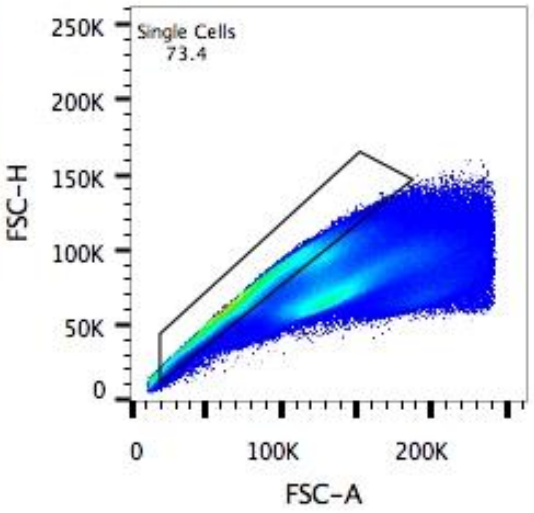
- CaMKK2 KO mice with EG7 tumor
- OT2-GFP T cells injected into EG7 bearing mice

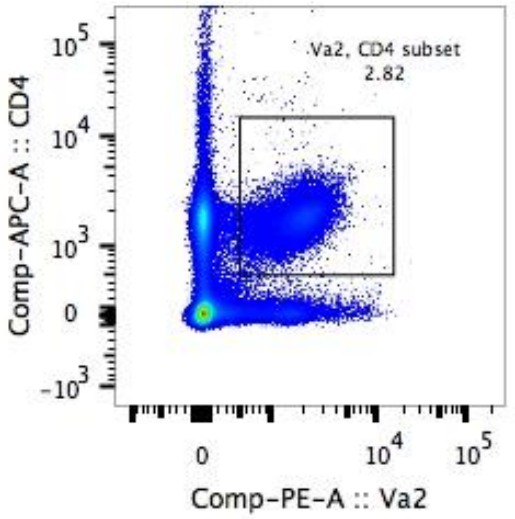


Tumor detectable

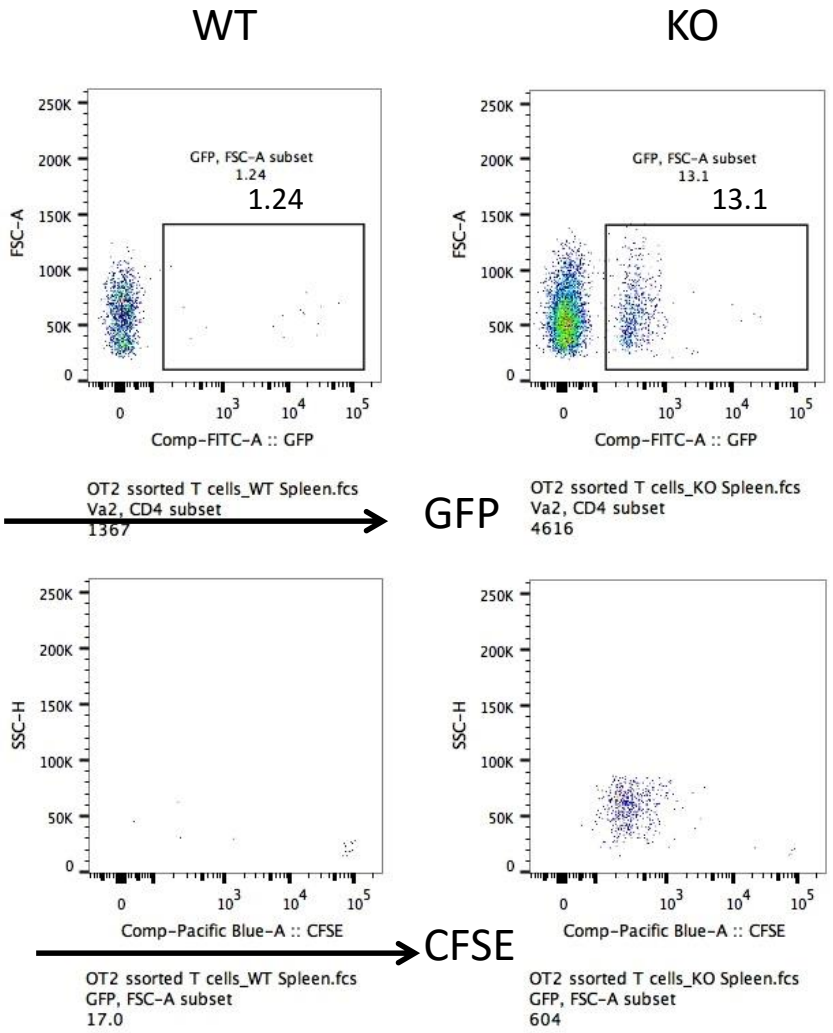
OT2-GFP, i.v.







OT2 cells have more proliferation in EG7-bearing CAMKK2 KO mice



Summary 3

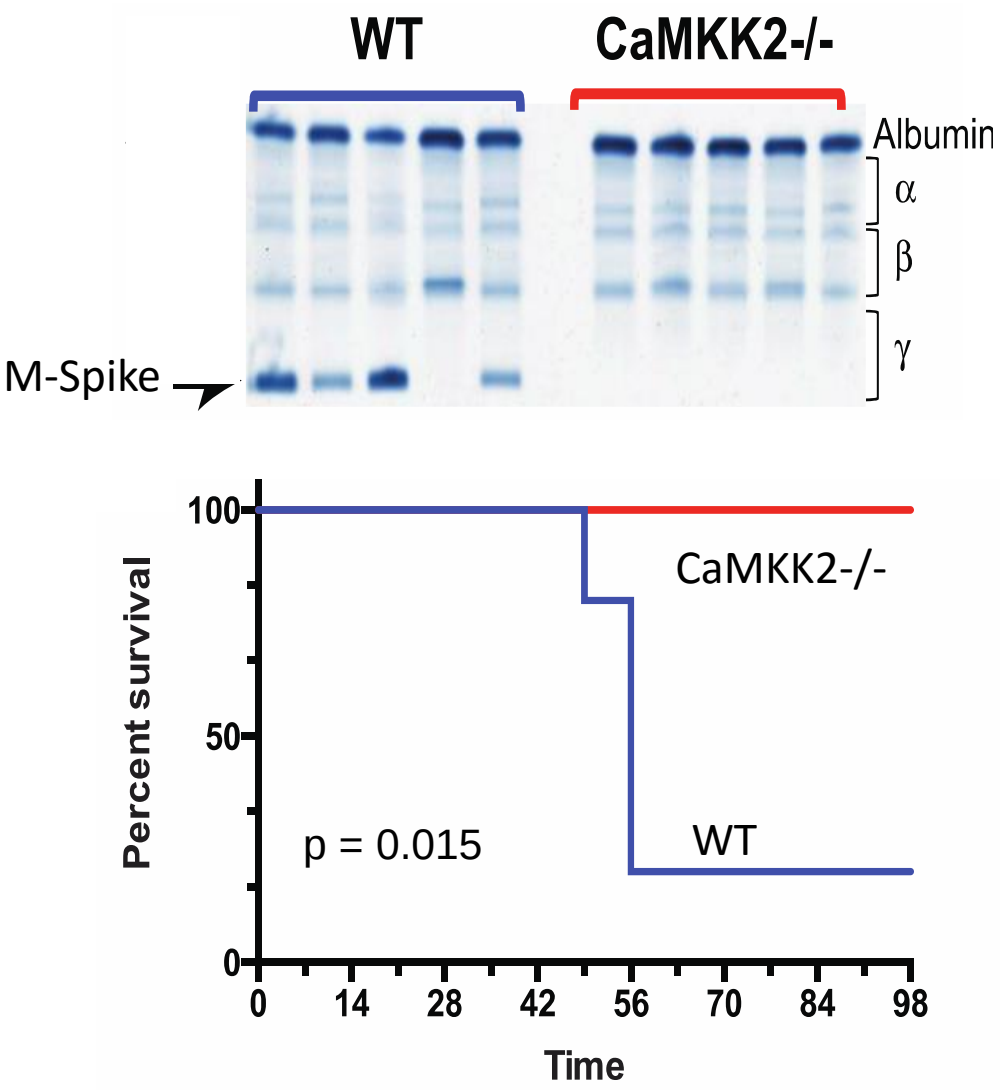
- CaMKK2 KO DCs stimulates T-cell activation more robustly
- Antigen-specific OT2 T cells have better proliferation in CaMKK2 KO tumor-bearing mice.
- WT DCs-mediated T cells activation can be blocked by anti-IL-12 mAb, while KO DCs are not sensitive.

Conclusion

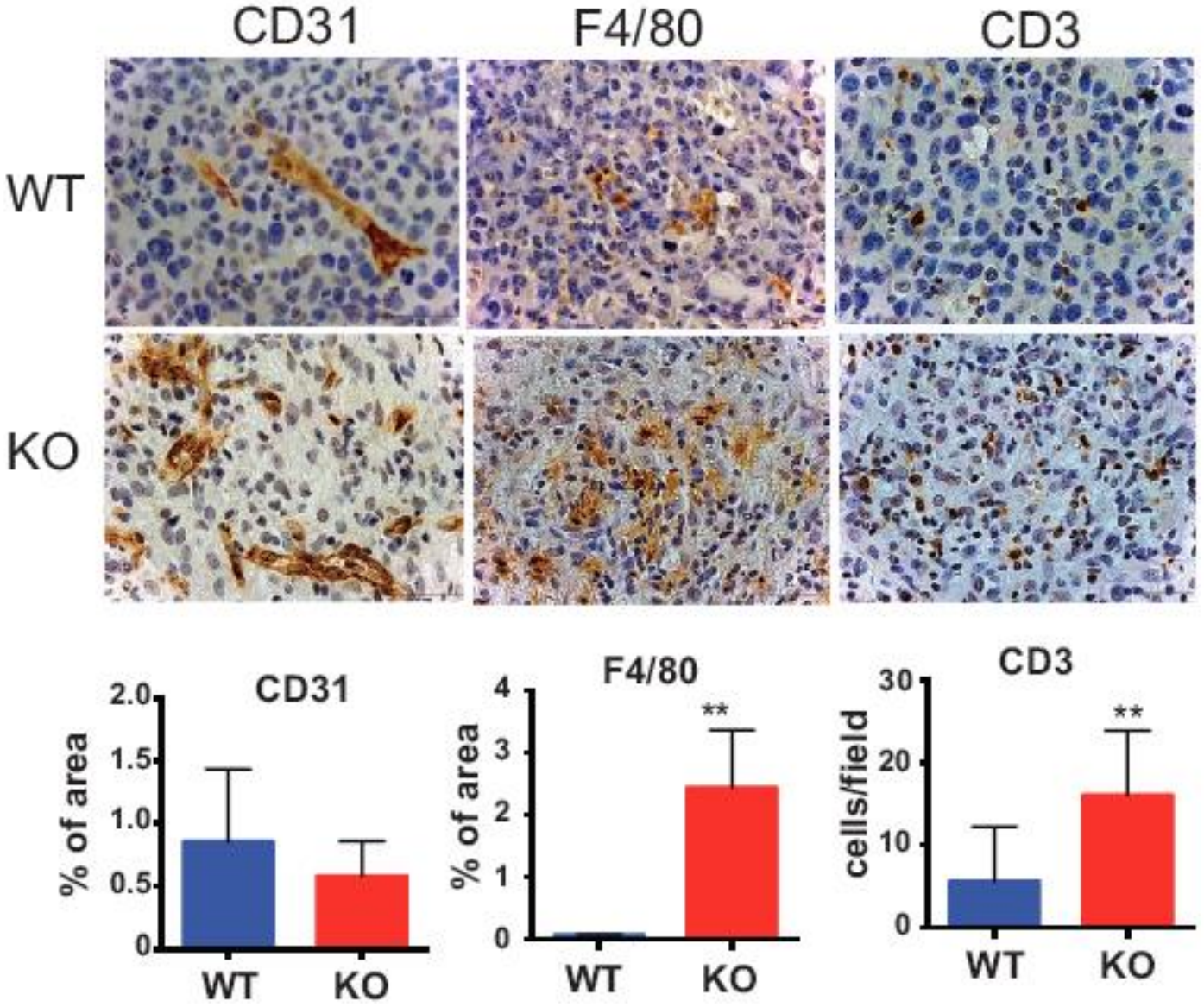
CaMKK2 is a new regulator in the lymphoma microenvironment

- CaMKK2 ablation suppresses lymphoma growth.
- CaMKK2-/- environment promotes myeloid cell maturation with higher MHC II and costimulatory molecules expression.
- CaMKK2-/- DCs stimulate T-cell activation in a more robust manner.
- STO609 is a small molecule that can inhibit CaMKK2 and could be useful in the treatment of lymphomas

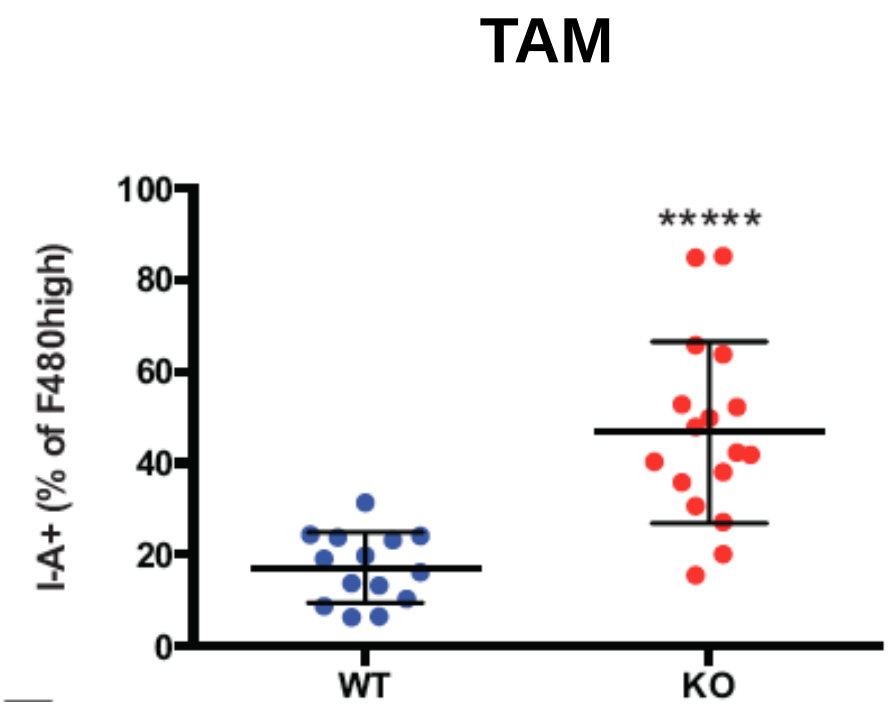
CaMKK2-/- mice are resistant to Vk*MYC myeloma

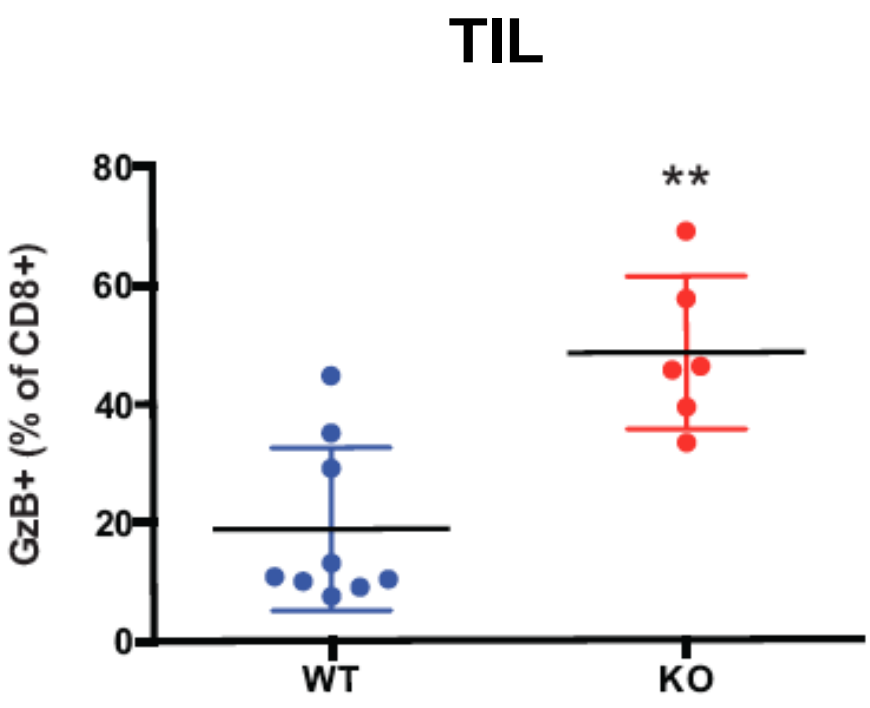


E0771 mammary tumors in WT and CaMKK2-/- mice

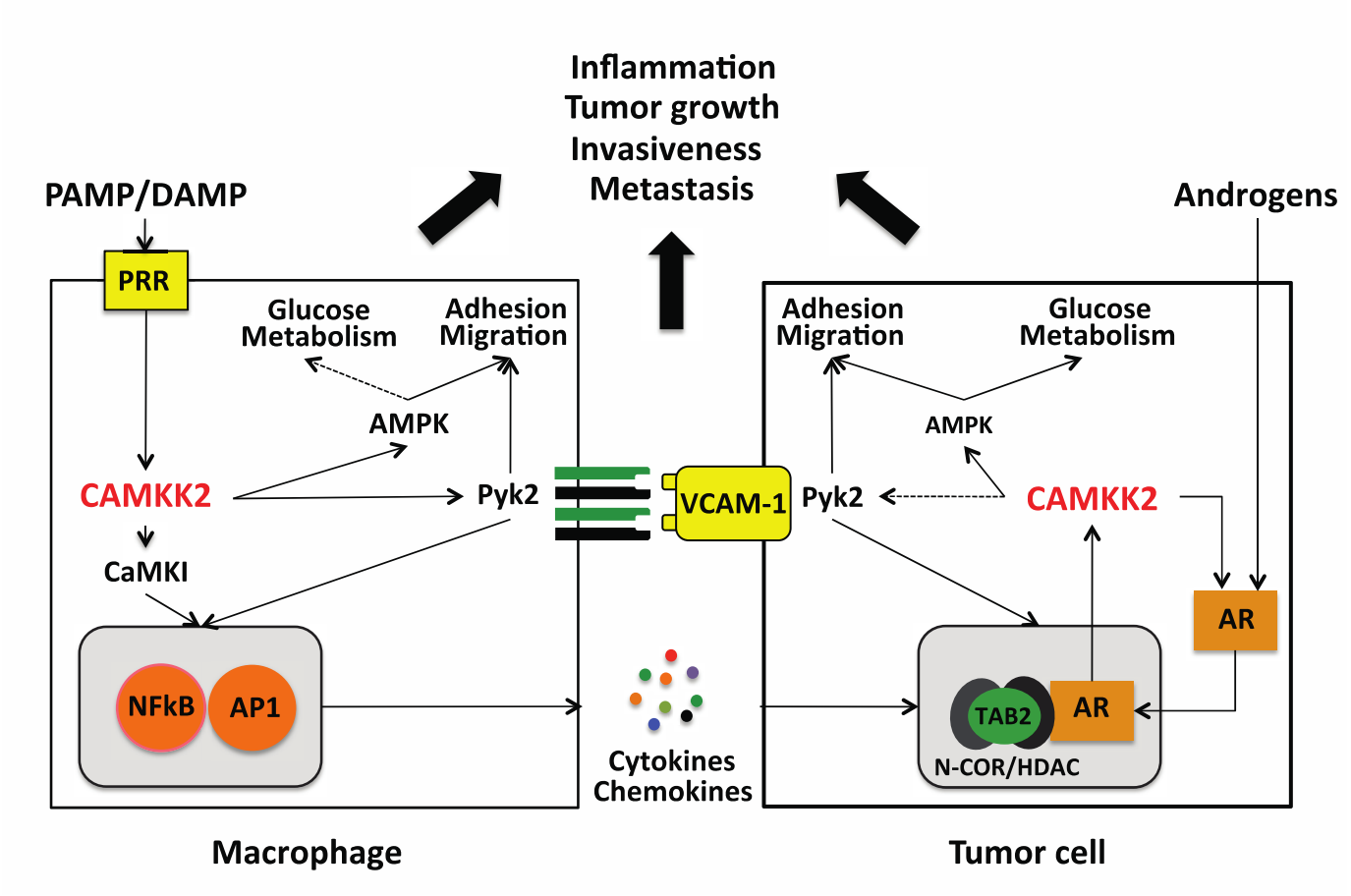


Immunophenotype of immune cells infiltrating mammary tumors of WT and CaMKK2-/- mice





CaMKK2 and Tumor ecosystem

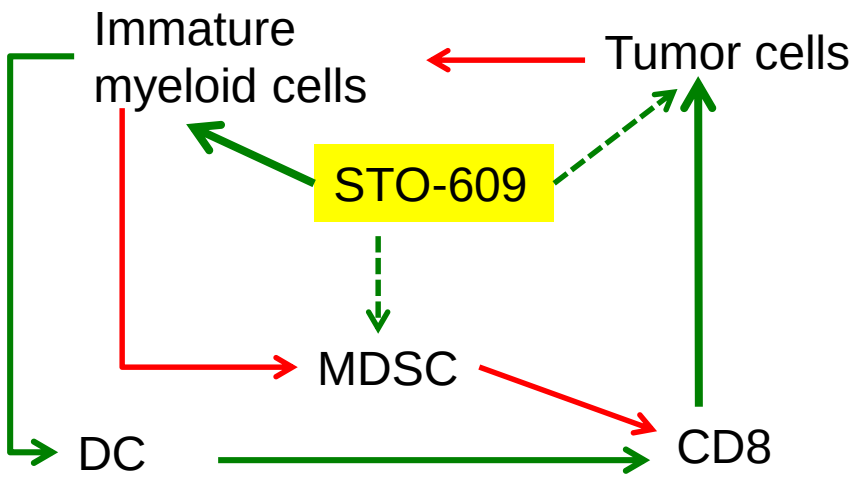


Luigi Racioppi (2013) Trends in Molecular Medicine

More functional assays are needed to investigate the molecular mechanism.

CaMKK2 is a potential therapeutic target for cancer treatment

- Immune regulation:
  - Limits MDSC generation
  - Promotes DC maturation & function: DC vaccines, CAR-T therapy
  - Increases T-cell infiltration: immune checkpoint blockade (anti-PD1/PD-L1 mAbs)
- Metabolic regulation
  - Specifically inhibits AMPK in myeloid cells
  - Promotes DC activation
  - Limits MDSC function



Future plan

- CaMKK2 and myeloid development:
  - Molecular mechanism: AMPK
    - p-AMPK level changes by Western blot
    - AMPK pathway RT-PCR array
    - Sh-RNA for AMPK
    - AMPK activator and inhibitor
- DC-mediated antigen-specific T-cell proliferation
- CTL assay
- T-cell infiltration in early tumor

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Sadhna Piryani, BS