Evaluation of Immunotoxicity

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Overview

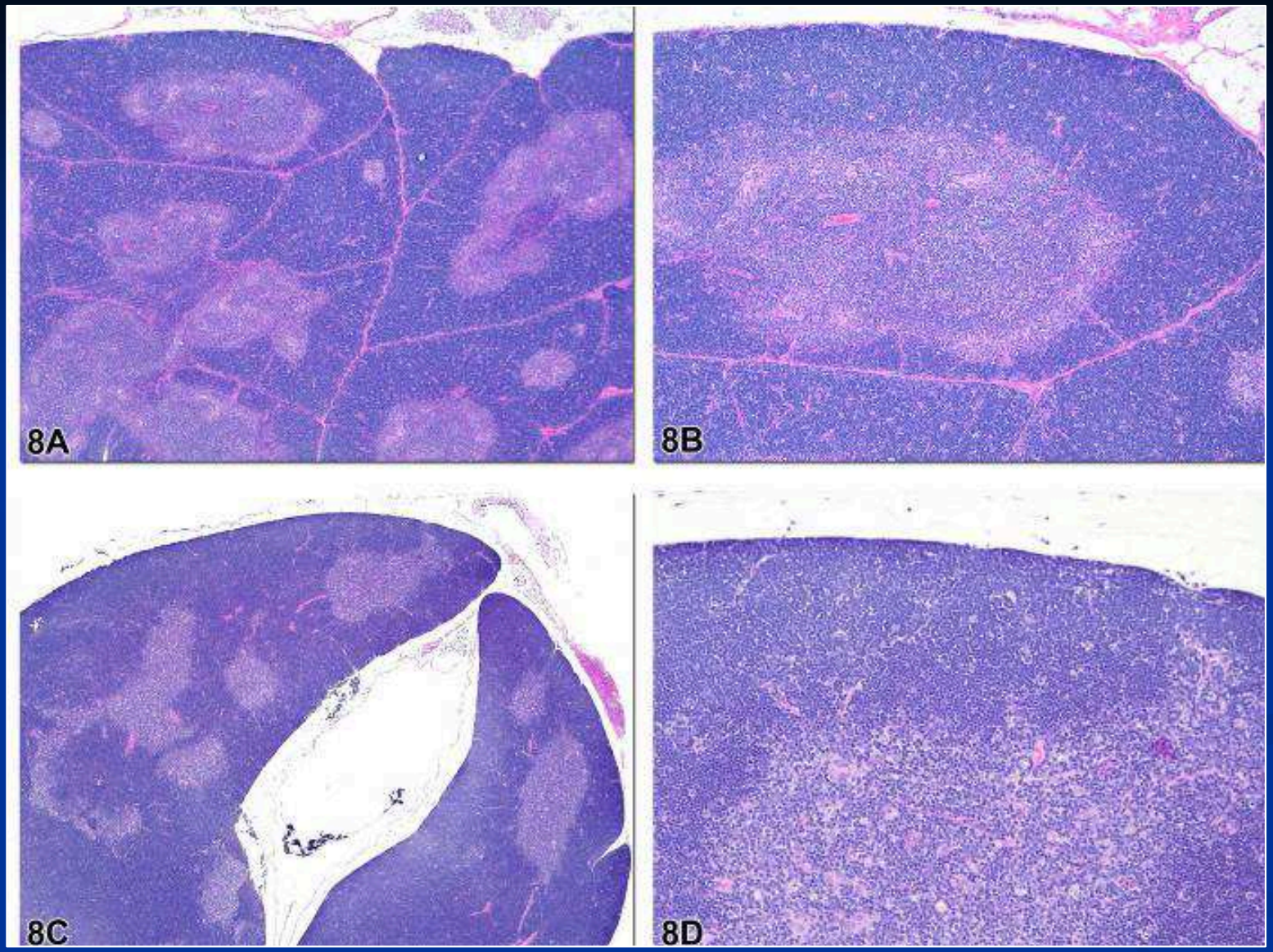
- Immune system
  - Lymphoid tissues
  - Differentiation, maturation, and selection
  - Cell distribution
- Thymic involution
- Stress and the immune system
  - Predicting immunotoxic potential
- Immunotoxicity
  - Suppression
  - Enhancement
- What is the "issue"
- Lymphoid tissues
- Best Practice Guidelines

Immune System

- Comprises complex cellular and physiologic mechanisms to protect the host
- Primary and secondary lymphoid tissues
  - Thymus, bone marrow
  - Spleen, lymph nodes, tonsils, "ALT's"
- Specific and non-specific responses
  - Innate immunity
  - First line of defense, antigen non-specific
    - Neutrophils, macrophages, IL-1, IL-6, TNF-α, TLR
  - Adaptive immunity
  - Second line of defense, antigen specific
    - Lymphocytes, CMI, antibody production

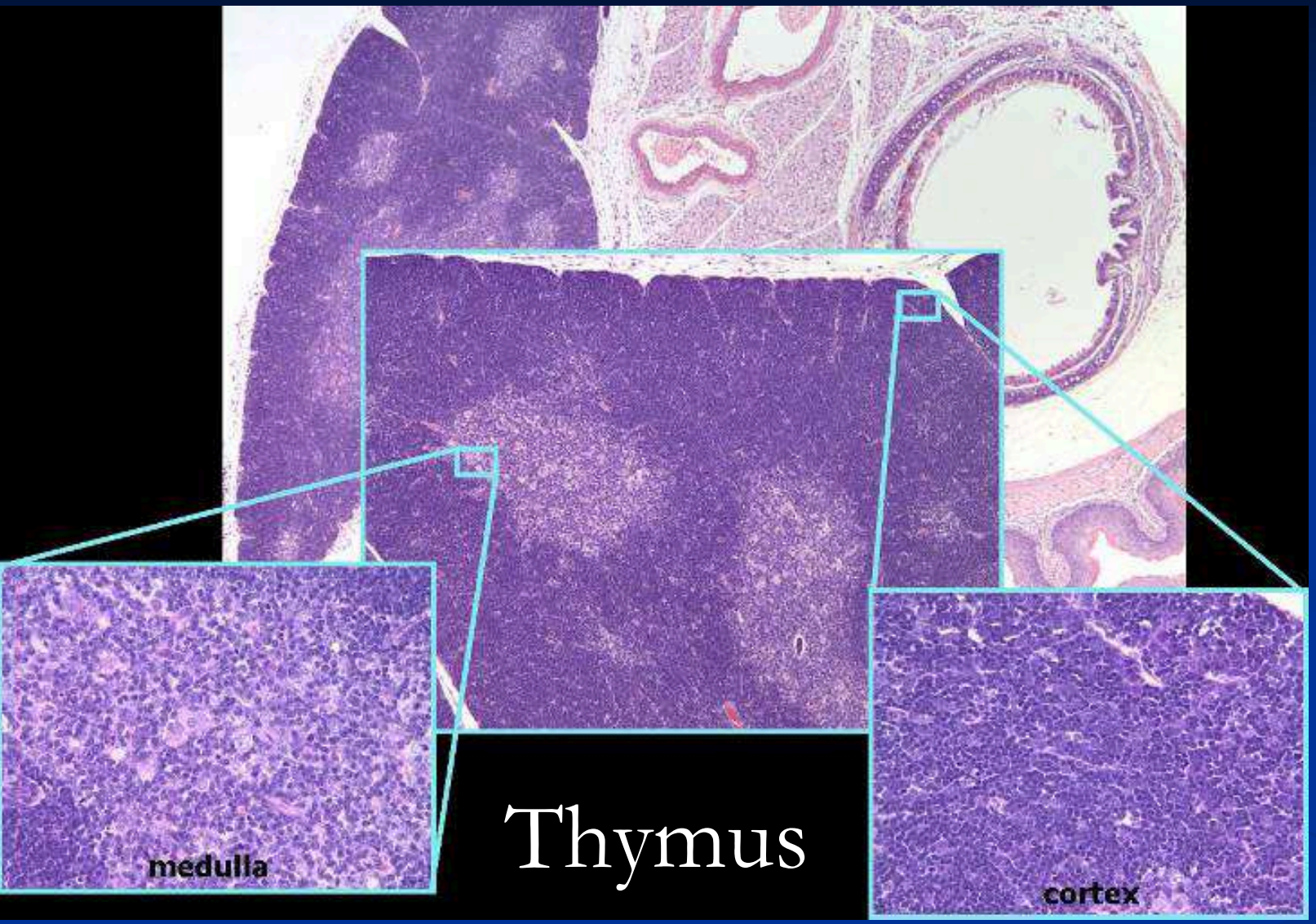
Lymphoid tissues

- Primary lymphoid tissues
  - Generation of B and T lymphocytes
  - Antigen-independent proliferation
  - Include
    - Thymus
    - Fetal liver, bone marrow
- Secondary (peripheral) lymphoid tissues
  - Initiation of antigen-specific immune response
  - Antigen-dependent proliferation
  - Include:
    - Spleen (white pulp)
    - Lymph nodes
    - Peyer's patches and solitary lymphoid nodules
    - NALT, tonsils, BALT

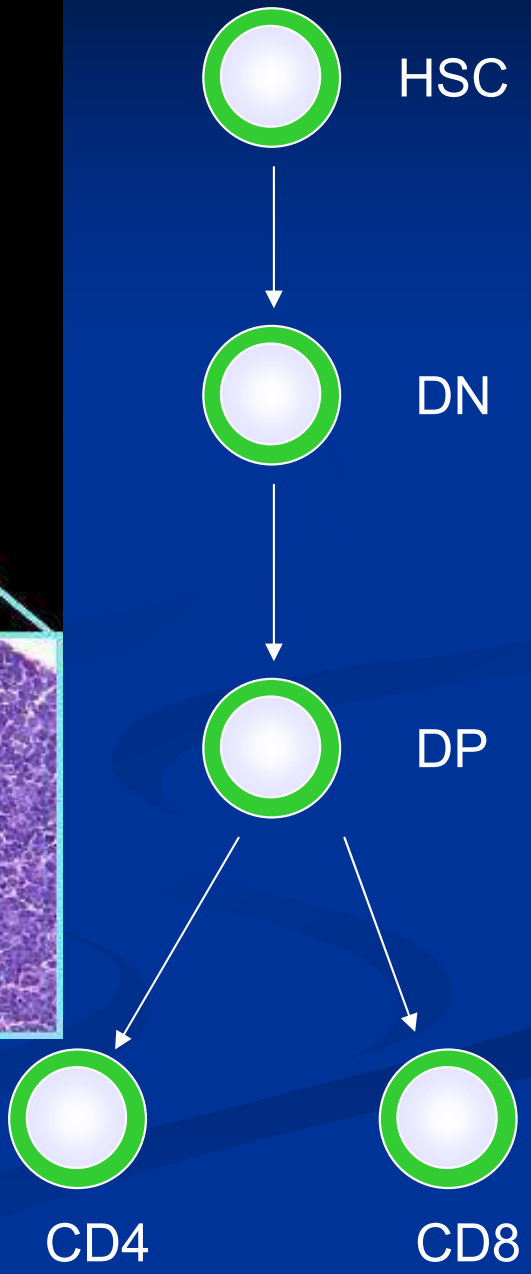


Rat, thymus

Mouse, thymus

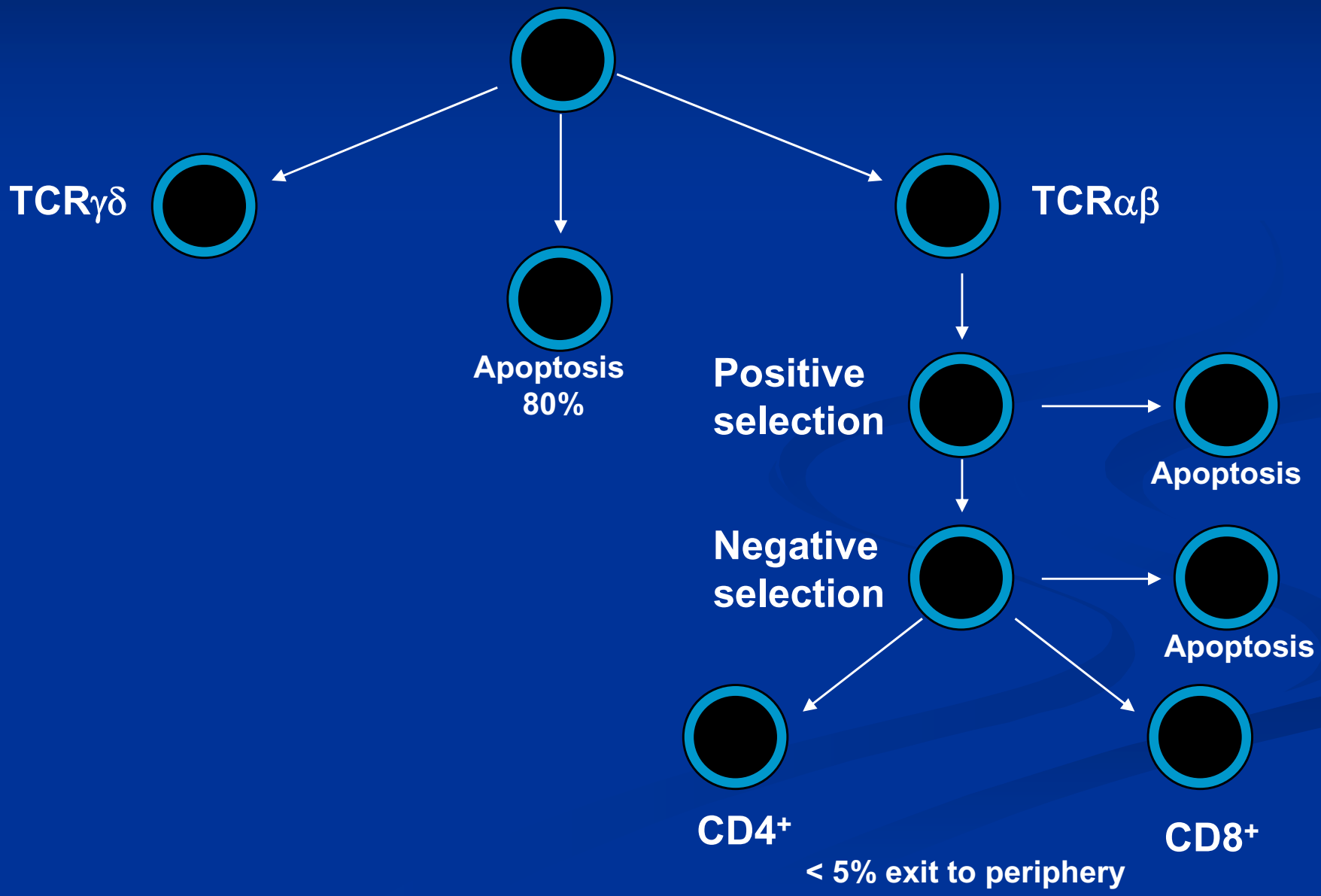


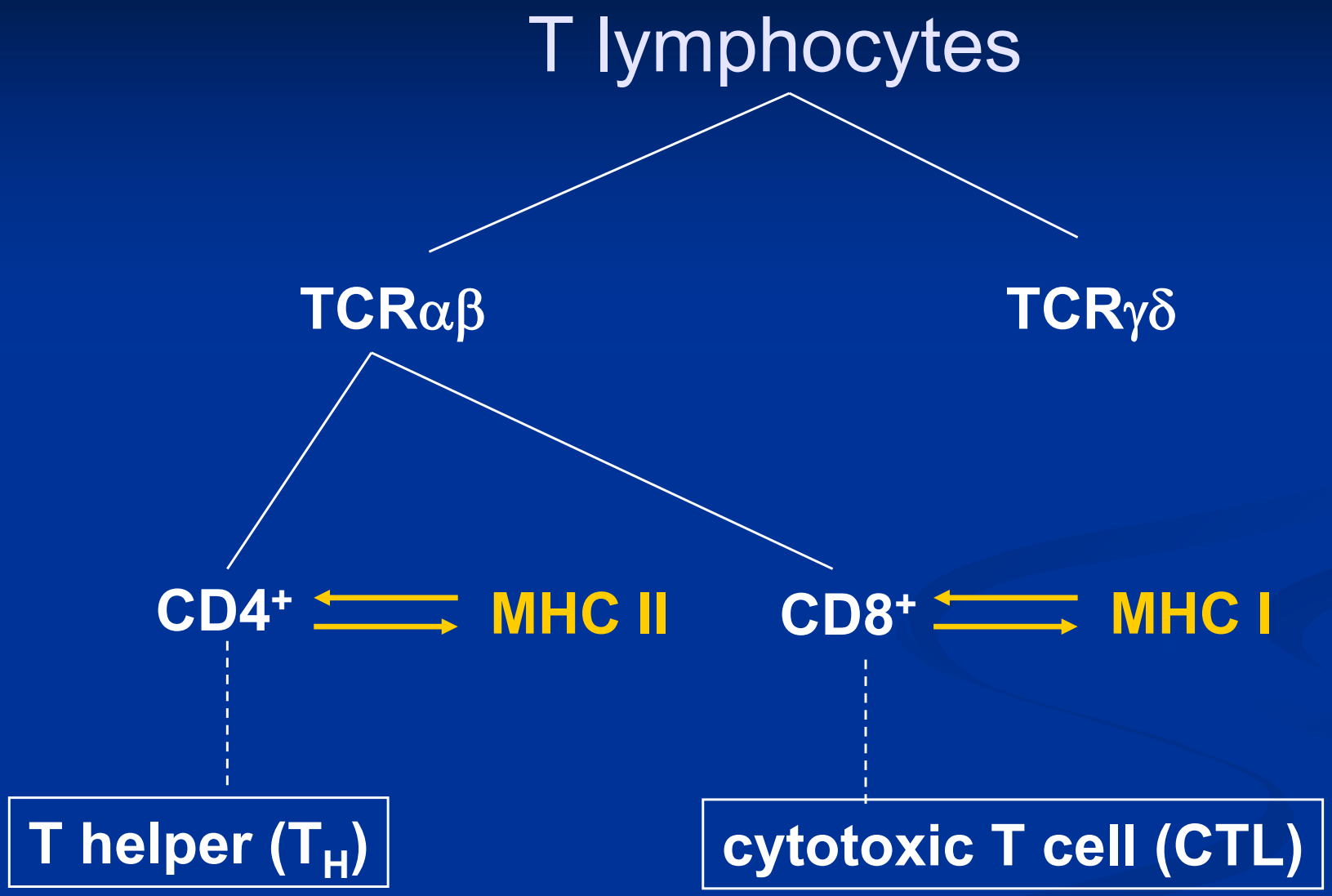
Thymus



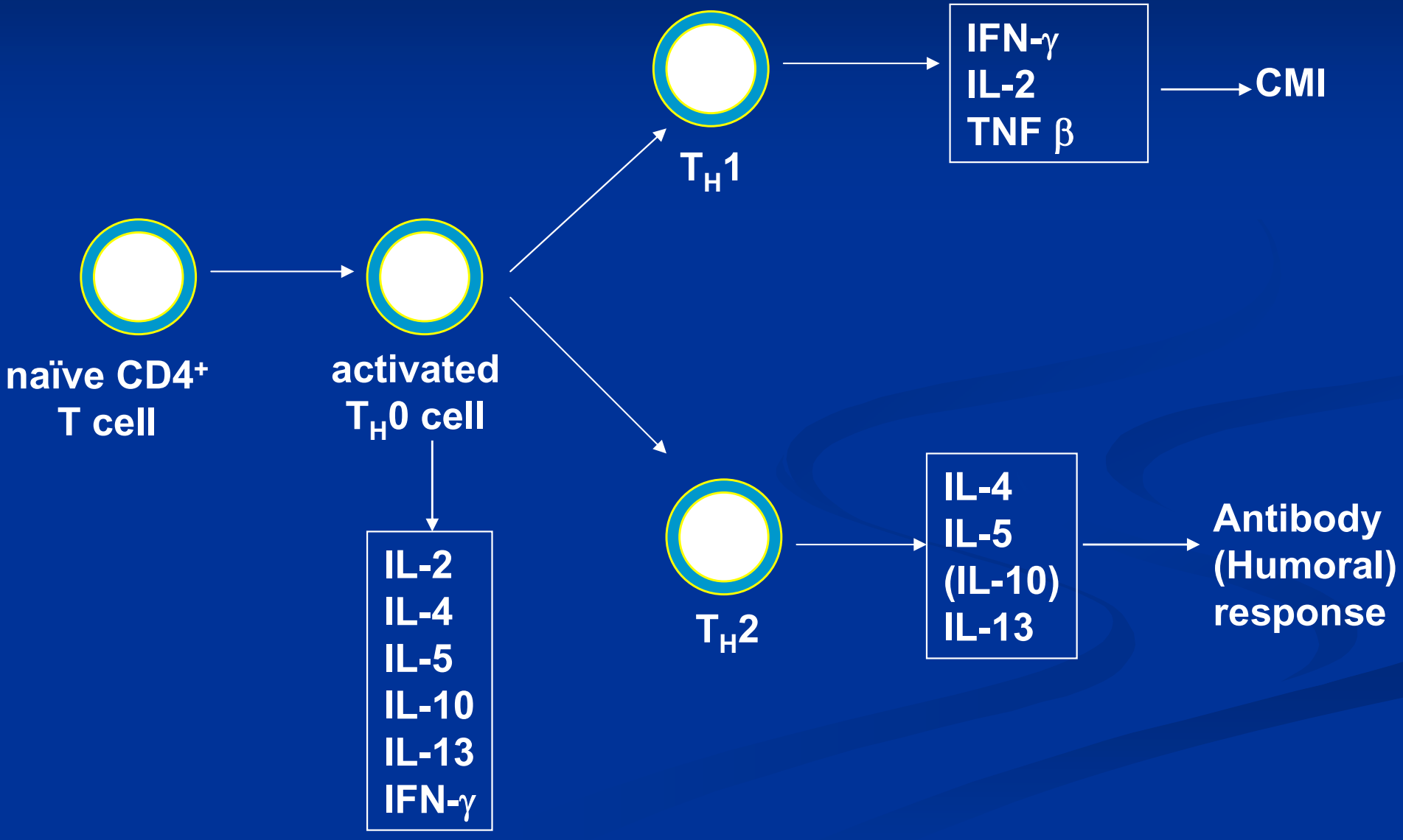
(< 5%, 10^6/day)

T cell maturation and selection

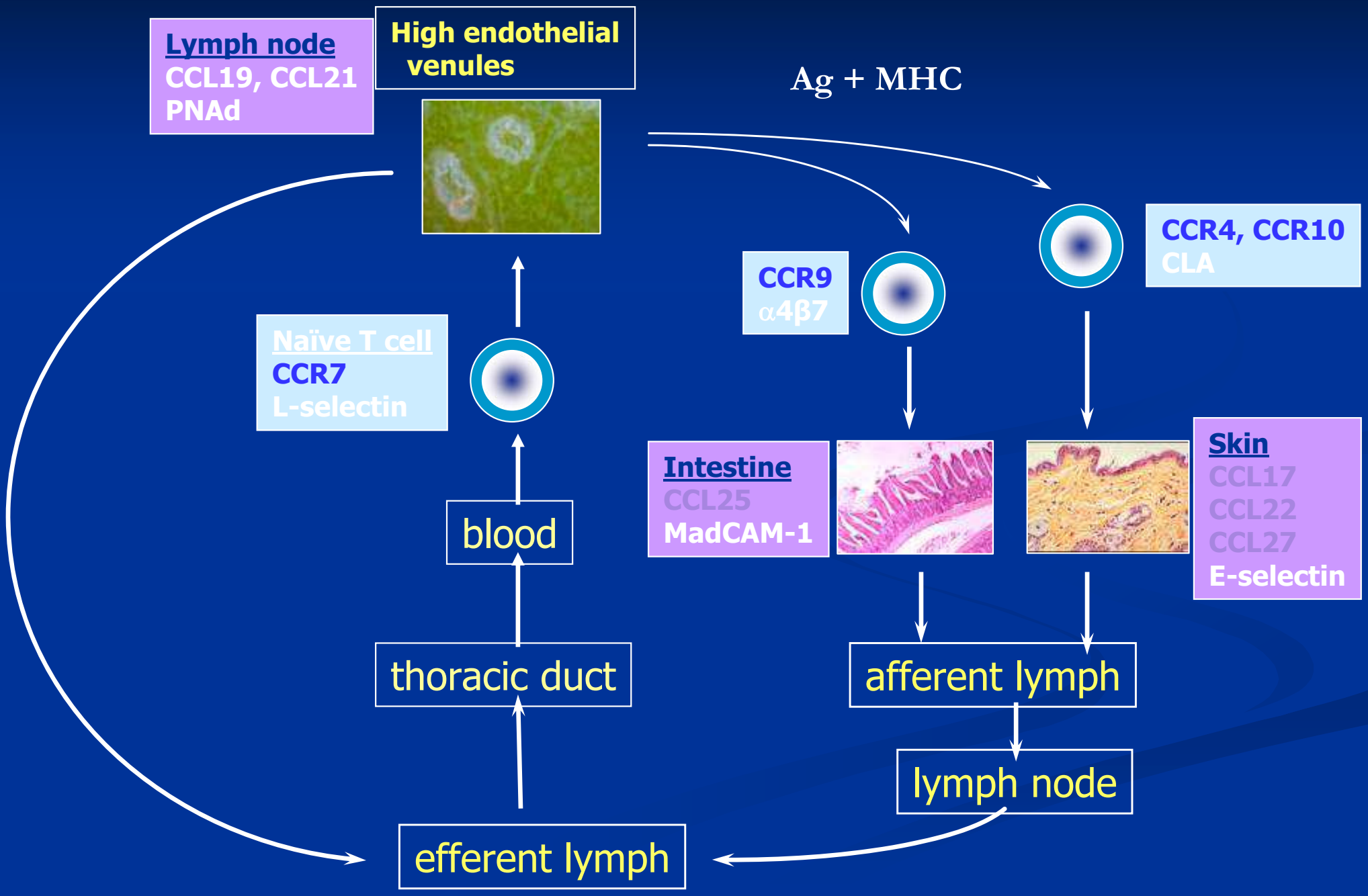



T lymphocytes
TCRαβ
TCRγδ
CD4+
MHC II
CD8+
MHC I
T helper (TH)
cytotoxic T cell (CTL)

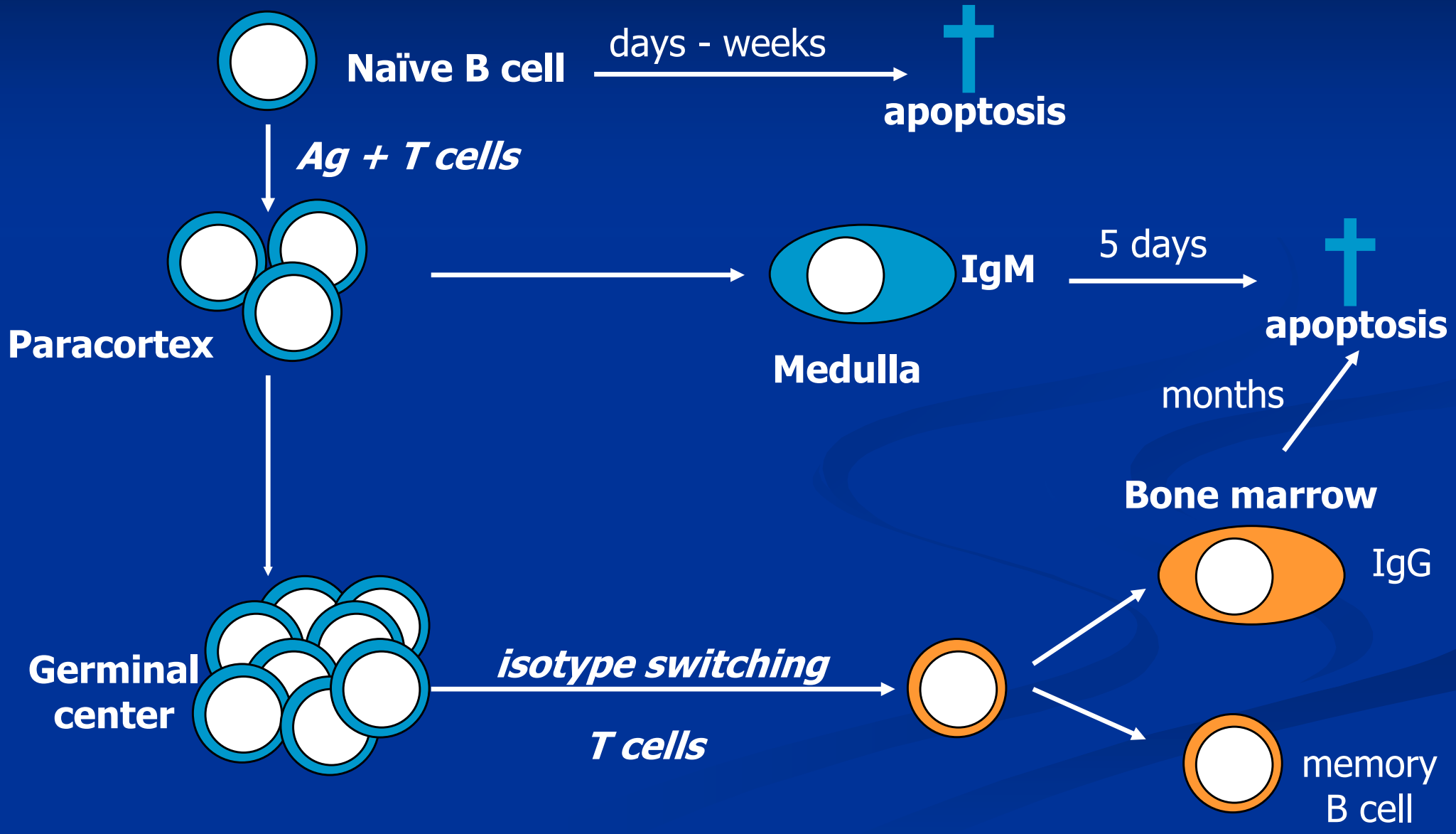
Differentiation of $CD4^+$ T cells



Lymphocyte migration

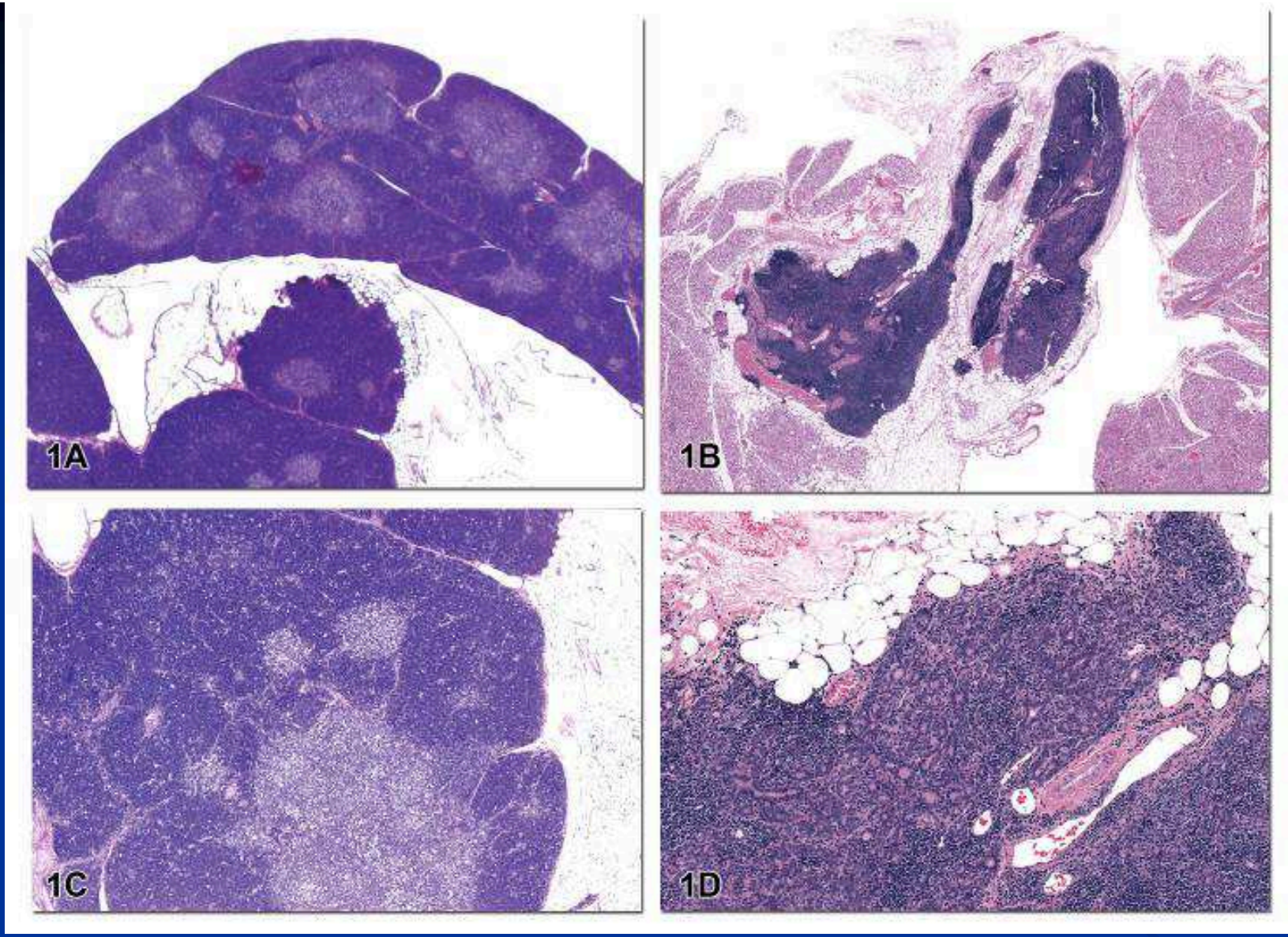


B cell development in lymph nodes



Thymic involution

- Normal physiologic process
  - Progressive decrease in size and relative weight
  - Decreased proliferative capacity and increased sensitivity to apoptosis
- Begins at sexual maturity
  - Rate and extent is species, strain and sex dependent
- In mice glucocorticoids can delay the progression of thymic involution
- Can confound interpretation



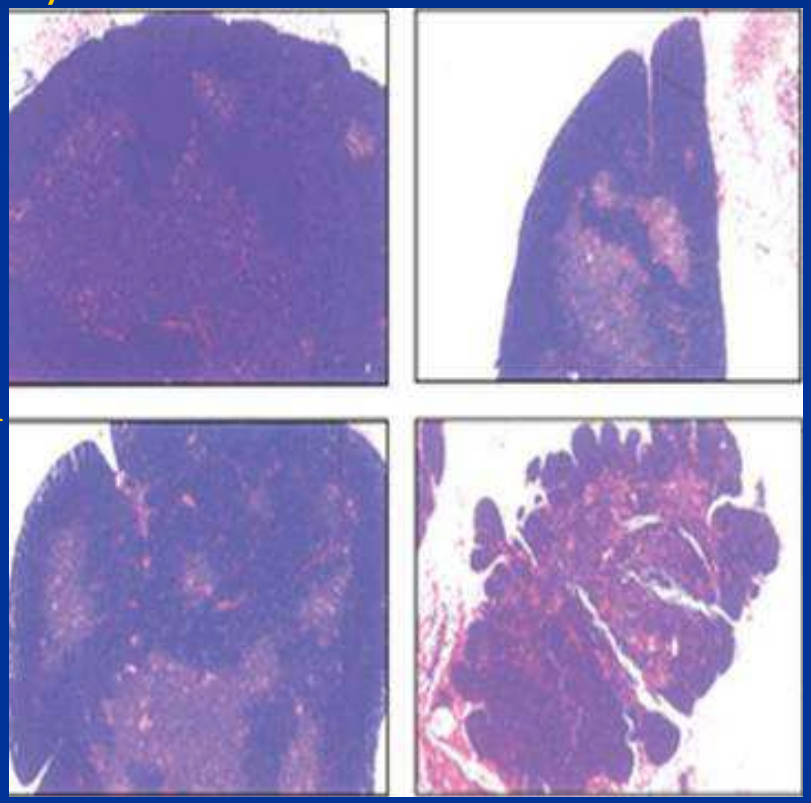
Thymus, 31 week old rat

Thymic involution, 2 yr old rat

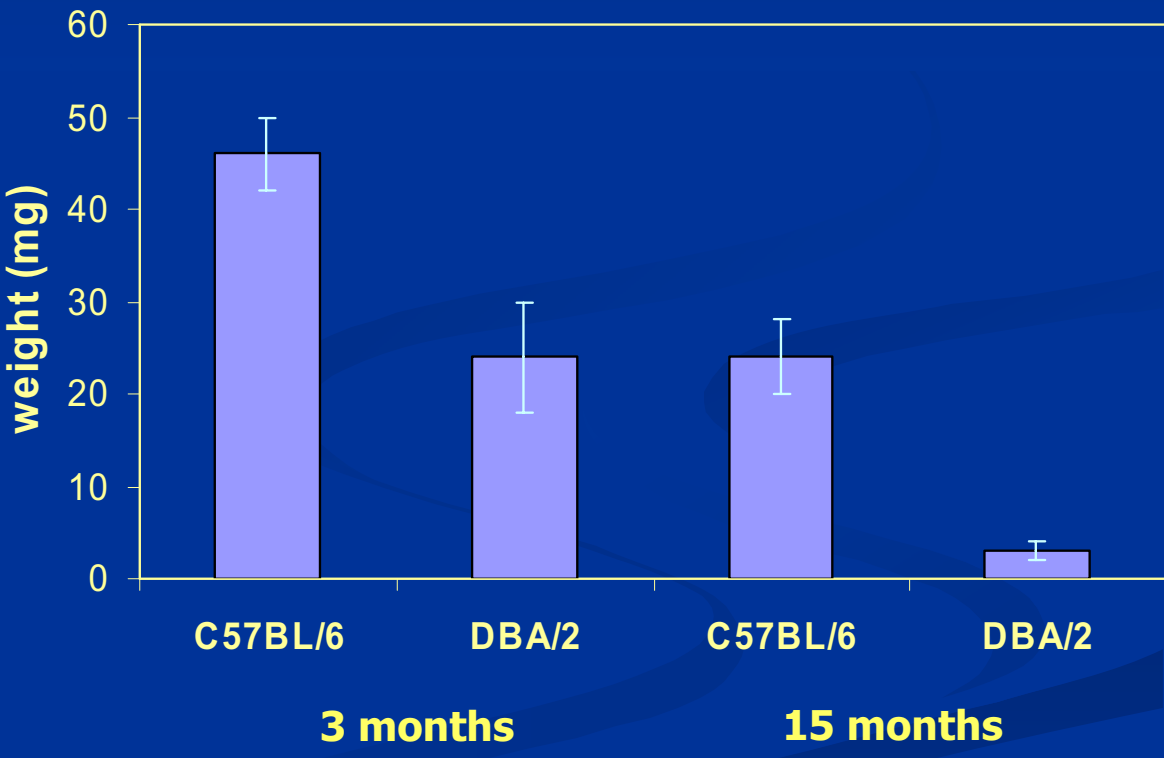
Genetic influence on thymus size and thymic involution: C57BL/6 vs. DBA/2

C57BL/6

DBA

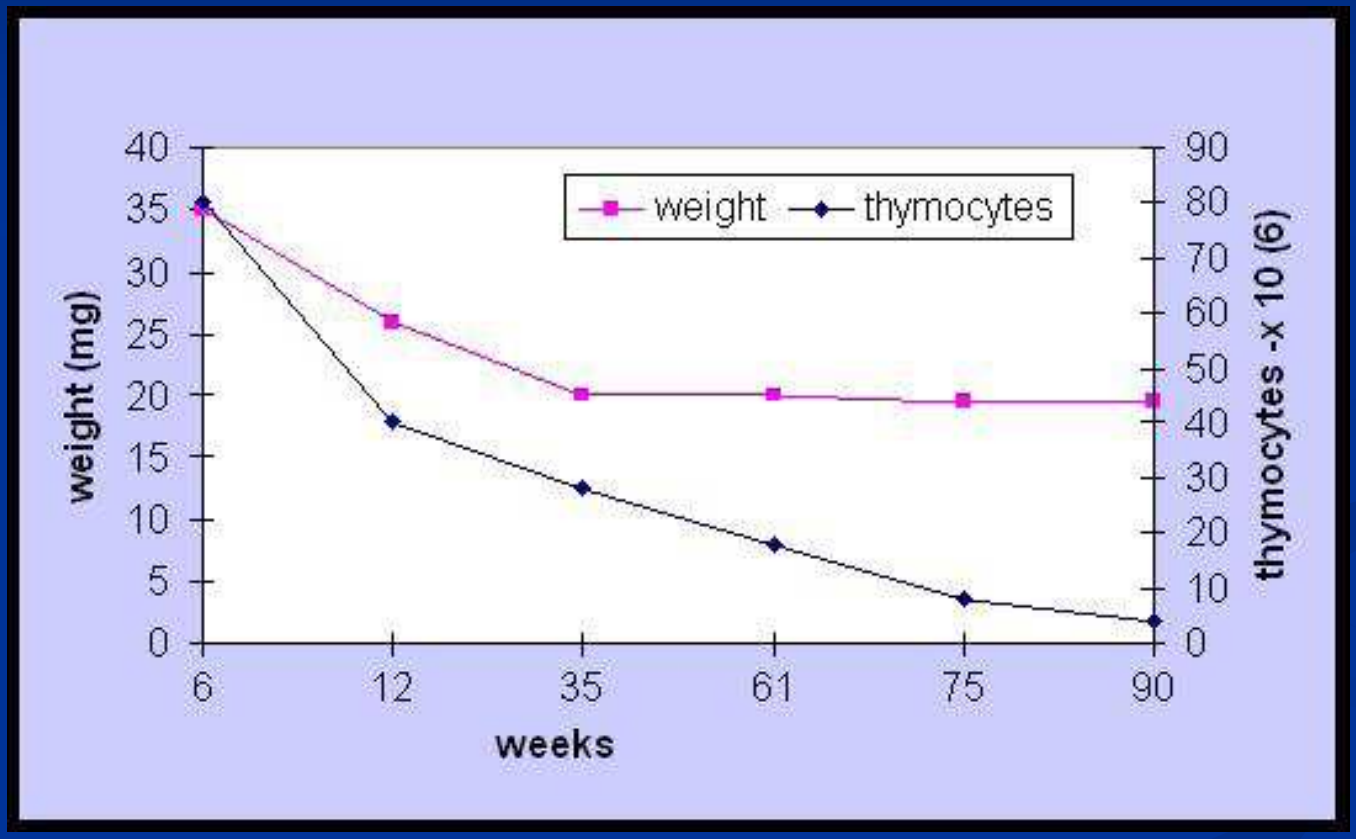


3 mo. 15 mo.

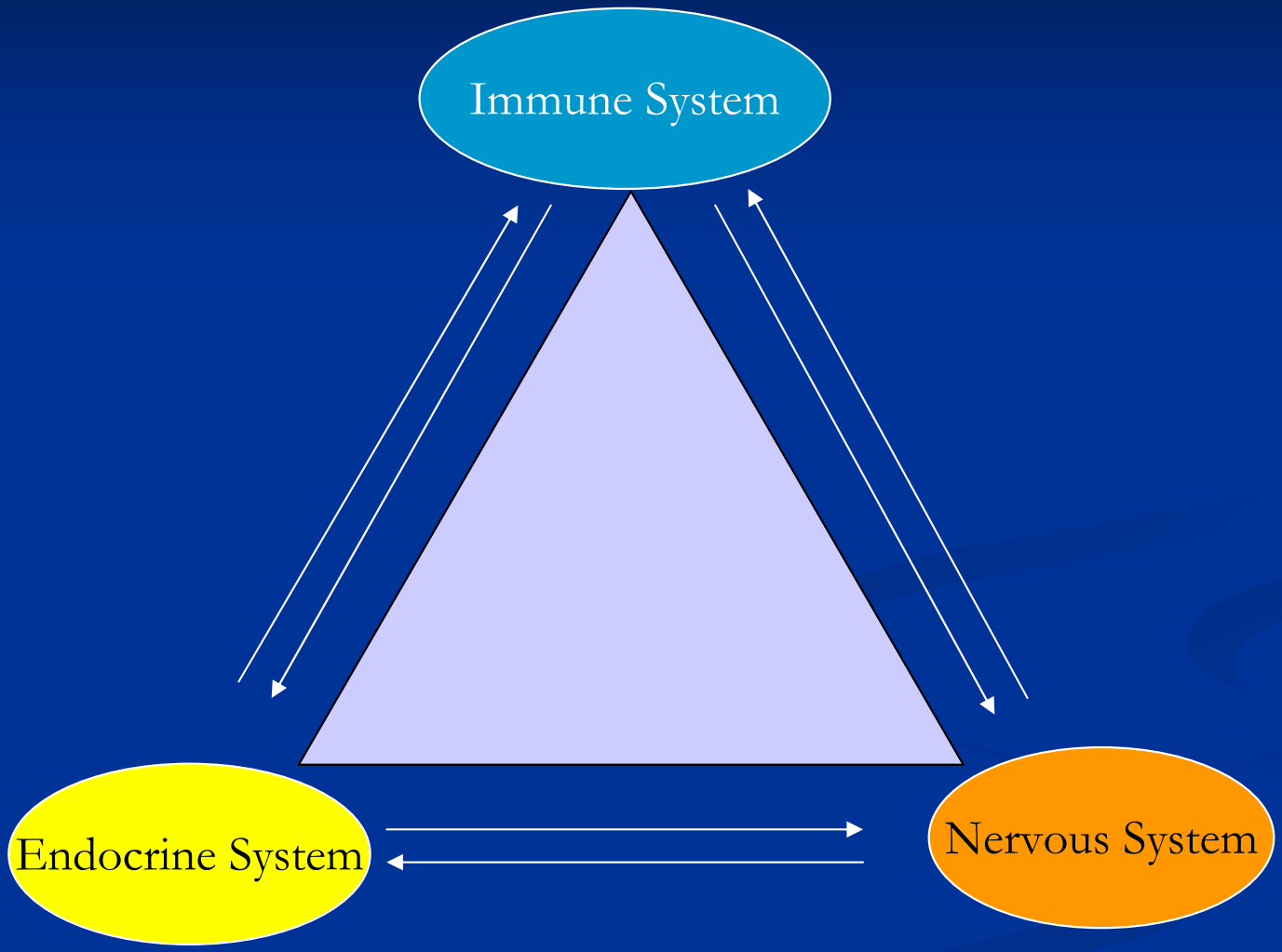


Adapted from Scan J Immunol 57:410, 2003

Thymus involution in BALB/c mice



Adapted from Mol Immunol 38: 841, 2002

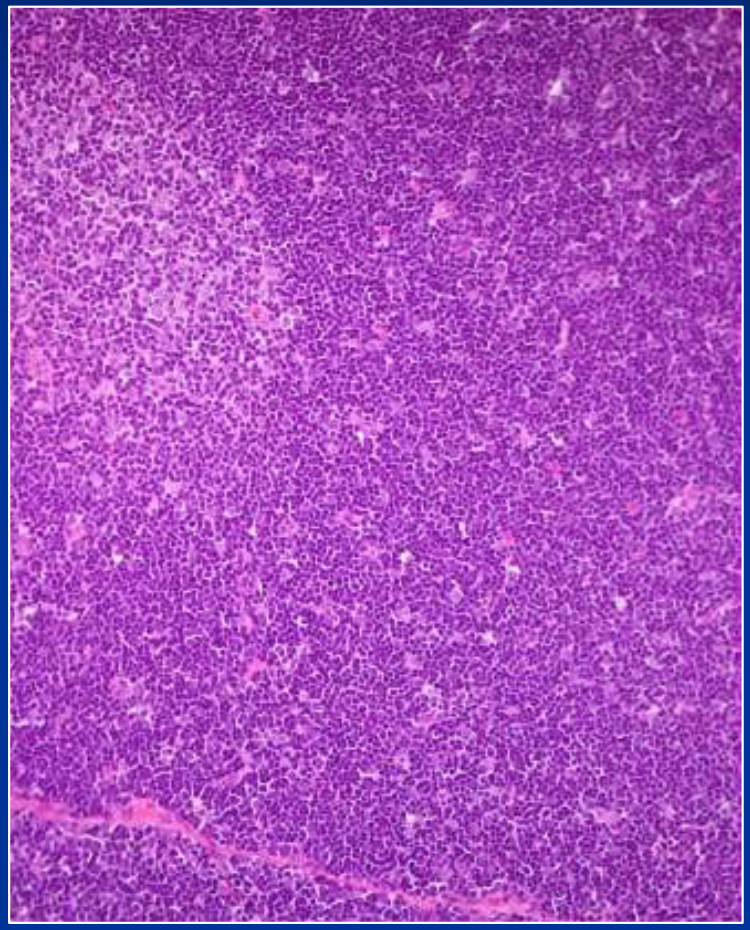


Adapted from Burn-Naas et al, pg 430, 2001

Stress in routine preclinical safety studies

- Hallmarks
  - Decreased body weights
  - Decreased feed consumption
  - Decreased thymic weights
  - Increased adrenal gland weights
  - Increased monocytes and neutrophils
  - Decreased lymphocytes and eosinophils
- Additional findings

Thymus



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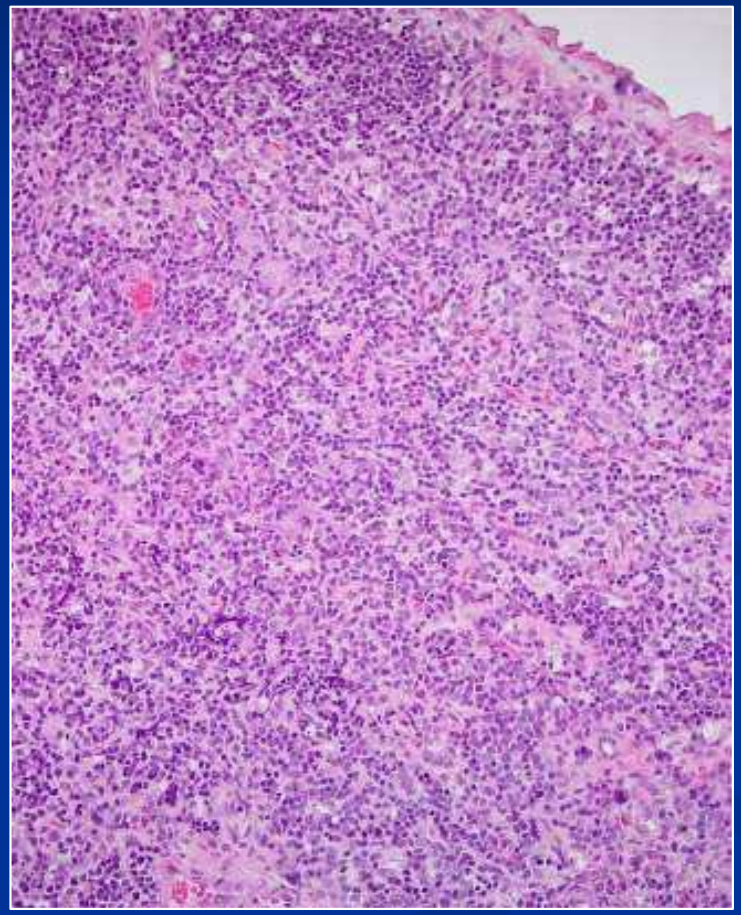
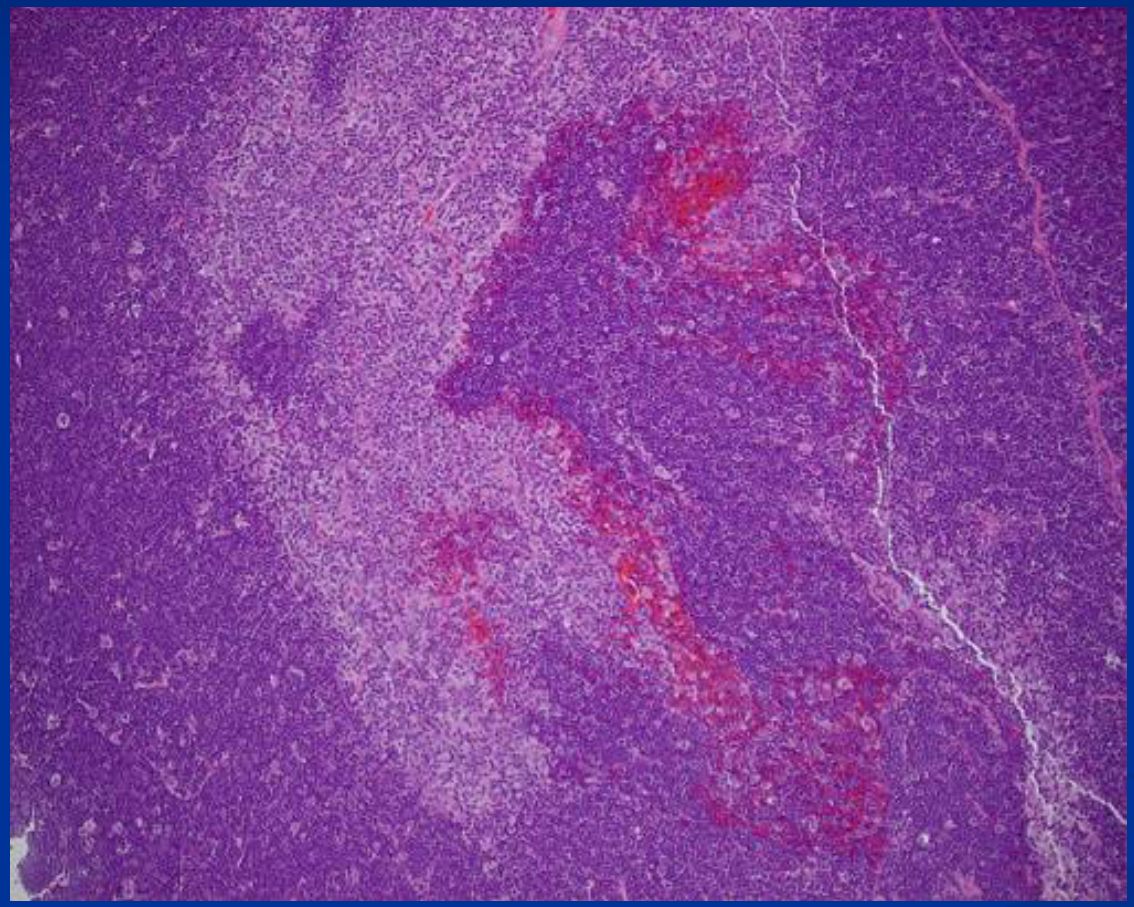


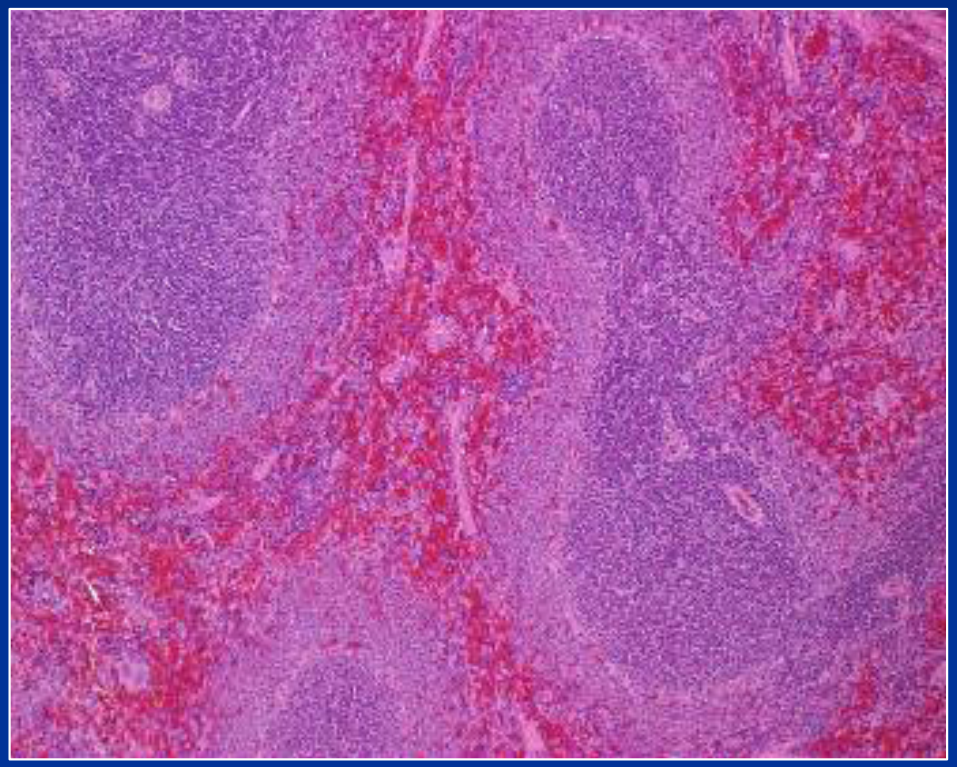
Stress-related

Thymic hemorrhage



Acute stress effect

Spleen



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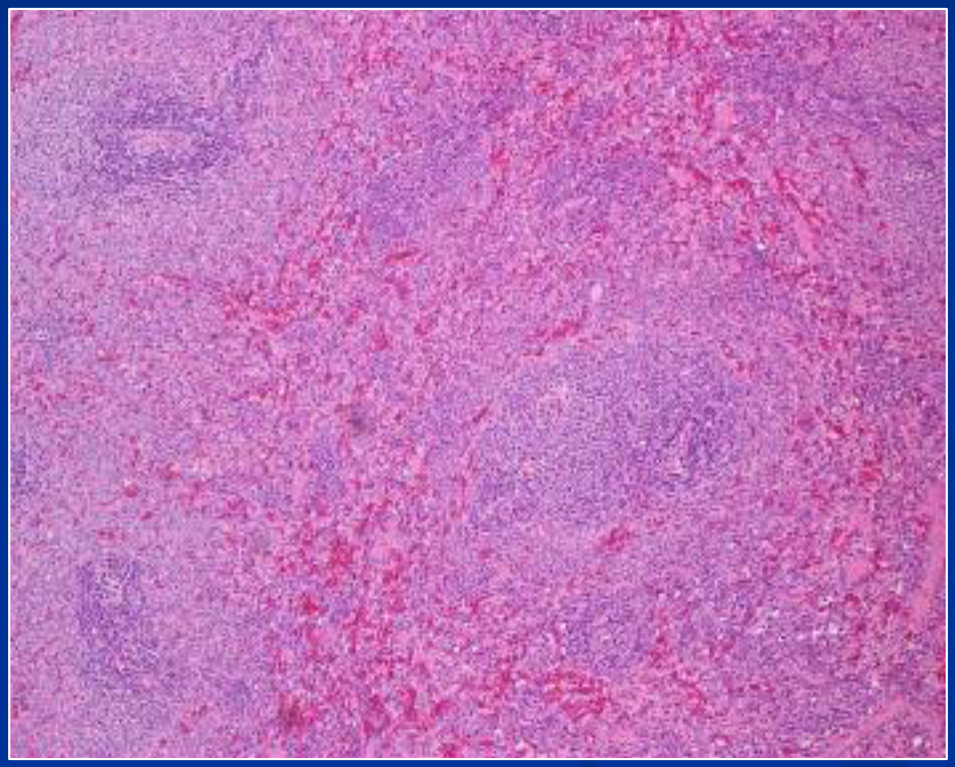


Stress-related

Acute stress and the immune system

- Biological effects are dependent on level and duration of mediators
- Acute stress:
  - Enhances innate immunity
    - ↑ NO production by macrophages and neutrophils
    - ↑ Pro-inflammatory cytokine production
    - ↑ Acute phase protein synthesis
    - ↑ Complement activity
  - Suppresses adaptive immunity
    - ↓ Ag-specific antibody responses
    - ↓ T cell proliferation
    - ↓ Cytotoxic T cell responses

Chronic stress and immunity

- Immunosuppression
- Chronic restraint stress increased lymphocyte apoptosis via ↑CD95 expression
  - 35-40% ↓ splenic lymphocytes
  - opioid-dependent
  - adrenalectomy had no effect (spleen < sensitive to corticosterone??)
  - Interestingly testis Sertoli cells express high levels of CD95L
- Sensitivity to chronic stress
  - Mature T cells > B cell
- Confounded by other physiologic responses
    - Anorexia
    - Decreased body weight
- Short-term toxicity studies (28 day)
    - Stress early
    - Habituation or tolerance later

Literature Review for Rats: Sensitivity of Various Systems to Stress

- Thymus and spleen > lymph node
- Effects on peripheral blood lymphocytes earlier than thymic lymphocytes
- Sensitivity of organ weights:
  - Thymus = adrenal (many)
  - Thymus > adrenal (1 paper)
  - Adrenal > thymus (2 papers)
- Body weight gain and corticosterone generally most sensitive
- Histological changes attributable to stress between animals can be significantly varied

Chemicals associated with stress effects in rodent models

- Organophosphorous compounds
- Trimethyltin
- Chlorimeform
- PCBs
- Unleaded gasoline
- Cadmium
- Mirex
- Propanil
- Deltmethrin
- Carbaryl
- Gallium arsenide
- Morphine
- Ethanol
- Haloperidol
- Phenytoin
- Paraquat

Chemicals associated with stress effects in rodent models

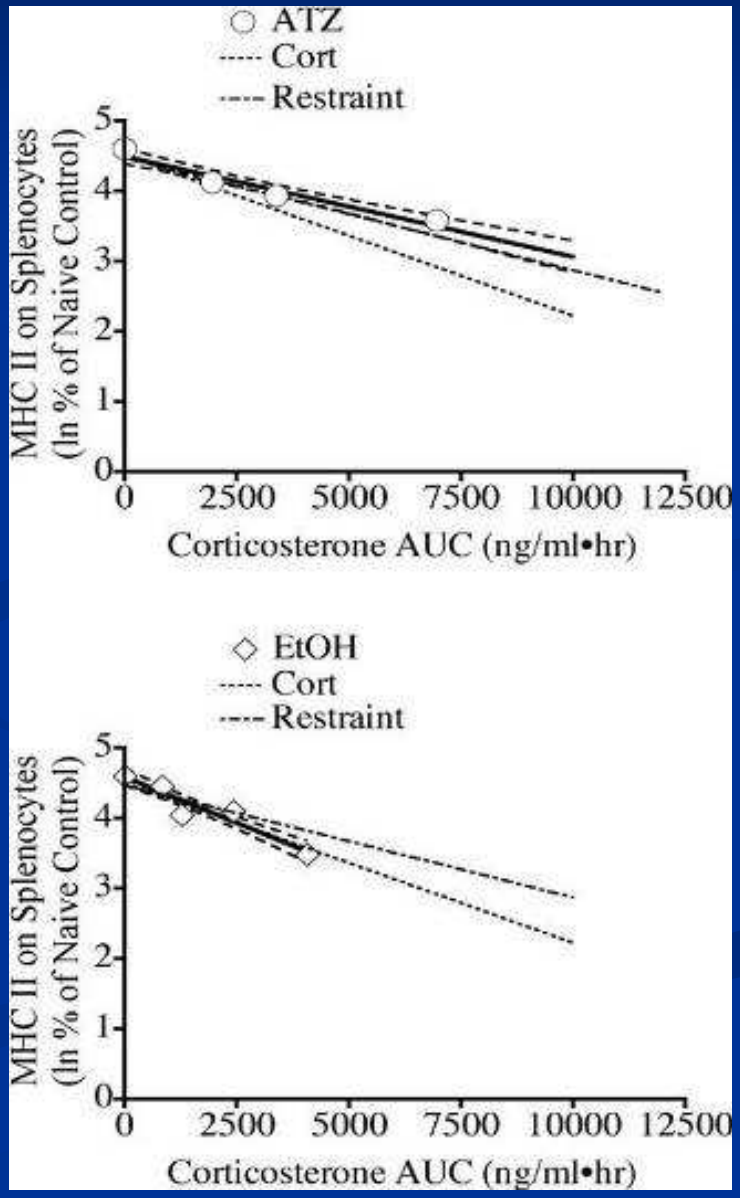
- Rodent models
  - Increased corticosterone levels
  - Degree and level of increases determines biological effect
    - Enhance immune responses
    - Suppress immune responses
  - Quantitative relationship between neuroendocrine mediators and immunosuppression
- Dose
  - MTD's only
- Biological effects
  - Decreased spleen &/or thymus cellularity
  - +/- detectable functional changes

Predicting stress-induced immunosppression

- Drugs and chemicals at high doses can induce immunosuppressive stress responses in mice
- Quantitatively consistent effects on parameters by chemical and physical stressors at comparable corticosterone AUC values
  - Spleen, thymus and blood
  - Chemical stressors in SP and TY are more like restraint stress than exogenous corticosterone
  - Exogenous corticosterone and chemical stressors have a more dramatic effect on blood parameters (e.g. decreased lymphocytes, increased neutrophils) than restraint stressors
- Values for immunological effects of the chemicals were calculated from the dose-response line for each chemical at the dosage yielding 50% suppression of MHC class II. These are compared to the values predicted using corticosterone AUC values induced by restraint at the AUC value yielding 50% suppression of MHC class II.

Predicting stress-induced immunosppression

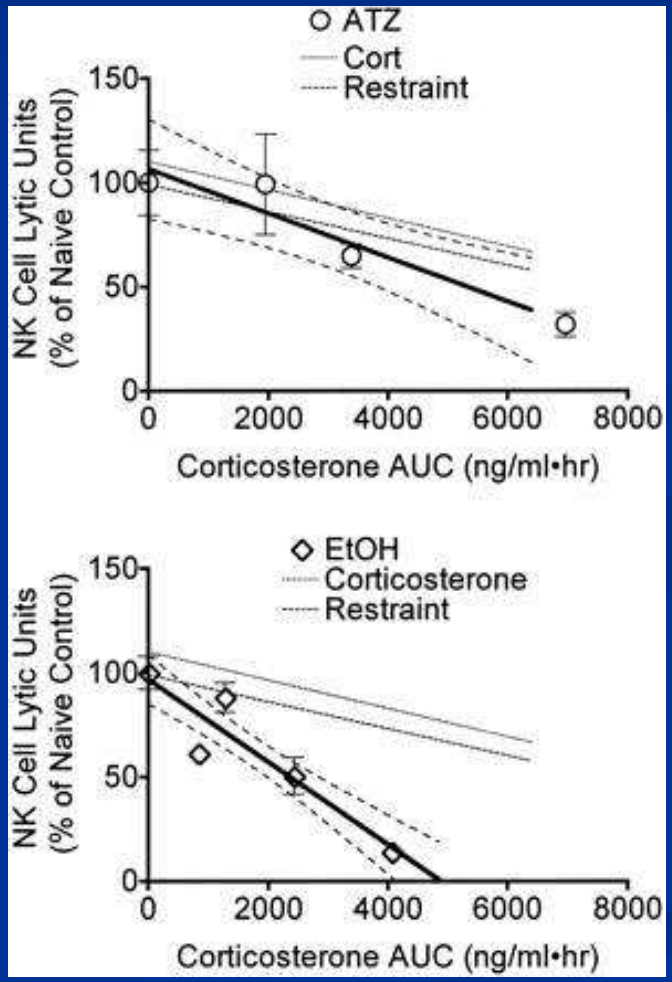
- ↓ MHC II expression on leukocytes
  - Peritoneal macrophages
  - Splenic B lymphocytes
  - Thymic lymphocytes



Pruett et al Tox Sci 75, 343-354, 2003

Predicting stress-induced immunosppression

- ↓ NK cell activity



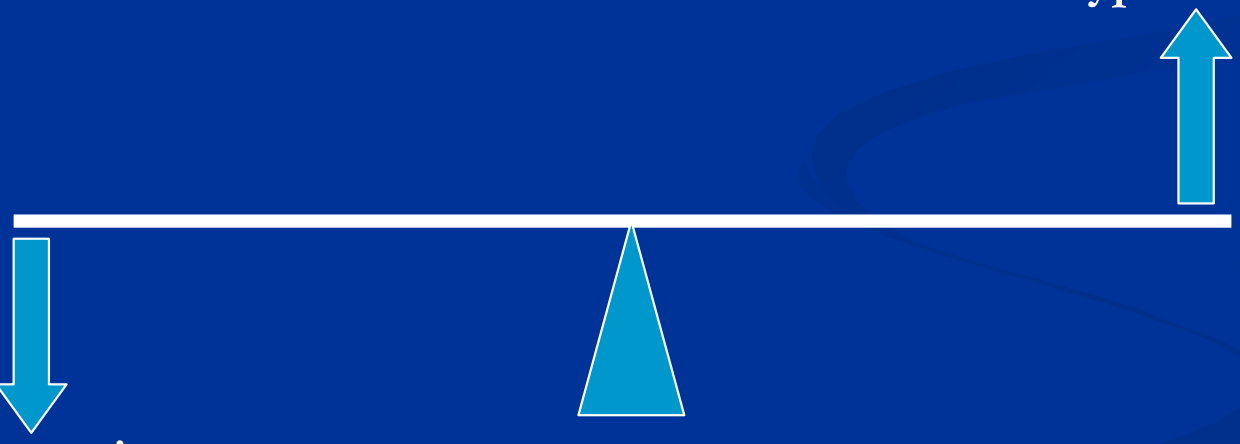
Pruett et al Tox Sci 75, 343-354, 2003

Predicting stress-induced immunosppression

- MHC II expression is more sensitive vs NK cell activity
- MHC II expression predicted effect of chemical stressors but not NK cell activity
- ↓ MHC II expression not known to be associated with other stressors
- ↓ MHC II expression not known to be associated with drugs or chemical exposures that don't induce stress response
- Only applicable to acute stress effects of a single dose of chemical stressor
- Rat immune parameters are < sensitive to corticosterone compared to mice
  - Not because the response to stress in mice > rats

Immunotoxicity

- Direct or indirect adverse effects of the immune system



Immunotoxicity

- Whole animal
  - Increased incidence of disease
- Tissue level
  - Organ weights
  - Cellularity
- Cellular level
  - Function
  - Surface markers
  - Products

Routine evaluations

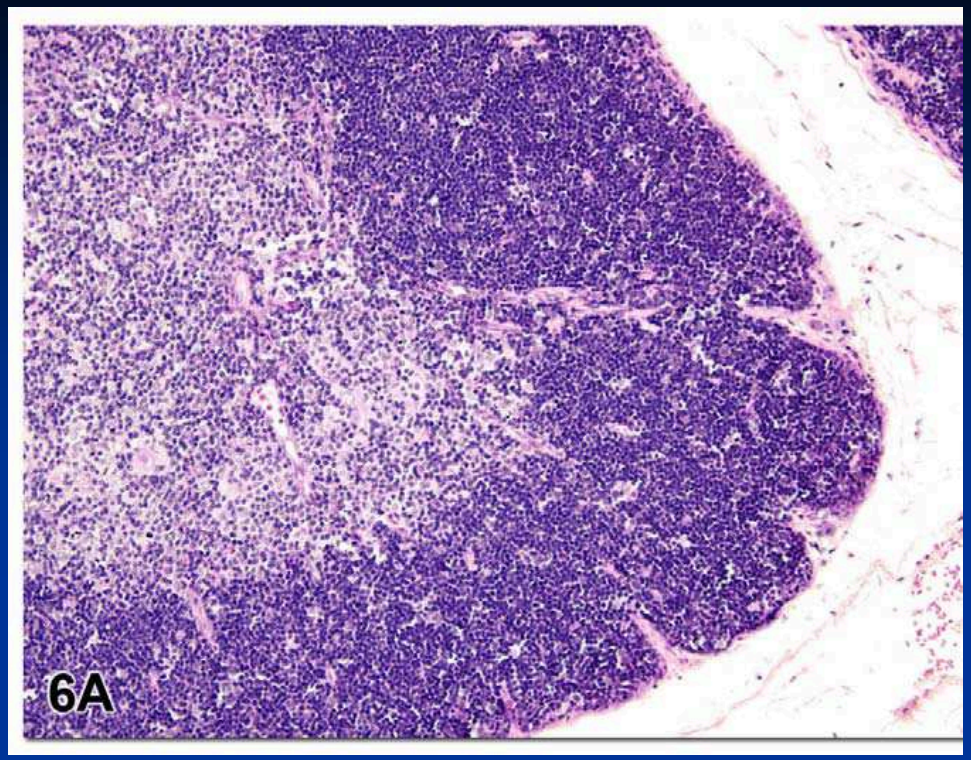
- Hematological assessments
- Lymphoid organ weights
  - Thymus
  - Spleen
- Histopathology
  - Thymus
  - Spleen
  - Bone marrow
  - Lymph nodes
- Immunotoxicity studies
- Immunophenotyping
  - Peripheral lymphocytes
  - Tissue lymphocytes

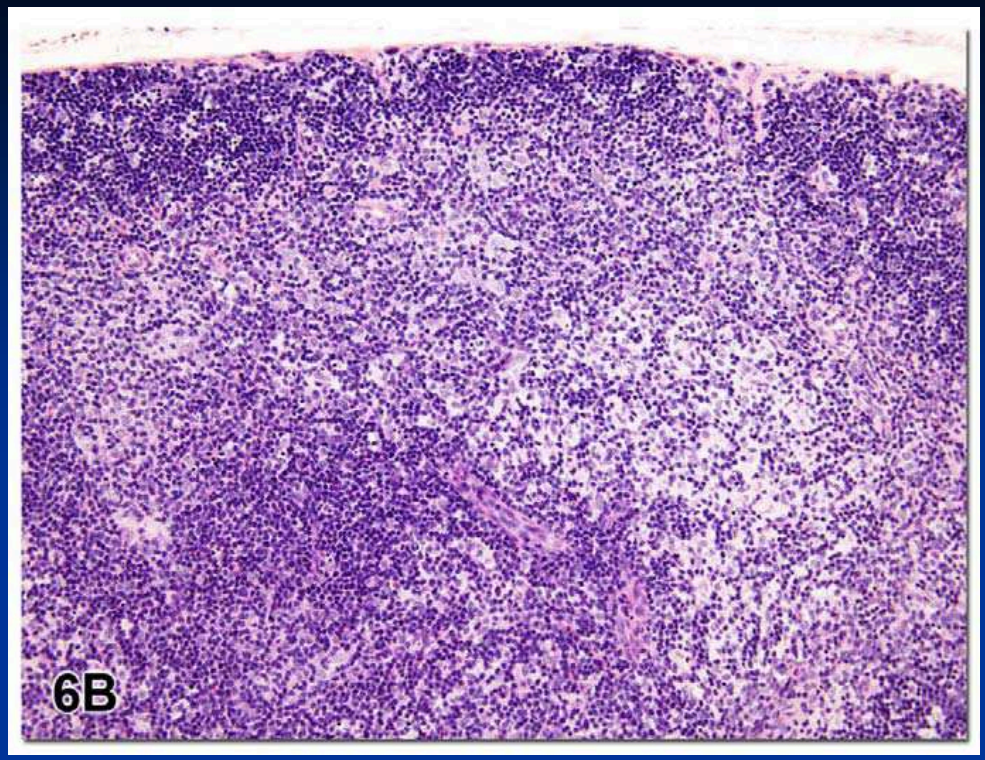
Pathologic Changes in the Immune System

- Like most tissues, lymphoid tissue has a limited repertoire of possible responses to damage or stimuli
  - Hyperplasia
  - Atrophy
  - Necrosis
  - Neoplasia
- Some changes are merely a reflection of the function of the lymphoid tissue
  - Filtering of lymph
    - Antigens
    - Particulates (foreign material; RBCs ⇒sinus erythrocytosis)
    - Cells

Xenobiotics & Suppression

- Increased susceptibility to infections
- Halogenated aromatic hydrocarbons
  - genetic basis for susceptibility
  - Ah-R
  - TCDD
  - severe lymphoid atrophy, thymus





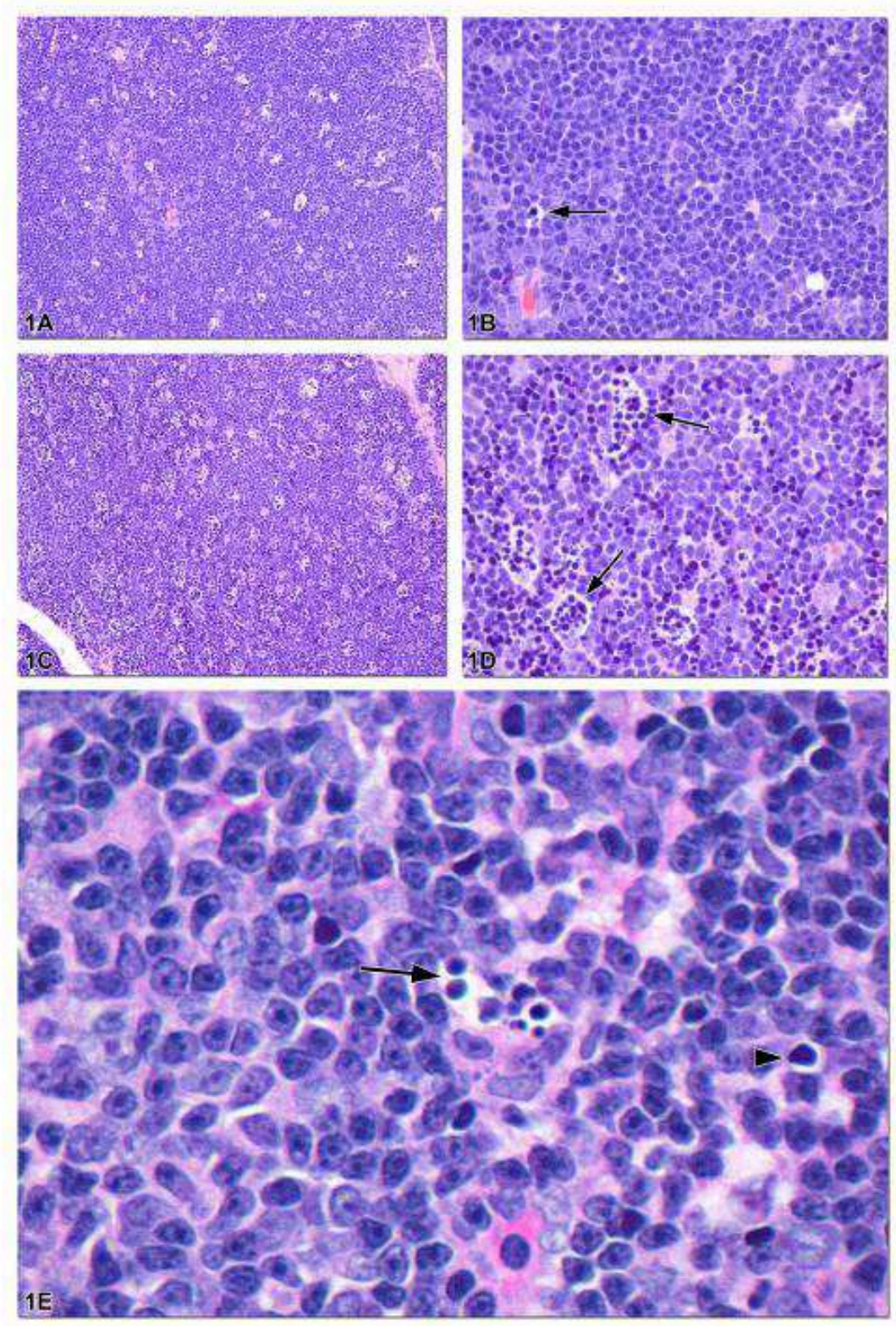
Female Sprague-Dawley rats: Control (6A) and Treated (6B). 31 weeks of treatment with a low dose of dioxin

Xenobiotics & Suppression

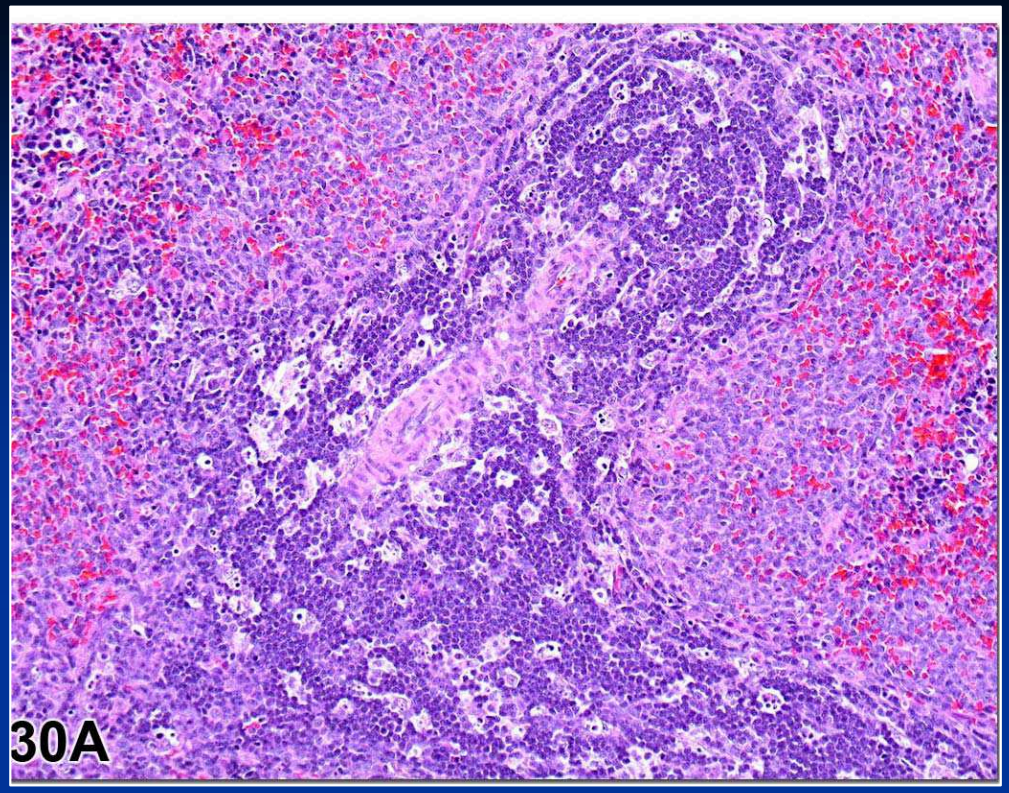
- Polycyclic aromatic hydrocarbons
  - environmental contaminants
- Metals
  - lead, arsenic, mercury, cadmium
- Organic solvents
  - benzene - myelotoxic
  - toluene
  - carbon tetrachloride

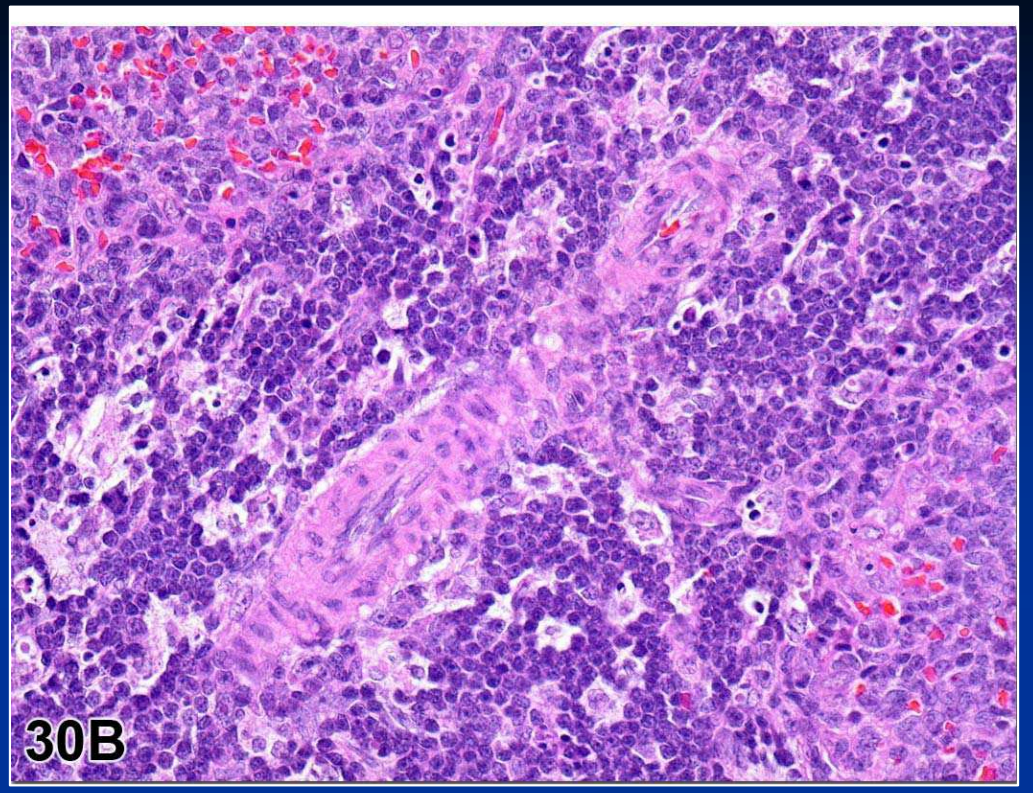
Xenobiotics & Suppression

- Therapeutics
  - alkylating agents, cyclophosphamide
  - corticosteroids
  - cyclosporin - inhibits IL-2 gene transcription
  - macrolides - FK506
- Drugs of abuse
- UV-B radiation



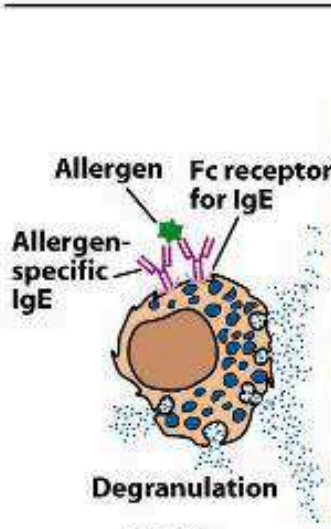
30 Day old Sprague-Dawley rat treated three hours previously with dexamethasone (1 mg/kg)

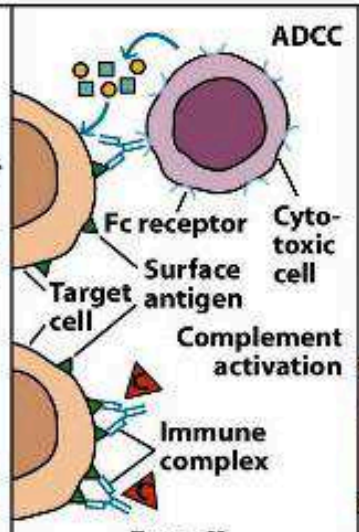


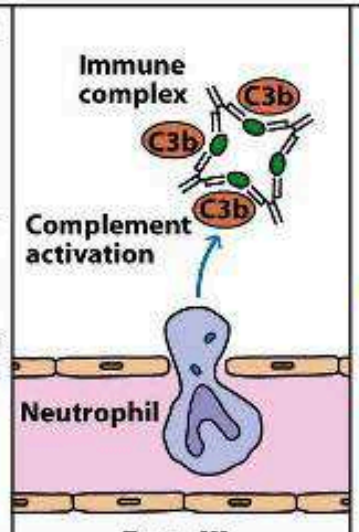


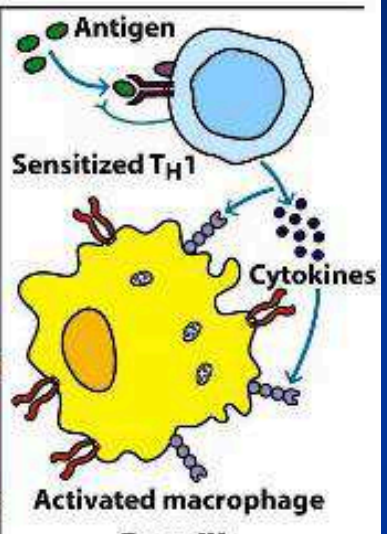
F344 rat treated with cyclophosphamide 48 hours earlier.
Marked apoptosis in the PALS of the spleen.

Hypersensitivity reactions









Type I	Type II	Type III	Type IV
IgE-Mediated Hypersensitivity	IgG- or IgM-Mediated Cytotoxic Hypersensitivity	Immune Complex–Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
Ag induces cross-linking of IgE bound to mast cells and basophils with release of vasoactive mediators.	Ab directed against cell surface antigens meditates cell destruction via complement activation or ADCC.	Ag-Ab complexes deposited in various tissues induce complement activation and an ensuing inflammatory response mediated by massive infiltration of neutrophils.	Sensitized T_H1 cells shown above release cytokines that activate macrophages or T_C cells that mediate direct cellular damage. T_H2 cells and CTLs mediate similar responses.
Typical manifestations include systemic anaphylaxis and localized anaphylaxis such as hay fever, asthma, hives, food allergies, and eczema.	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis, and autoimmune hemolytic anemia.	Typical manifestations include localized Arthus reaction and generalized reactions such as serum sickness, necrotizing vasculitis, glomerulonephritis, rheumatoid arthritis, and systemic lupus erythematosus.	Typical manifestations include contact dermatitis, tubercular lesions, and graft rejection.

Figure 15-1
Kuby IMMUNOLOGY, Sixth Edition

Xenobiotics & Hypersensitivity

- Polisocyanates (eg. toluene)
  - inhalation and skin
- Acid anhydrides (eg. TMA)
  - inhalation and skin
- Metals
  - platinum, nickel, beryllium
  - type IV

Xenobiotics & Hypersensitivity

- Drugs
  - 10% of all adverse effects
  - type I - IV
- Pesticides
  - contact & immediate hypersensitivity
- Cosmetics
  - contact dermatitis
- Formaldehyde
  - contact hypersensitivity

Autoimmunity

- Reflects a loss of immunologic tolerance
- Mechanisms
  - Auto-antibodies
  - Immune complex deposition
  - Sensitization of effector T cells
- Immunoregulatory abnormality most likely centered on T helper cell CD4+ T cell
  - TH1 > TH2 imbalance
- MHC
  - certain MHC alleles
- TCR
  - V beta regions

Xenobiotics and Autoimmunity

- Methyl dopa - antihypertensive
- Hydralazine, isoniazid & procainamide
  - SLE - like disease
- Halothane
  - autoimmune hepatitis
- Vinyl chloride
  - collagenous tissues

Xenobiotics and Autoimmunity

- Mercury
  - direct injury
  - autoimmune glomerular nephropathy
- Silica
  - adjuvant
- Multiple chemical sensitivity syndrome
  - ? immune component

What is the "Issue"

- Regulatory guidance on immunotoxicity
  - *CPMP: Note for Guidance on repeated dose toxicity*
  - *FDA: Guidance for industry, immunotoxicology evaluation of investigational new drugs*
- Immunotoxicity testing should be performed on all new investigational drugs or medicinal products
- Initially - gross and microscopic evaluations of lymphoid tissues

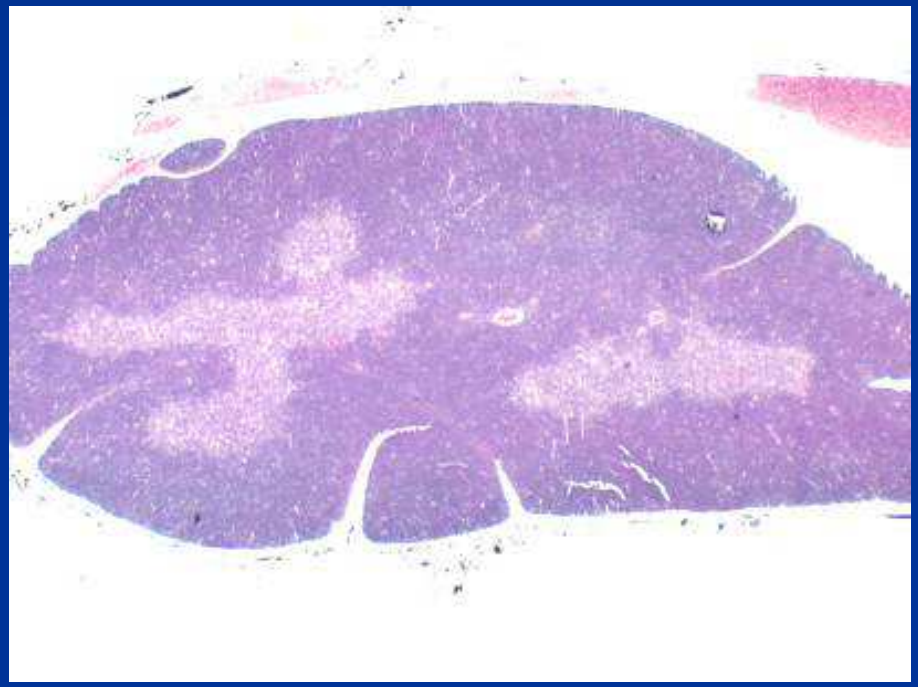
Immunopathology

- Two important requirements of the CPMP and FDA guidances
  - Lymphoid organ weights
    - ✓ Thymus and spleen
    - ✓ Draining and distant lymph nodes
  - Enhanced histopathology
    - ✓ Thymus, spleen, bone marrow, and draining and distant lymph nodes
- Standard 28-day repeat dose toxicity studies are recommended for immunotoxicity testing
- Procedures should be GLP compliant

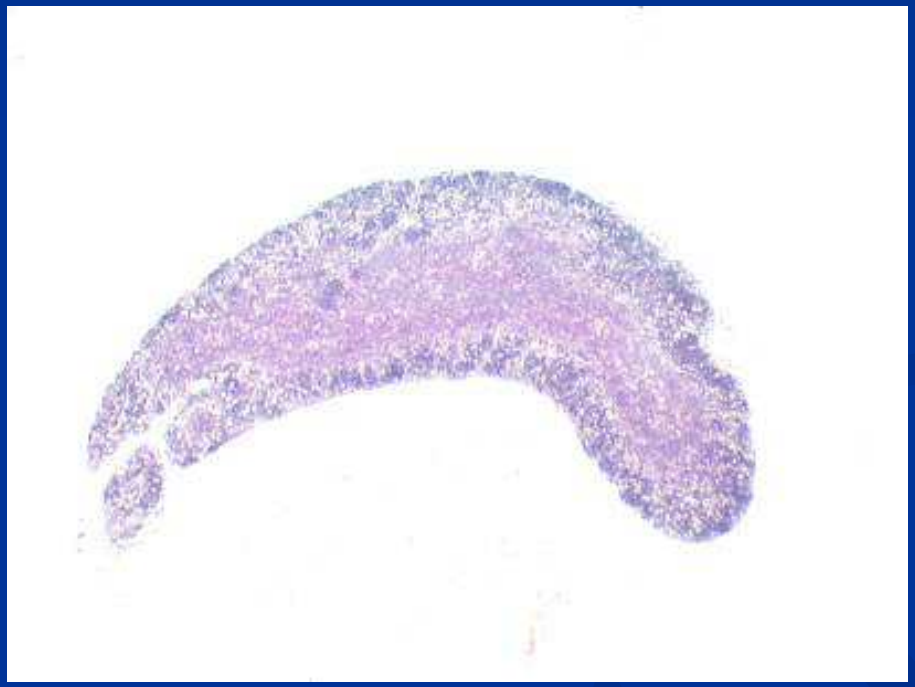
General considerations

- Initial phase – nonfunctional endpoints
- If there are indications of immunotoxicity –then specific immunological end points
- Terminology used
  - Descriptive vs interpretive
- Interpretation of the findings must take into consideration other toxicities and the health status of the animal
- Differentiating stress effects vs direct toxicities

Experimental Dexamethasone Treatment – Mouse Thymus



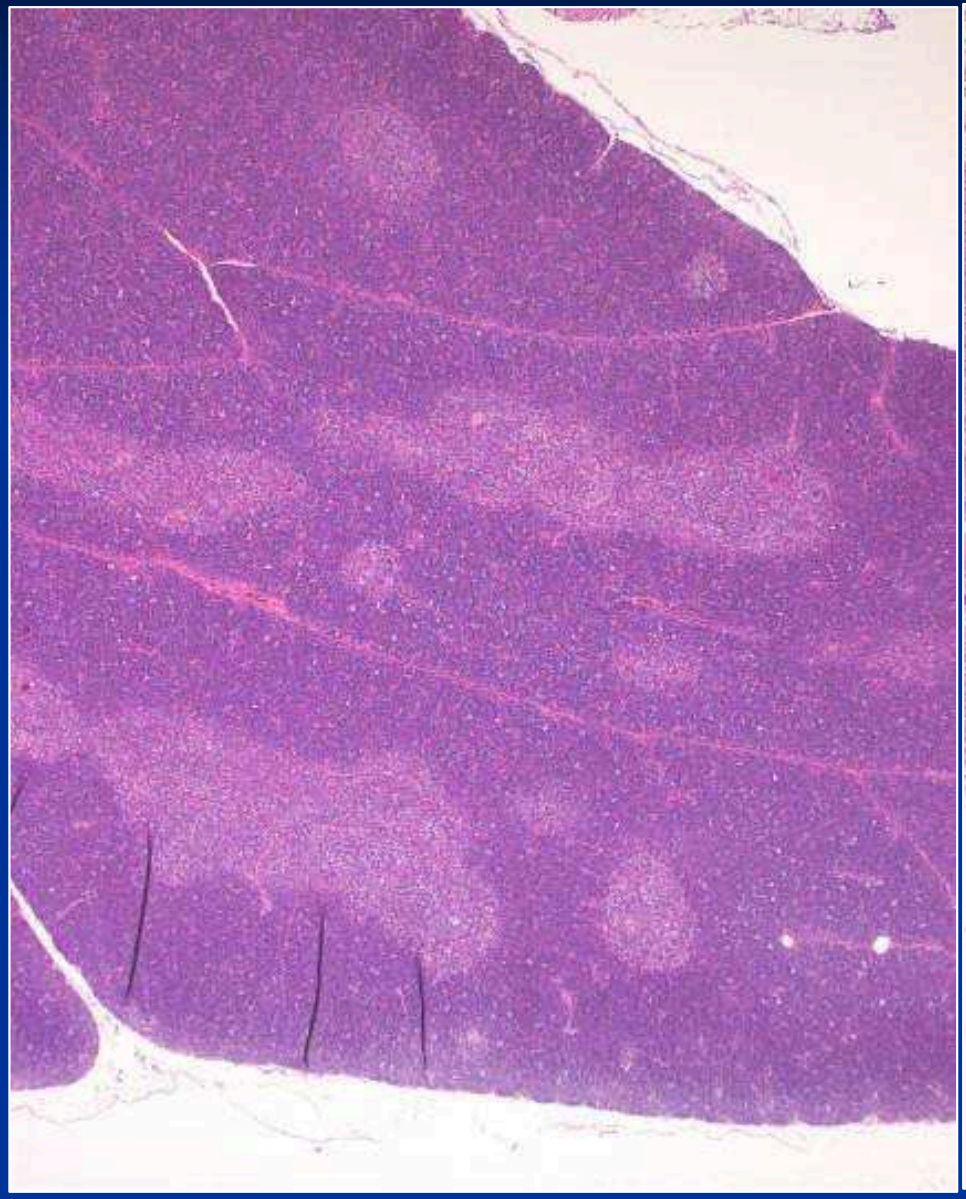
Control



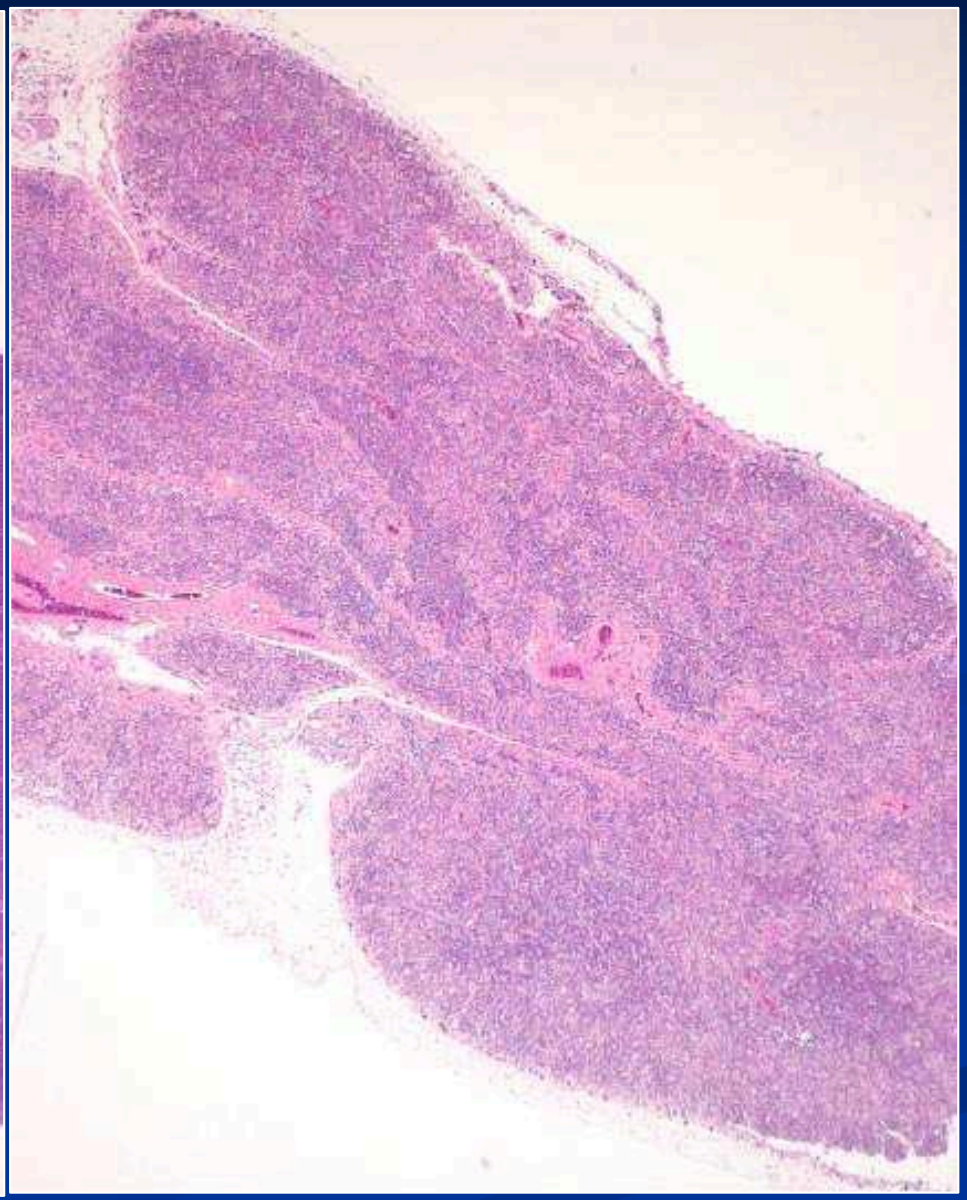
24 hr

J. Schuh, Applied Veterinary Pathobiology PLLC

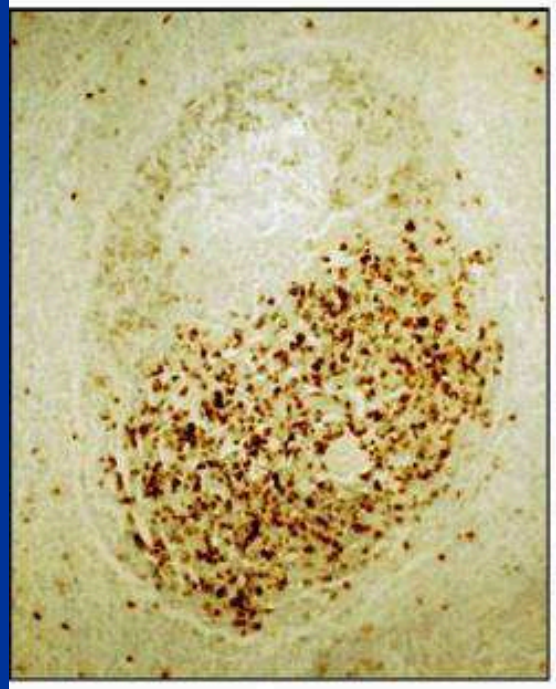
Thymus

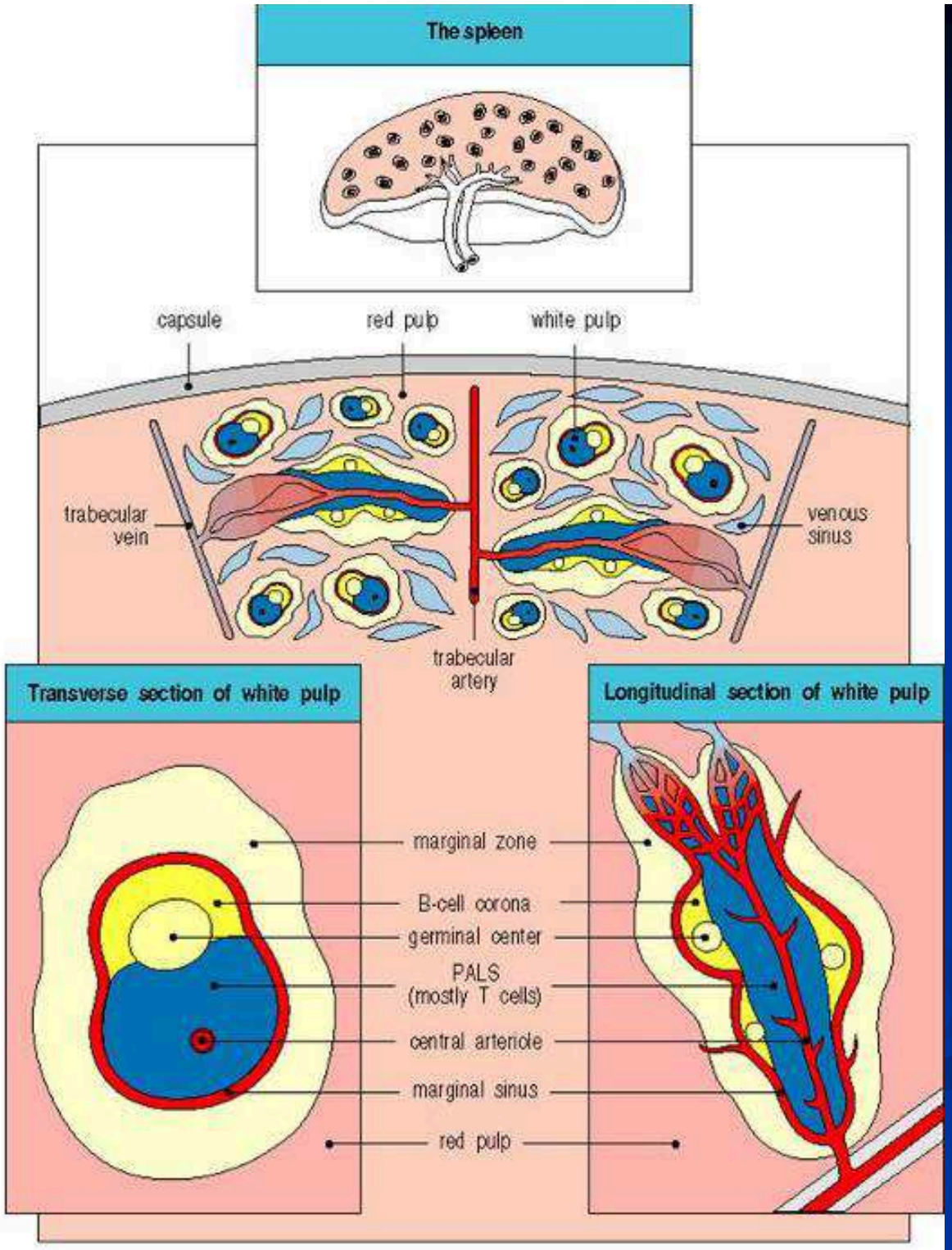


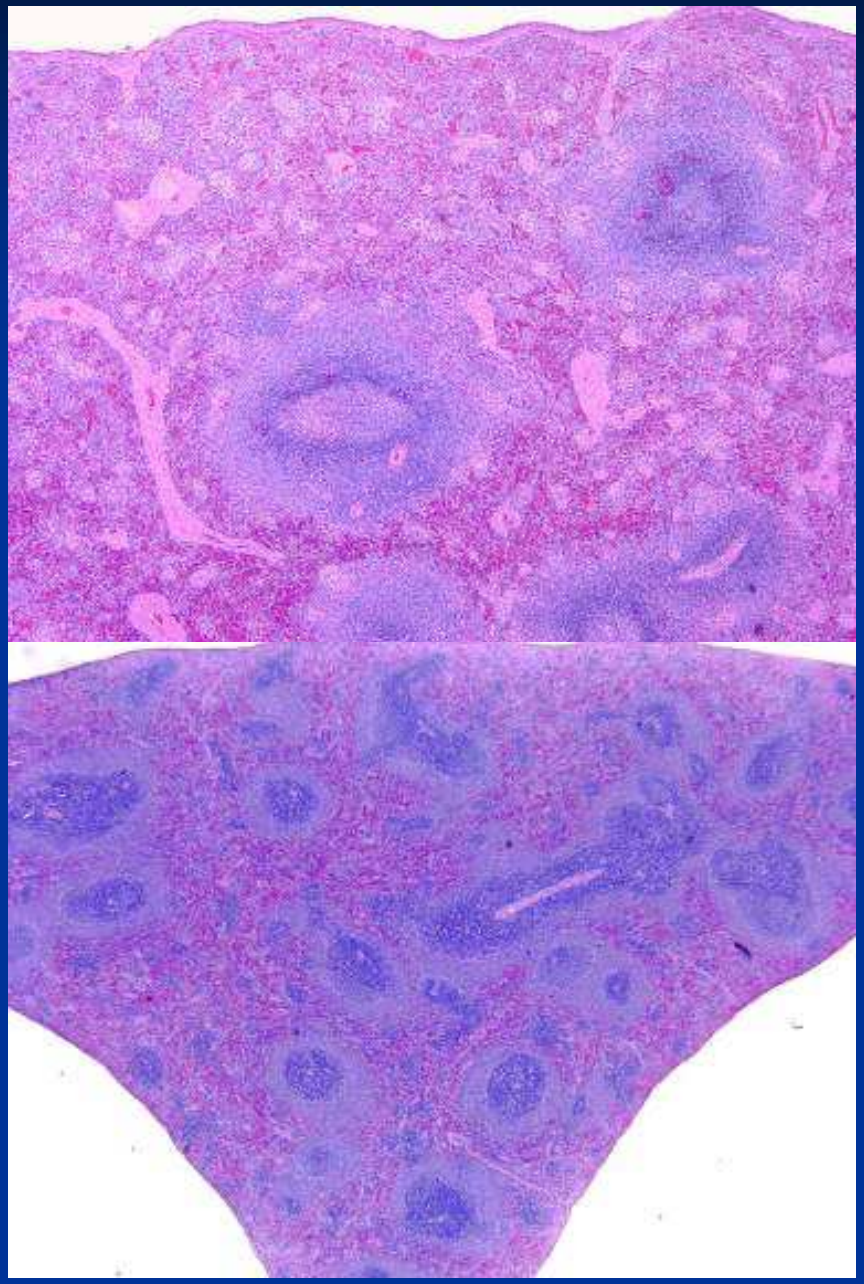
Control Rat



Treated Rat







Dog

Rat

Storage spleen

- Thick capsule and many trabeculae
- Prominent smooth muscle
- Relatively poorly developed white pulp
- Dogs, cats, horses

Defense spleen

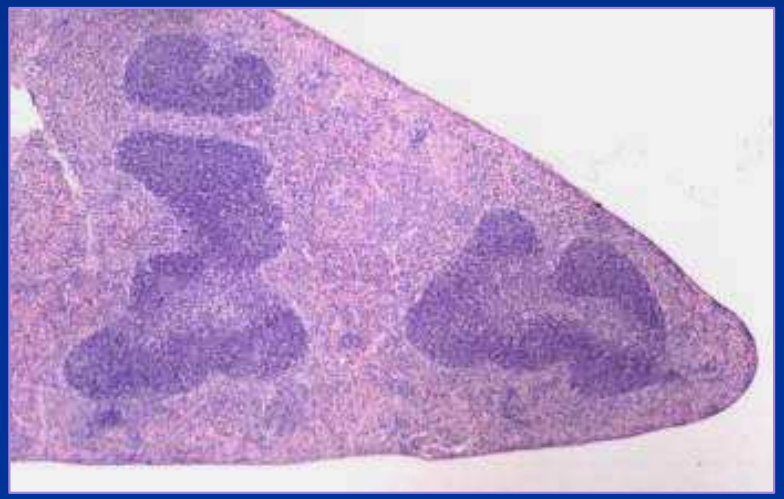
- Well developed lymphoid tissue
- Less smooth muscle
- Rats, mice, humans

Intermediate spleen

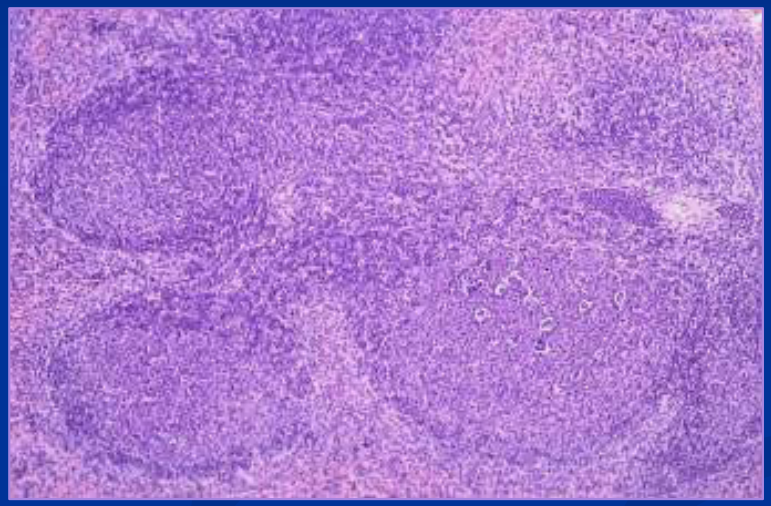
- Cattle, swine

Microarchitecture of the spleen

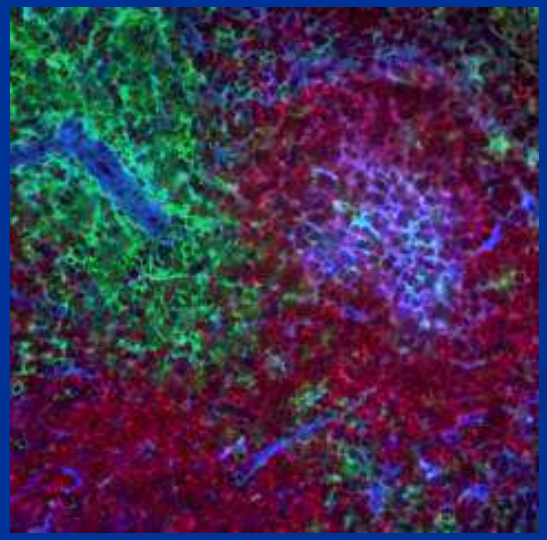
naïve (4x)



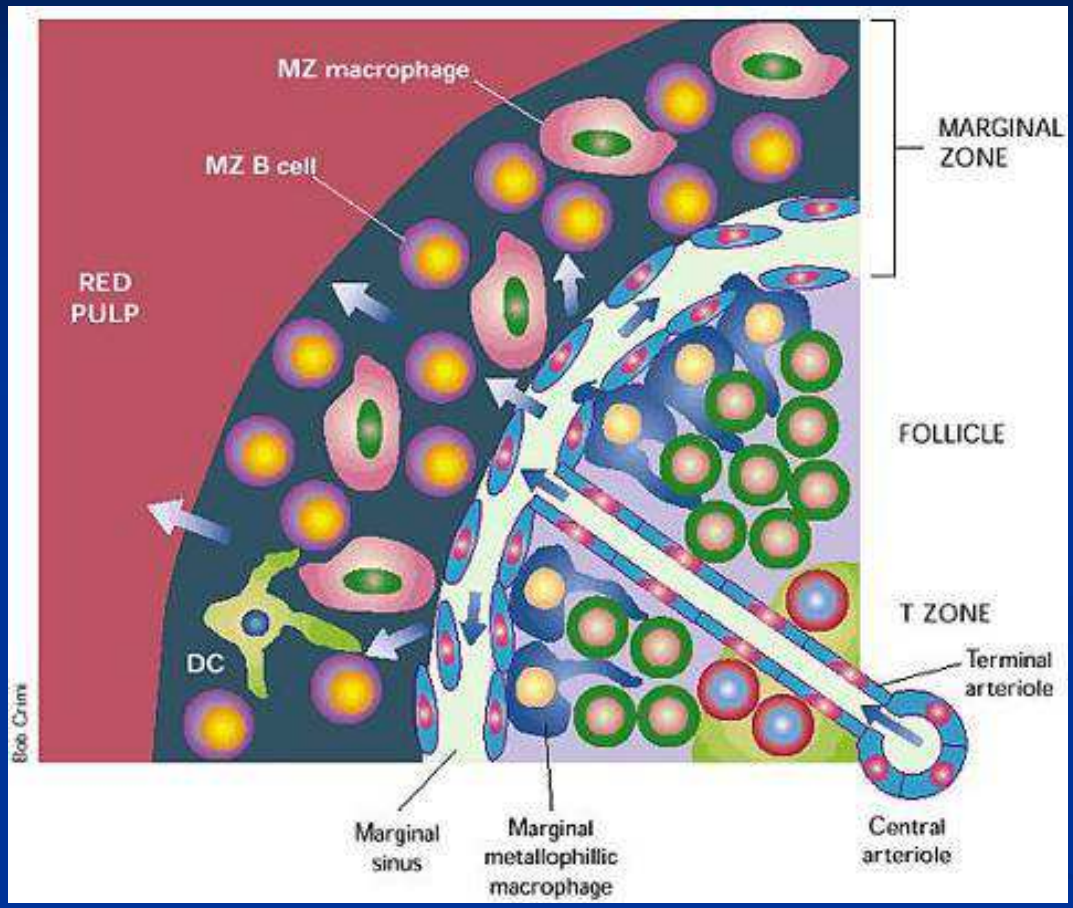
antigen-stimulated (10x)



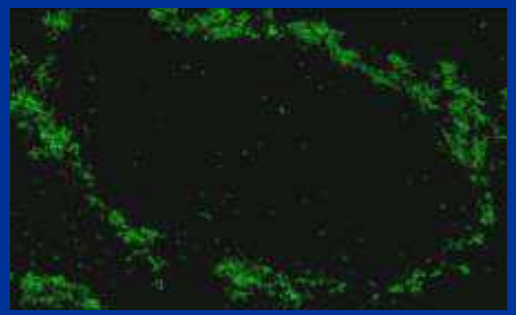
T cells
B cells
germinal center

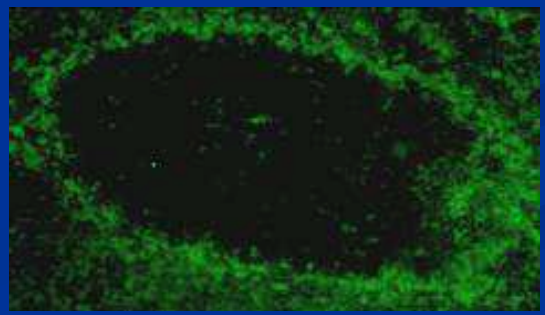


Marginal zone



ER-TR9
MZM

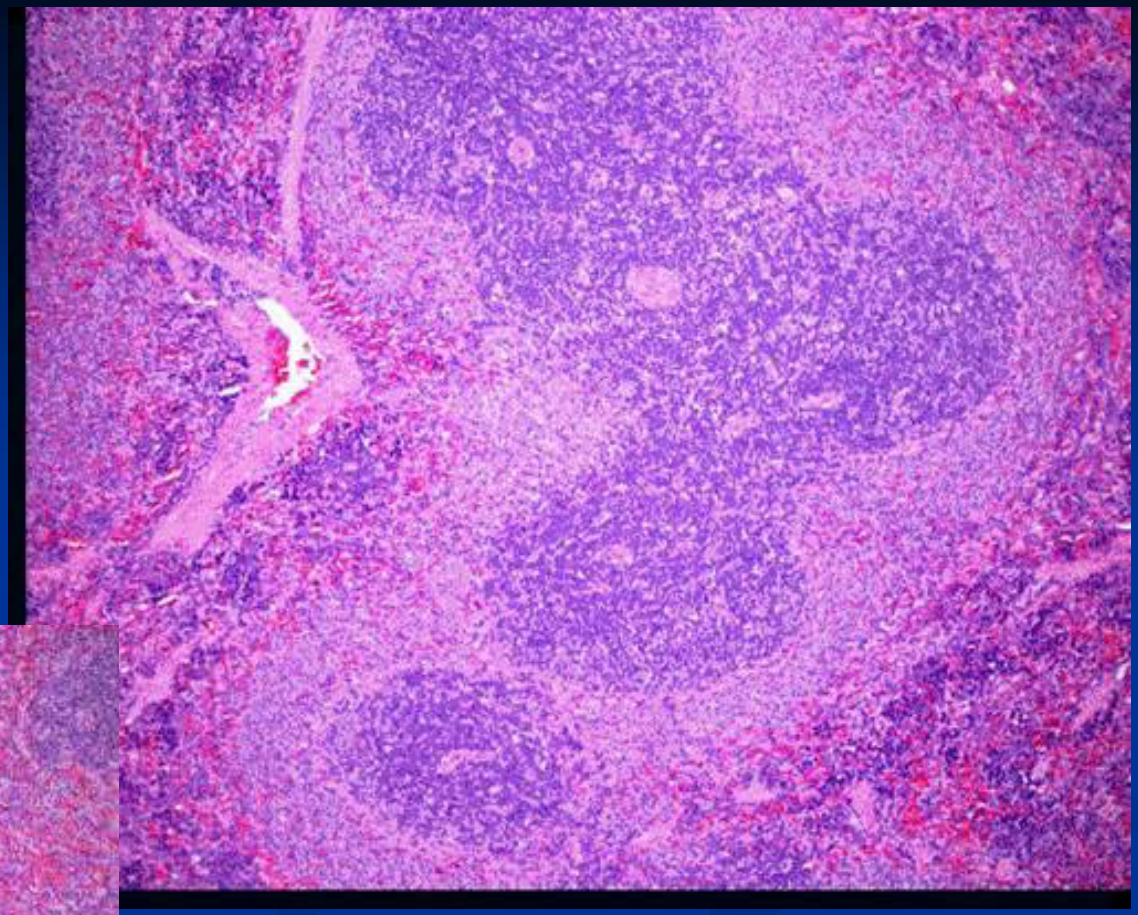


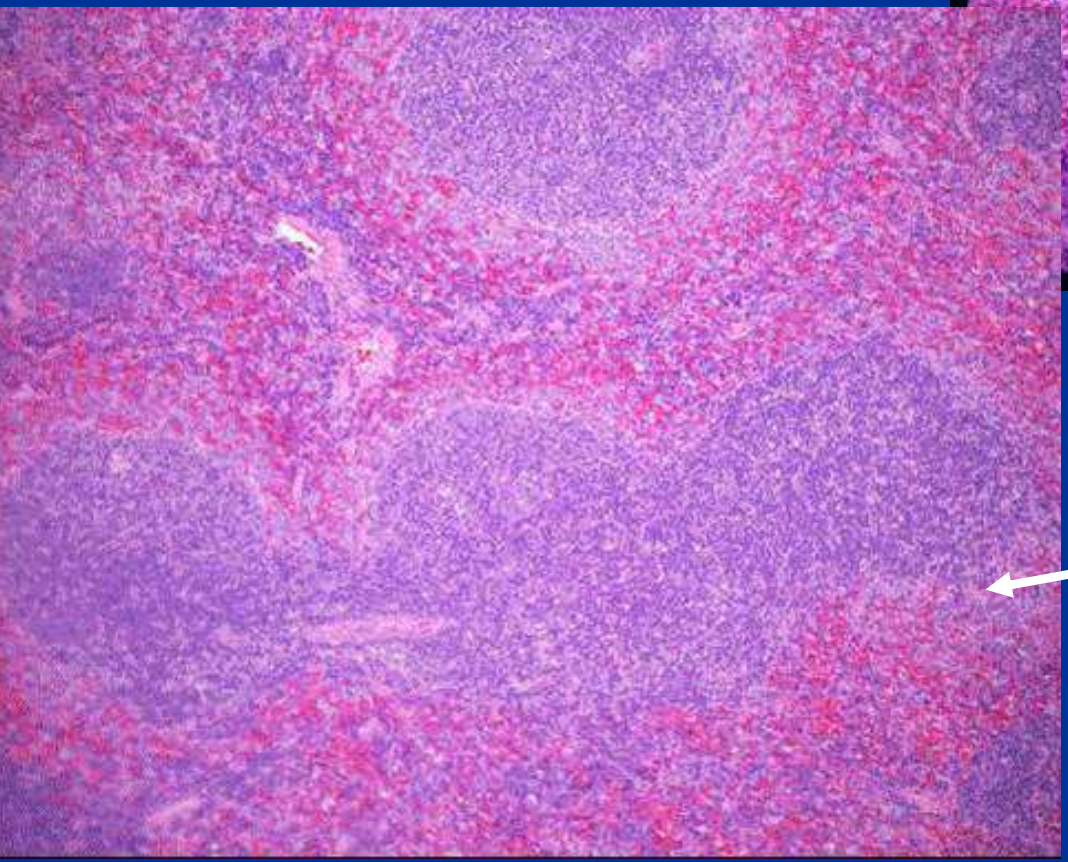


MOMA-1
(MMM)

Rat Spleen

Control 10x

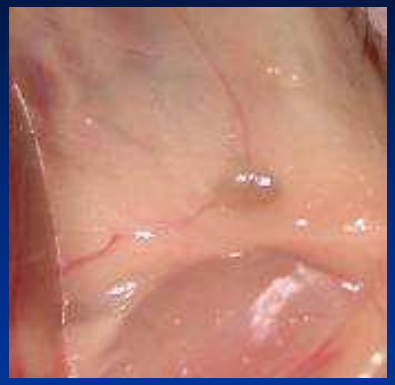




Treated 10x

Note loss of marginal zone lymphocytes

Lymph nodes - mouse



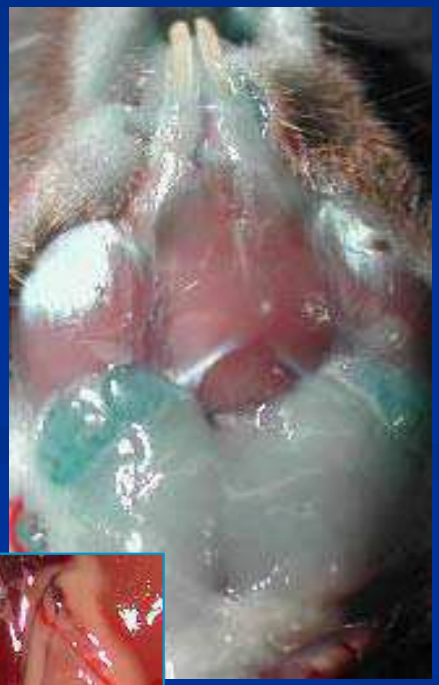
Evan's blue

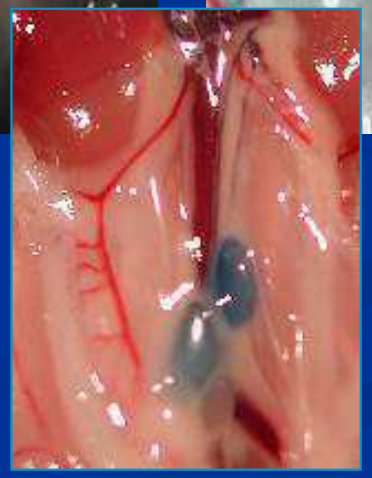
Pontamine sky blue

Inguinal LN

Renal and iliac LN

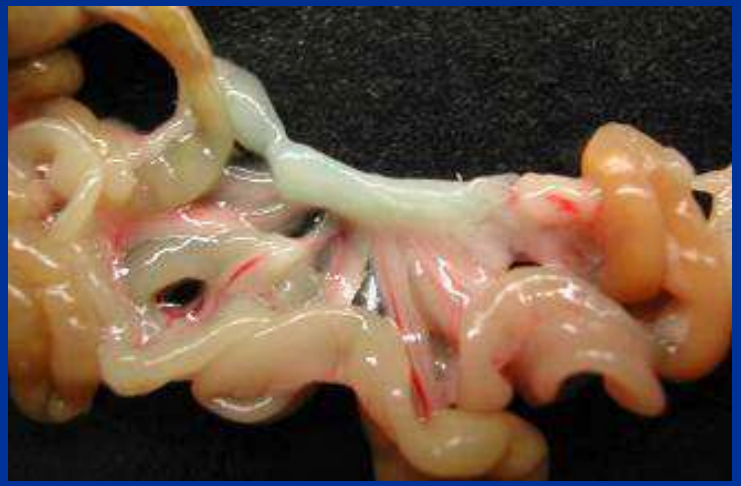
Mandibular LN

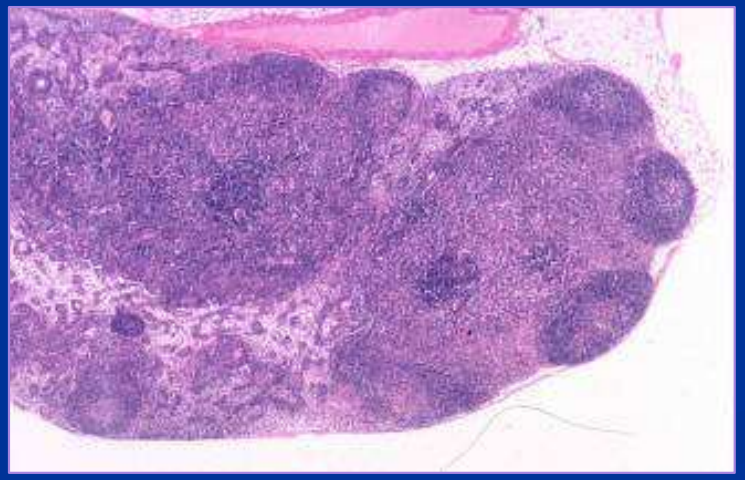




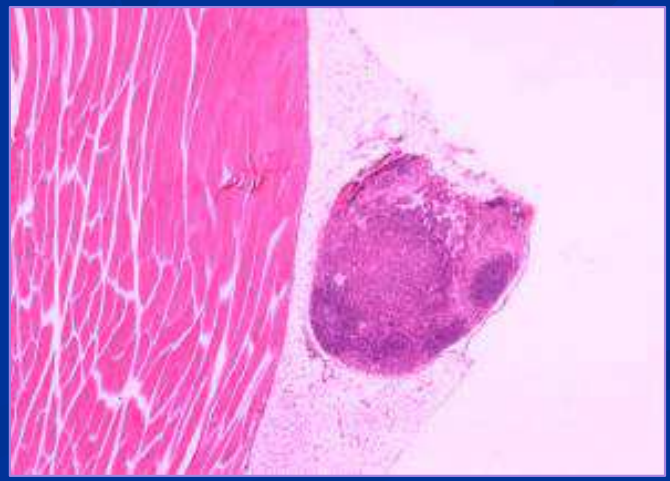
Lymph nodes

Mesenteric LN

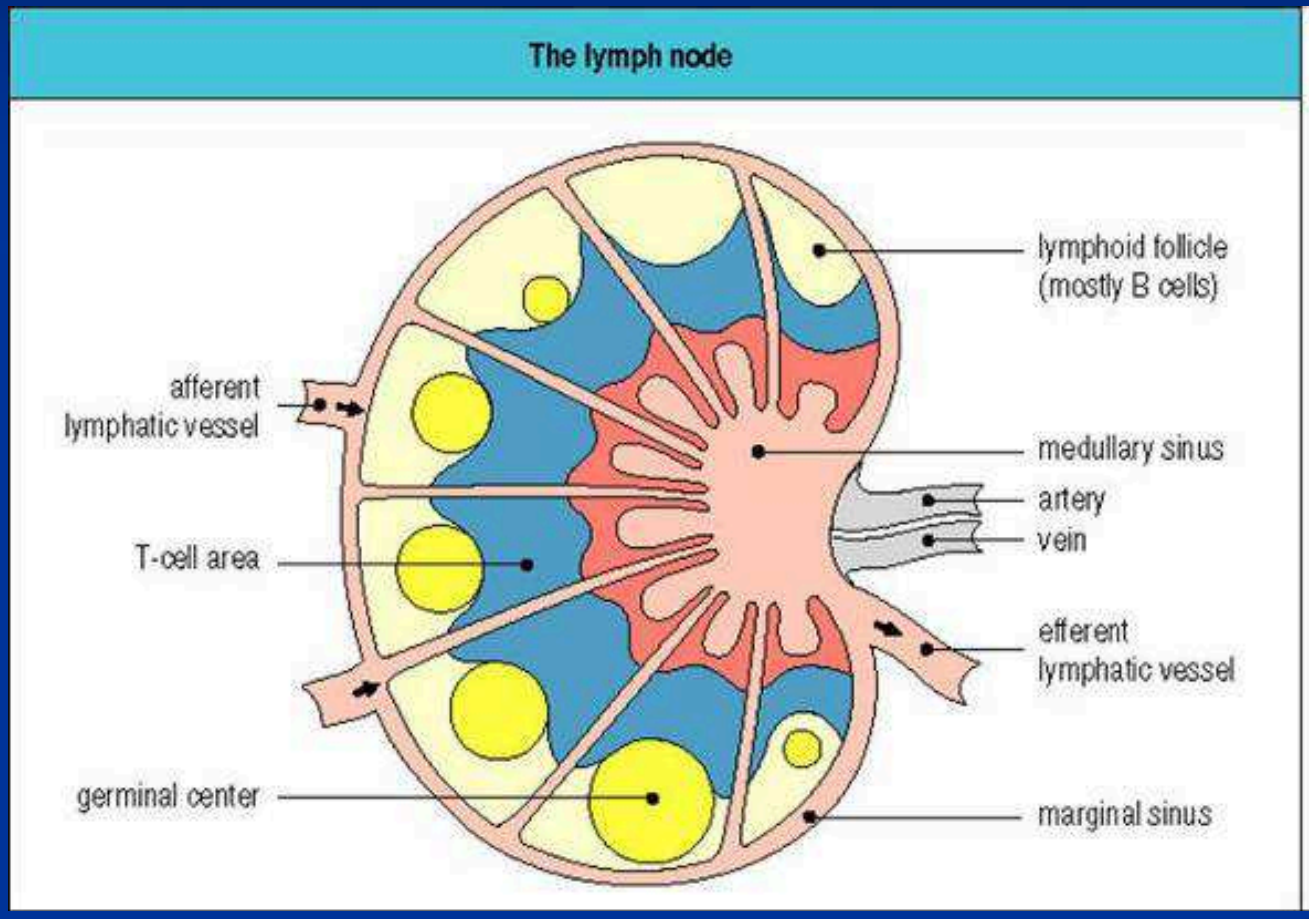


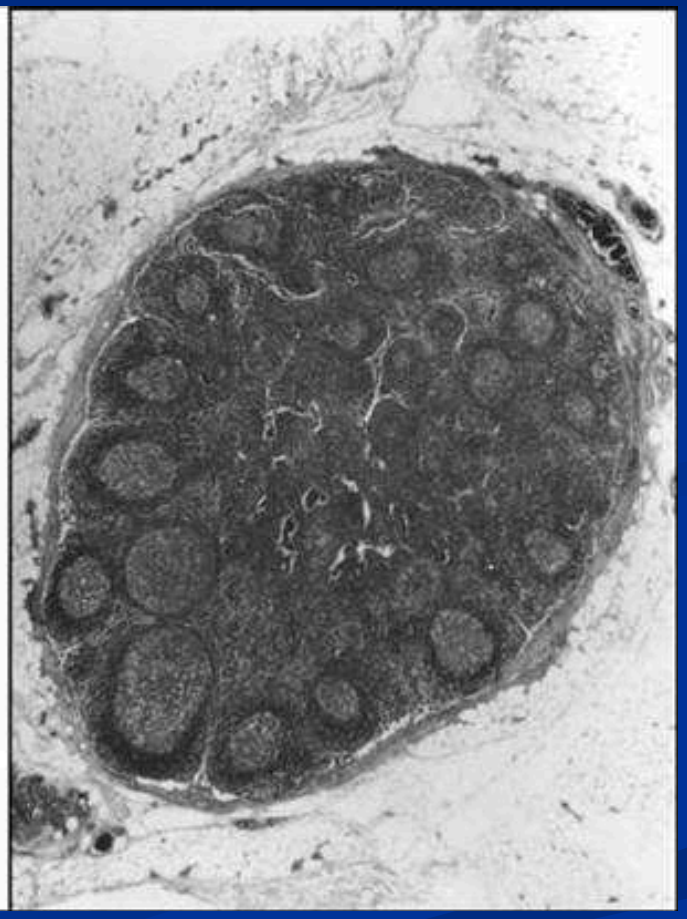


Popliteal LN

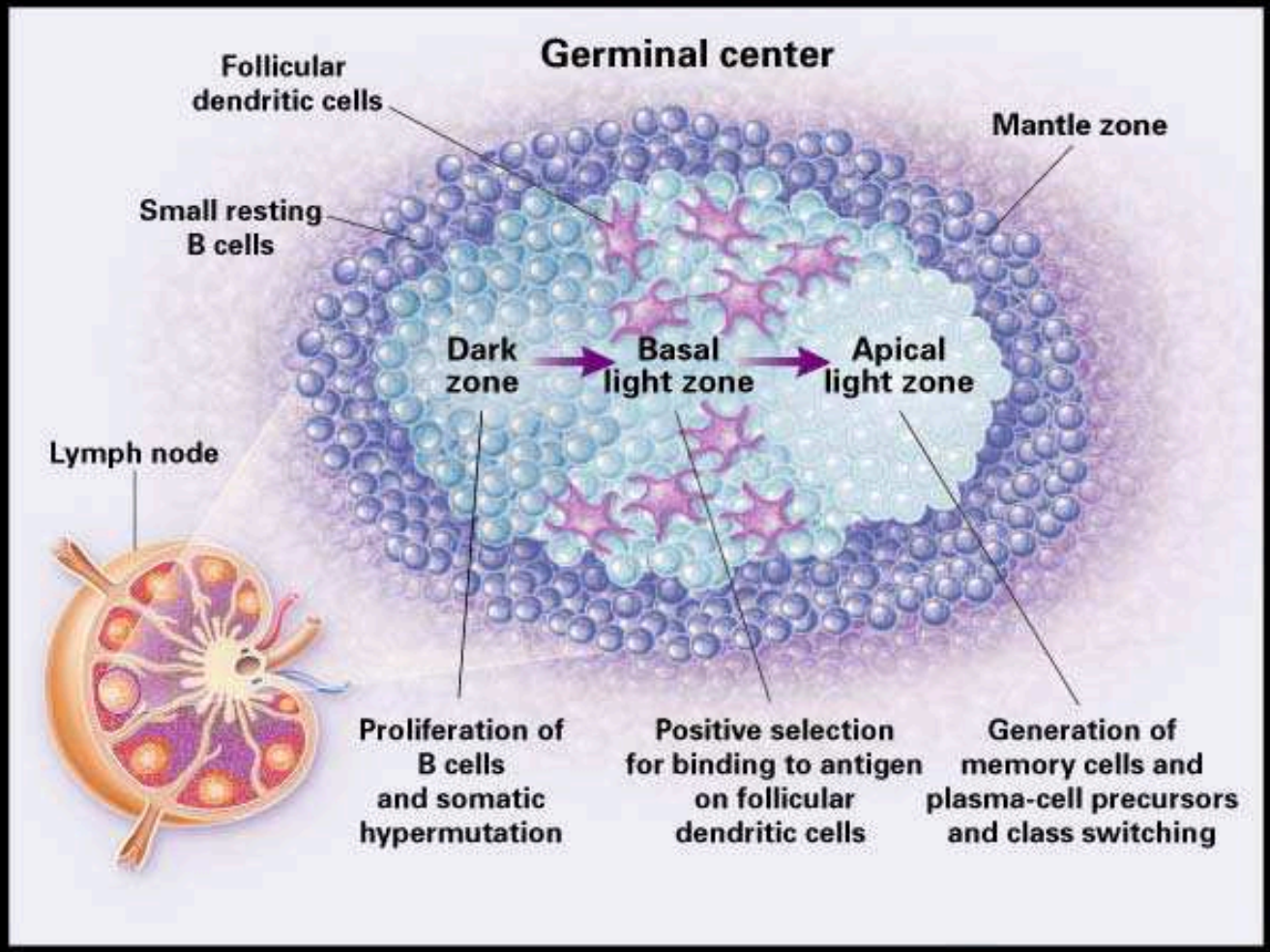


Lymph node


The lymph node
lymphoid follicle
(mostly B cells)
afferent
lymphatic vessel
medullary sinus
artery
vein
T-cell area
efferent
lymphatic vessel
germinal center
marginal sinus



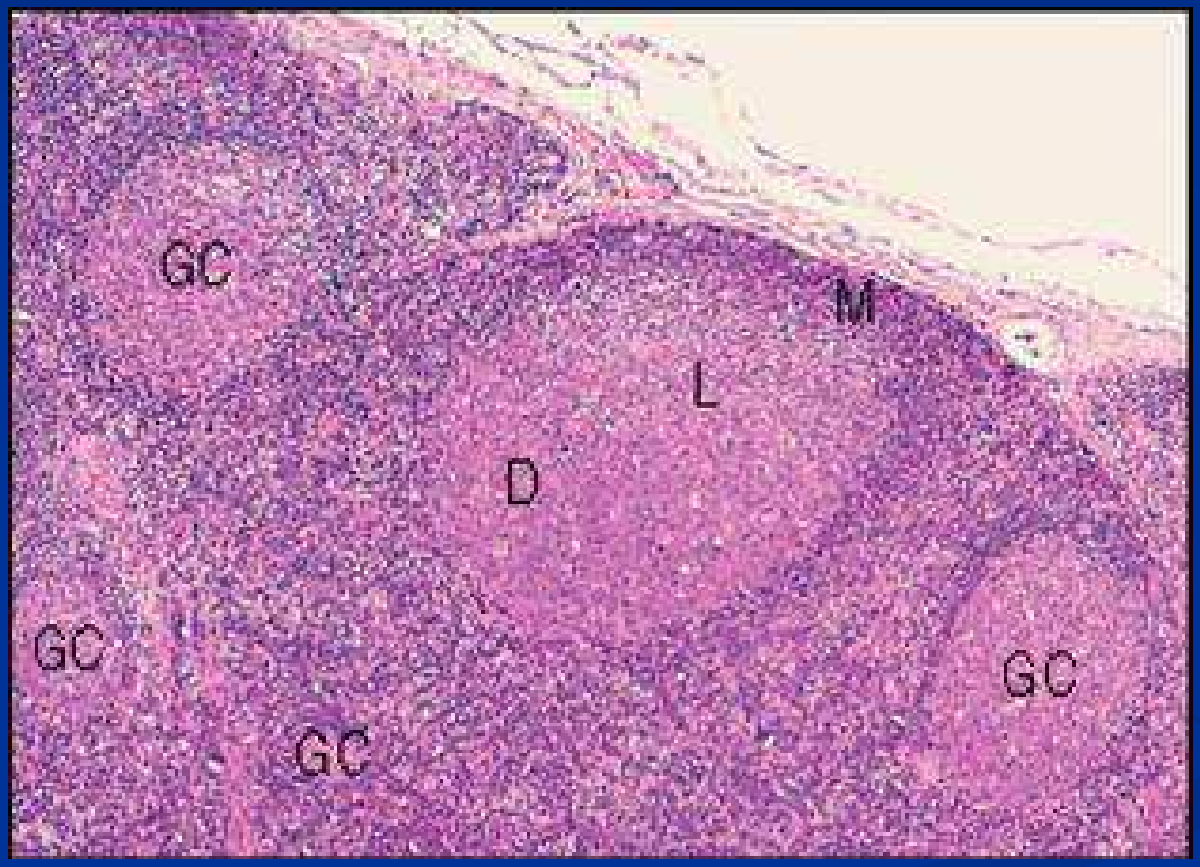
The Germinal Center



Delves PJ, Roitt IM. The Immune System (Part 2). N Engl J Med 2000;343:108-17.

The New England Journal of Medicine

Lymphoid follicles



Lymph node histology

- Extreme variability:
  - Species
  - Strain
  - Husbandry/environment
  - Location
  - Age

Best Practice Guideline for the Routine Pathology Evaluation of the Immune System

STP Immunotoxicology Working Group

P. Haley[1] (chair), R. Perry[2] (cochair), D. Ennulat[3],
S. Frame[4], C. Johnson[5], J-M Lapointe[6],
A.Nyska[7], P. Snyder[8], D.Walker[2], G. Walter[9]

Toxicologic Pathology, 33:404–407, 2005
(http://www.toxpath.org/Position_Papers/Immune_System.pdf)

[1]AstraZeneca, Wilmington DE; [2]Wyeth Research, Chazy NY; [3]GlaxoSmithKline, King of Prussia, PA [4]DuPont Company, Haskell Lab for Environmental Sciences Toxicology & Industrial Medicine, Newark DE; [5]Amgen, Inc. Thousand Oaks, CA [6]Pfizer Global Research & Development, Groton CT; [7]National Institute of Environmental Health Sciences, Research Triangle Park, NC; [8]Purdue University School of Veterinary Medicine, West Lafayette, IN; [9]Independent Consultant, Kalamazoo

Best Practices:Immunopathology

- **Collection and Weighing of Lymphoid Tissue**
  - Recording and evaluating thymic and splenic weights should be continued
  - Interpretation of these organ weights should only be done in the context of all other clinical, histopathology, and clinical pathology data from the study
  - Alterations of spleen and thymus weights (along with histopathology) are reasonable indicators of systemic immunotoxicity
  - Spleen and thymus weights are likely to be more reliable indicators than are changes in the weight of peripheral lymph nodes

Best Practices: Immunopathology

- Routine Best Practice for Histopathologic Examination of Lymphoid Tissues as Indicators of Systemic Immunotoxicity
  - Each animal should receive a thorough macroscopic examination of the the spleen, thymus and lymph nodes
  - Thymus, spleen, draining lymph nodes, bone marrow *in situ*, and any gross lesions of a lymphoid organ represent the minimum of tissues for routine evaluation of the lymphoid system

Best Practices:Immunopathology

- Routine Best Practice for Histopathologic Examination of Lymphoid Tissues as Indicators of Systemic Immunotoxicity
  - The most proximal regional lymphoid tissues that drain the drug application site can and should be examined microscopically
  - Orally given drugs: Peyer's patches and mesenteric lymph nodes
  - The most proximal draining peripheral lymph nodes is appropriate in cases of cutaneous, subcutaneous, or intradermal application

Best Practices: Immunopathology

- Alterations of spleen, thymus, and bone marrow histology are likely to be more reliable indicators of systemic immunotoxicity than are changes in distal peripheral lymph nodes

Best Practices:Immunopathology

- **Semi-Quantitative Description of Lymphoid Tissue Changes**
  - 'Best Practice' for lymphoid tissue microscopic examination involves "a semi-quantitative description of changes in compartments and/or microenvironments of specified lymphoid organs."
    1) each lymphoid organ has separate compartments that support specific immune functions
    2) these compartments can and should be evaluated individually for changes
    3) descriptive, rather than interpretative terminology, should be used to characterize changes within these compartments

Recommendations

- Morphological and functional compartments specific to each tissue
- Each compartment should be evaluated for substantive changes
- Substantive changes should be reported using standardized descriptive nomenclature rather than interpretative terminology
- Example
  - "thymus, cortex, decreased lymphocytes, marked" would be preferable to "thymic involution"

Immunopathology

Descriptive vs. Interpretative Terms

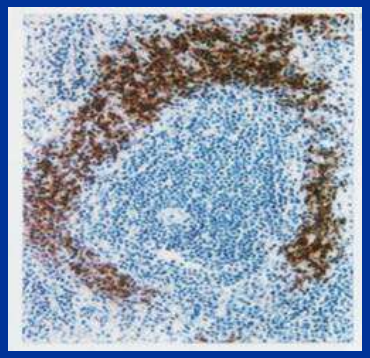
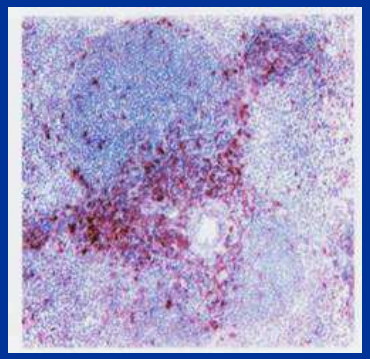
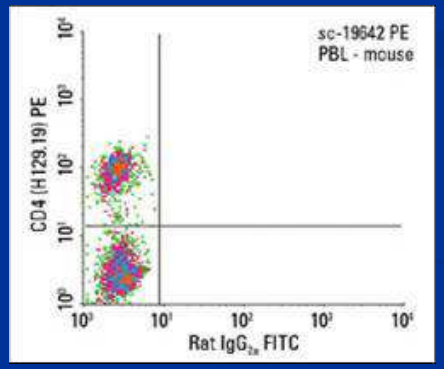
Descriptive	Interpretative
Decreased cellularity	Atrophy Lymphoid depletion Involution Hypoplasia
Increased cellularity	Hypertrophy Hyperplasia Proliferation

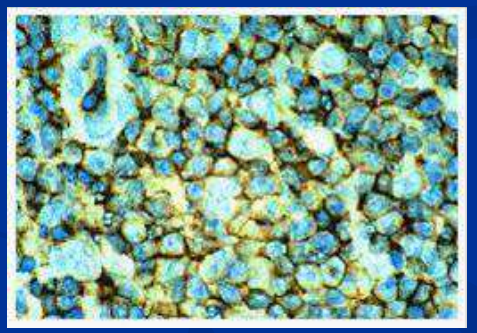
Recommendations

- Changes observed in the lymphoid tissues should be interpreted in the context of all the findings
- Interpretation of lymphoid findings should be in the Discussion section of the report

Best Practices: Immunopathology

- Specialized techniques are done AFTER the initial assessment shows a change has occurred
  - Lymphoid tissue immunohistochemistry
  - Blind scoring of lymphoid tissues
  - Morphometry of lymphoid tissues
  - Flow cytometry of lymphoid tissue cell suspensions



- These procedures should be directed at answering a specific scientific question; they should <u>not</u> be used as routine screening tools.