Regular Office Hours: Tuesdays 11-12
Extra office hours: Wed, Feb 7 12-1pm
Thurs, Feb 8 11am-12
Fri, Feb 9 2-4pm
I WILL NOT BE HOLDING OFFICE HOURS ON TUESDAY Feb 13!!

Dina, Tim, and I encourage all confused students to come to our office hours and discussion sections so we can try to help un-confuse you.

No class on Tuesday Feb 13.

First midterm: Thurs Feb 15 at 6pm in 155 Dwinelle (not 2050 VLSB as listed in the original schedule).
Midterm will focus on material covered in lectures and will be designed to be taken in 90 min. (We have the room till 8pm.)
The GSIs will conduct a review session in our regular class period on Thursday Feb 15 (or Tues Feb 13, we will let you know).

A result of V(D)J recombination every mature B cell expresses a unique antibody. Encounter with an antigen leads to clonal expansion of B cells with a particular specificity.

Last time: Immunological Techniques

Monoclonal Antibodies

Radioimmune Assay (RIA)
Enzyme Linked Immune Sorbant Assay (ELISA)
Western blot
Immunoprecipitation
Flow cytometry
(Expression cloning)

Where we have been and where we are going

Innate Immunity

Antibodies and antigens I (emphasis on antibody structure)

Antibodies and antigens II
(emphasis on antigen-antibody binding interactions)

Techniques based on antibodies

V(D)J recombination

B cell development and function
V(D)J Recombination

Discovery of Ig gene rearrangements

Structure of antibody genes (RSS)

Role of RAG proteins and DNA repair machinery

The puzzle of antibody diversity

- Limitless array of Ig sequences (too large to be encoded in genome)
- Variation limited to V domain.
- Identical V segment could be associated with two different C regions (myeloma protein with γ and μ chains)

- Germ-line vs somatic variation models
- Dreyer and Bennett (1965) the 2 gene model; a violation of the "one gene, one polypeptide" rule
- 1976: the emerging tools of molecular biology open the way for the breakthrough...
Review of Southern blot method

Restriction endonucleases cleave at specific sequences in DNA and can be used to generate a physical map of DNA. (e.g. EcoRI cleaves at the sequence: 5'-GAATTC-3')

Southern blot patterns seen with DNA isolated from different tissues from an individual (or an inbred mouse strain) are all the same.

Surprising Southern blot

Different Southern blot patterns are seen using a Cκ probe and DNA isolated from B cells because the antibody-coding DNA in B cells has been altered.  Somatic Recombination!!!!

Recombination:

process in which chromosomes (DNA) are broken and rejoined in different configurations.

Crossing-over during meiosis: germ line recombination

versus

V(D)J: Somatic DNA Recombination
Detecting Ig gene rearrangement using Southern blot

V(D)J Recombination

Discovery of Ig gene rearrangements

Structure of antibody genes (RSS)

Evidence for role of RAG proteins and DNA repair machinery

Multigene organization of Ig genes: light chain genes

Light chains encoded by 2 gene loci: kappa and lambda

Each light chain encoded by 3 kinds of gene segments:
V (variable), J (joining), C (constant)

A V and J segment are brought together by somatic DNA rearrangement process: “V(D)J recombination”

Multigene organization of Ig genes: heavy chain genes

Heavy chains encoded by a single gene locus.

Each heavy chain encoded by 4 kinds of gene segments:
V(variable), D (diversity), J (joining), C (constant)

V, D, and J segments are brought together by somatic DNA rearrangement process: “V(D)J recombination”
Multigene organization of Ig genes

V(D)J Rearrangement: light chain

V and J gene segments brought together in DNA before transcription. (RNA slicing removes introns.)

V(D)J Rearrangement: heavy chain

V, D, and J gene segments brought together in DNA before transcription. (RNA slicing removes introns.)
Junctional diversity (flexible joining of segments, P and N region additions at junctions) also contributes substantially to the total diversity of antibodies.

### Gene rearrangement juxtaposes promoter and enhancers

Promoters: relatively short nucleotide sequences within ~200 bp of transcriptional start site that initiate transcription in a certain direction.  
**Enhancers:** nucleotide sequences located up-stream or down-stream of a gene that activated the promoter in an orientation independent manner.
Ig promoters are actively transcribed when they are brought close to enhancers due to gene rearrangement.

Allelic exclusion of Ig genes: ensures that most B cell will express a single antibody specificity (allele: two or more alternative forms of a gene.)

Rearranging gene segments are flanked by a conserved "rearrangement signal sequence"

Also known as “one turn RSS” and “two turn RSS”.

Rearranging gene segments are flanked by a conserved “rearrangement signal sequence”
The 12/23 Rule

Only gene segments flanked by RSSs with dissimilar spacers can undergo V(D)J recombination with one another.

Ensures that V segments don’t join with other Vs, that V_H don’t join with J_H, etc.

V(D)J Recombination: Reactants & Products

Flexibility in joining of gene segments contributes to junctional diversity.

Note most rearrangements are non-productive! (Only 1/3 rearrangements preserves the correct reading frame of the J segment.)
P and N nucleotide addition also contribute to junctional diversity.

(a) P-nucleotide addition

Hairpin

Cleavage of hairpin generates sites for the addition of P-nucleotides

Repair enzymes add complementary nucleotides

(b) N-nucleotide addition

Hairpin

Cleavage of hairpin generates sites for the addition of P-nucleotides

TdT adds N-nucleotides

Repair enzymes add complementary nucleotides

P nucleotides are generated by resolution of hairpin structures. N nucleotide are added by an enzyme called terminal deoxynucleotidyl transferase (TdT).
VDJ rearrangement can lead to deletion or inversion of DNA depending on the orientation of the gene segments being joined.

Genetic Defects leading to Immunodeficiency

- RAG mutants-- mice & men (Omenn’s Syndrome)
- Scid mice-- DNA-PK mutation
- Scid humans-- multiple genes including ADA, IL-7Rα chain, and Artemis
- Ataxia-telangiectasia-- AR disorder with frequent chromosomal translocations and inefficient V(D)J recombination
V(D)J Recombination

Discovery of Ig gene rearrangements
Structure of antibody genes (RSS)
Evidence for role of RAG proteins and DNA repair machinery

Both lymphocyte-specific and general factors participate in VDJ recombination.

Recombination activating genes (rag genes) are expressed only in lymphocytes and target Ig gene segments (and TCR gene segments) for rearrangement.

DNA repair machinery is used by all cells to repair DNA damage. This machinery has been co-opted by the VDJ recombination pathway to participate in BCR and TCR gene rearrangement.

Mutations in rag genes or components of the DNA repair machinery lead to defective VDJ recombination and blocks in B (and T) cell development.

Cloned 2 linked genes: RAG-1 and RAG-2 (for Recombination Activation Genes).

Expression of RAG genes is sufficient to convert a fibroblast that cannot carry out V(D)J rearrangement into one that can.

RAG genes are expressed only in developing T and B cells Mutation of rag genes leads to a complete block in B and T cell development.
Unusual features of the RAG locus

- Physical linkage
- Convergent transcription
- Single, large coding exon

**Hypothesis:** the V(D)J recombinase is the descendant of a transposable element system (transposon)

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Identification of a third gene required for V(D)J recombination: The *scid* mouse

- Spontaneous, autosomal recessive mutant
- No serum immunoglobulin
- No mature B cells or T cells
- Attempts V(D)J recombination but makes large deletions in stead of coding joints
- Fibroblasts from *scid* mice also shown to be defective in dsDNA break repair

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**V(D)J Recombination**

**Discovery of Ig gene rearrangements**

**Structure of antibody genes (RSS)**

**Evidence for role of RAG proteins**

**Evidence for role of DNA repair machinery**

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**Table 3.1: Mutations in Genes Required for V(D)J Recombination**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rag-1&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>No V(D)J recombination, No B and T cell development beyond pro-B and pro-T stages.</td>
<td>Mooberry et al., 1992</td>
</tr>
<tr>
<td>Rag-2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>No V(D)J recombination, No B and T cell development beyond pro-B and pro-T stages.</td>
<td>Shinkai et al., 1992</td>
</tr>
<tr>
<td>Scid&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination, defective DNA repair. Defect in coding joint formation. Impaired B and T cell development.</td>
<td>Bowta et al., 1983</td>
</tr>
<tr>
<td>DNA PKcs&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination and DSB repair. Both coding and signal joints affected. Block in B cell development. Only partial block in T cell development. Also required for Ig class switching.</td>
<td>Tuccilo et al., 1998</td>
</tr>
<tr>
<td>Ku70&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination and DSB repair. Both coding and signal joints. Block in B and T cell development.</td>
<td>Gao et al., 1998a</td>
</tr>
<tr>
<td>Ku80&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination and DSB repair. Both coding and signal joints. Block in B and T cell development.</td>
<td>Mann et al., 1998</td>
</tr>
<tr>
<td>XRCC-4&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination, defective DSB repair. Block in B and T cell development. Late embryonic lethality due to defect in CNS development.</td>
<td>Nissenwolczew et al., 1996</td>
</tr>
<tr>
<td>DNA Ligase IV&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Impaired V(D)J recombination, defective DSB repair. Block in B and T cell development. Late embryonic lethality due to defect in CNS development.</td>
<td>Zou et al., 1996</td>
</tr>
<tr>
<td>TdT&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Lack of N regions in antigen receptor genes.</td>
<td>Komori et al., 1993</td>
</tr>
<tr>
<td>Griffis et al., 1993</td>
<td></td>
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</tr>
</tbody>
</table>
**Lymphocyte differentiation**

1. Early differentiation (bone marrow for B cells, thymus for T cells)
2. Naïve cell (sometimes called a resting or quiescent B or T cell.)
3. Effector cell (rapidly dividing, fully functional.)
4. Memory cell

**B cell development**

**The stages of B cell development**

- Ordered gene rearrangements
- A model for allelic exclusion
- The role of the preBCR in B cell development
- B cell tolerance

**Bone marrow stromal cells provide secreted and cell surface factors that promote B cell maturation.**

In vitro cultures of bone marrow stromal cells and progenitor B cells can accurately recapitulate the normal steps of B cell development.
Stages in B cell development:

- **proB cells**: no HC or LC expression
- **preB cell**: HC (cytoplasmic), but no LC expression
- **B cell**: surface HC and LC expression

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**Flow Cytometric analysis of cells stained with 2 different labeled antibodies**

**1 parameter histograms:**

<table>
<thead>
<tr>
<th>Cell number</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1% positive</td>
<td>Unstained cells (negative control)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell number</th>
<th>Fluorescence intensity (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% positive</td>
<td>Stained cells</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell number</th>
<th>Fluorescence intensity (green)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% positive</td>
<td>Stained cells</td>
</tr>
</tbody>
</table>

**2 parameter dot plot:**

<table>
<thead>
<tr>
<th>Fluorescence intensity (red)</th>
<th>Fluorescence intensity (green)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% “double Positive”</td>
<td>80% red only</td>
</tr>
<tr>
<td>80% red only</td>
<td>20% “double Positive”</td>
</tr>
<tr>
<td>80% green only</td>
<td>80% green only</td>
</tr>
</tbody>
</table>

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**Isolating defined cell populations using Fluorescence Activated Cell Sorting FACS**

- **Ultrasound nozzle vibrator**
- **Cell suspension**
- **Sheath fluid**
- **Drop-charging signal**
- **Cell collector**
- **Flask for undeflected droplets**
Successive stages of B cell development can be distinguished by correlated expression of various cell surface markers.

Randy Hardy’s scheme for fractionating bone marrow B cells. Different populations were FACS sorted, cultured in vitro, and reanalyzed to establish developmental order.

**B cell development**
- The preB cell
- **Ordered gene rearrangements**
  - A model for allelic exclusion
  - The role of the preBCR in B cell development
  - B cell tolerance

V(D)J recombination can be detected by as simple PCR assay.
**PCR assay for gene rearrangement**

[Diagram showing PCR assay for gene rearrangement]

**Sequential gene rearrangement and regulated gene expression**

[Diagram showing gene rearrangement and expression]

**Sequence of Ig gene rearrangement**

as determined by FACS sorted developing B cells (and from studies using transformed “preB cell-like” cell lines).

\[
\text{DJ}_H, \ \text{VDJ}_H, \ \text{VJ}_k, \ \text{VJ}_\lambda
\]

**Sequence of Ig gene rearrangement**

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\[
\text{DJ}_H, \ \text{VDJ}_H, \ \text{VJ}_k, \ \text{VJ}_\lambda
\]

(proB cell), (preB cell), (B cell)
**B cell development**

The preB cell

Ordered gene rearrangements

**A model for allelic exclusion**

The role of the preBCR in B cell development

B cell tolerance

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**Ordered Gene Rearrangement and Allelic Exclusion**

µo = germline heavy-chain locus

κo = germline light-chain locus

Some B cell clones have VDJ rearrangements on both HC alleles.

In these clones, only one allele is productively rearranged!!

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**Allelic exclusion of Ig genes**

Flexibility in joining of gene segments contributes to junctional diversity.

(Note most rearrangements are non-productive!)
Why do antibody genes show allelic exclusion?

Because having a single B cell produce multiple antibodies would pose problems for regulating antibody production.

Because most gene rearrangements produce out-of-frame coding regions and do not encode functional proteins.

Because developing B cells sense the presence of productively rearranged antibody genes and respond by turning off V(D)J recombination.