M127/227
Lecture 9
T cell signaling and apoptosis
Antigen receptor signaling overview: adaptors

Adaptor proteins contain SH2 and SH3 domains, often arranged as shown.

Ligand binding clusters receptors, activating tyrosine kinases and leading to the phosphorylation of receptor cytoplasmic domains.

Adaptor proteins bind to phosphotyrosine via their SH2 domains.

Other signaling proteins bind to the adaptor protein via proline-rich sequences, becoming concentrated around the activated receptor.

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Ag receptor signals are propagated from the receptor through adapter proteins, which recruit other signaling proteins to the receptor.
The TCR doesn’t directly transduce signals. It associates with CD3 chains (ε,δ,γ,ζ); following binding to MHC + peptide, this cluster activates the phosphorylation of adapters.

How does this work?

CD3 chains have ITAMs (immunoreceptor tyrosine-based activation motifs), motifs that are present in many activatory immune receptors. When clustered by Ag, these ITAMs become tyrosine phosphorylated by Src kinases. This allows binding of a tyrosine kinase called ZAP70...

Types of TKs: Src family (e.g. Lck), Syk family (e.g. ZAP70)

Types of domains: SH2 (Src homology 2) bind pTyr SH3 bind poly-prolines in adaptors/kinases.
Because it associates with a coreceptor (CD4 or CD8), Lck becomes activated when the coreceptor is brought into this complex. Lck can then phosphorylate the ITAM-bound ZAP70. This activates ZAP70.
Complete TCR signaling requires clustering of many receptors
The Immunological Synapse (IS)

T cells are activated by clustering of multiple receptors and signaling molecules focused by ligands present on the surface of the APC.

T cell activation movie:
http://www.youtube.com/watch?v=Xt_y7f6KivI&feature=PlayList&p=7D18C93964A61F67&playnext_from=PL&index=22&playnext=1

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The IS: central vs. peripheral SMAC (supra-molecular activation clusters)
Changes in the constituency of the c-SMAC with time

0

5

15

Time (min)

TCR/CD3  CD45  Lck  PKCθ  TALIN

CD45

TCR/CD3
Control of Lck by Csk and CD45

- Csk kinase negatively regulates Lck through Y505.
- CD45 phosphatase removes phosphate at Y505, putting it in a state ready to become activated upon CD4 ligation.
T cell activation molecules in the IS: lipid rafts

Lipid raft = membrane microdomain that freely floats in PM; enriched in signaling molecules. High cholesterol in membrane holds rafts together.
But, how do the Src kinases Lck (and Fyn) get activated?

Initial activation of the T cell involves the clustering of large numbers of signaling molecules and receptors into a complex called the immunological synapse (IS). One of the molecules is called CD45, a phosphatase.

Lck and Fyn are normally tyrosine phosphorylated by Csk, turning their kinase activity off. But, during IS formation, CD45 is recruited, leading to dephosphorylation of this site and activation of the kinases.
**Activated ZAP70 causes tyrosine phosphorylation of adapters**

LAT: Adaptor that is recruited to plasma membrane via palmitoylation. Tyrosine phosphorylation via ZAP70 causes binding of SH2-domain containing proteins. Forms complex with SLP76 that is essential (see below). Also, recruits GADS (homolog of Grb2) which uses SOS to activate Ras.

SLP76: Another adapter protein that associates with LAT at the membrane. Is tyrosine phosphorylated by ZAP70. Allows recruitment of Tec family kinase Itk (this requires PI3K activity). SLP76 binds to PLCγ and Itk, within this complex, activates PLCγ. GEFs also activated.
Multiple signaling cascades activated downstream of SLP76 and LAT

- PLCγ cleaves phosphatidylinositol bisphosphate (PIP₂) to yield diacylglycerol (DAG) and inositol trisphosphate (IP₃)
- DAG and Ca²⁺ activate protein kinase C
- Protein kinase C activates a transcription factor, NFκB
- IP₃ increases intracellular Ca²⁺ concentration, activating a phosphatase, calcineurin
- Calcineurin activates a transcription factor, NFAT (nuclear factor of activated T cells)
- GEFs activate Ras, which in turn activates a MAP kinase cascade
- The Ras-induced kinase cascade induces and activates Fos, a component of the AP-1 transcription factor
- The transcription factors NFκB, NFAT, and AP-1 act to induce specific gene transcription, leading to cell proliferation and differentiation

Fig 6.15 part 2 of 2 © 2001 Garland Science
Fluorescence activated cell sorting (FACS) and cytometry
Mixture of cells is labeled with fluorescent antibody

Stream of fluid containing antibody-labeled cells

Green photomultiplier tube (PMT)

Red PMT

Side scatter

Forward scatter

Laser

CPU

Figure A-25 part 1 of 2 Immunobiology, 6/e. (© Garland Science 2005)
FSC vs. SSC
Analysis of cells stained with labeled antibodies

IgM

Dot plots

Contour maps

Standard

5% probability

IgD

Color density

With outliers

Figure A-25 part 2 of 2 Immunobiology, 6/e. (© Garland Science 2005)
Compensation

Fluoresceine (FITC)  Phycoerythrin (PE)
Sample with mixed population of cells - half positive for FITC (FL1) and all negative for PE (FL2)

- FL1 signal leaking into FL2
- Partially compensated
- Over compensated
Back-gating

Ungated

1d MZ

Live Gate

Live Gate

1d MZ

CD24-PE

CD1d-B-SA-APC

Forward Scatter

Side Scatter

10

100

1000

10

10

10

10
Measuring Cell Death

• Cell death is important at many stages of lymphocyte development (e.g. for blocking autoreactivity, maintenance of homeostasis, etc).

• Cells can die by two major mechanisms, apoptotic or necrotic.

• Apoptotic cells can be detected because they expose phosphatidyl-serine (PS) on their surface before they lose plasma membrane integrity.

• Necrotic cells lose membrane integrity without surface exposure of PS.

• Annexin-V binds to PS, so detection of Annexin-V binding on cells with intact plasma membranes indicates apoptotic cells.

• 7-actinomycin D (7-AAD) is a “vital dye” and normally cells exclude it if plasma membrane is intact.

• Necrotic cells (or late apoptotic) would have staining for both annexin-V and 7-AAD.
Lab Plan: Week #9 – Western blotting for tyrosine phosphorylation; FACS analysis of cell death

A: Phospho-tyrosine western blot
1. Next week: Wash blot 3X.
2. Incubate blot in primary antibody (4G10, phospho-tyrosine specific monoclonal Ab).
3. Wash 3X.
4. Incubate blot in secondary Ab (anti-mouse-HRP).
5. Wash blot 3X.
6. Add luminol-based enhanced chemiluminescence (ECL) reagent to blot to visualize bands.
7. Capture image of blot using image capture camera (3rd floor, McGaugh).
8. Determine the effect of TCR stimulation or phosphatase treatment on total tyrosine phosphorylation. Can you identify any potential adaptors or tyrosine kinases affected by TCR stimulation?

B: FACS staining to evaluate death of Jurkat cells.
1. Obtain Jurkat cultures: a) untreated, b) etoposide treated, c) sodium azide treated.
2. Wash 3X in FACS buffer; stain with annexin-V-PE and 7AAD.
3. Incubate 15 minutes.
4. Wash 2X in FACS buffer.
5. Analyze samples by FACS and produce 7AAD vs. annexin-V dotplots.