Diagnostic and Prognostic Immunohistochemistry of Neoplastic Diseases

Dr. med. vet. Matti Kiupel, MS, PhD, DACVP
Associate Professor, Section Chief Anatomic Pathology
Michigan State University
Diagnostic Center for Population and Animal Health
4125 Beaumont Road, Room 152A
Lansing, MI 48910, USA

Tel.: ** 517 432 2670
E-mail: kiupel@dcpah.msu.edu

Setting the Rules

- Never use IHC to diagnose a neoplasm without a solid morphological diagnosis based on the H&E!
- Always read the IHC in association with the H&E!
- Never read an IHC without the proper controls!
- Never base your diagnosis on the absence of staining only!
- Use antibody panels rather than single tests!
- Know your antibodies!
- Don’t be dogmatic, remember it’s a tumor!

Canine Round Cell Tumors

- Malignant Lymphoma
- Plasma Cell Tumors/Multiple Myeloma
- Mast Cell Tumors
- Cutaneous Histiocytoma
- Langerhans Cell Histiocytosis
- Cutaneous Reactive Histiocytosis
- Systemic Reactive Histiocytosis
- Histiocytic Sarcoma
- Periarticular Histiocytic Sarcoma
- Hemophagocytic Histiocytic Saroma
- Transmissible Venereal Tumor
- Melanoma

Obvious

Secrets

Confusing

Cluster Differentiation (CD) Designation for Surface Antigens of Canine Hematopoietic Cells

- **Primarily T-cell associated:**

CD3: thymocytes, T-cells, NK-cells, most T-cell lymphomas

CD4 (fresh tissue?): help/inducer subpopulation of T-cells

CD8 (fresh tissue?): suppressor/cytotoxic subpopulation of T-cells

- **Primarily B-cell associated:**

CD79a: appears in early B-cell maturation

CD20: in B-precursor cells throughout maturation

Bla.36: early/activated B-cells, Reed-Sternberg (mononuclear) cells

- **Present on all leukocytes:**

CD18: leuko-integrin beta 2 subunit

CD45: leukocyte common antigen

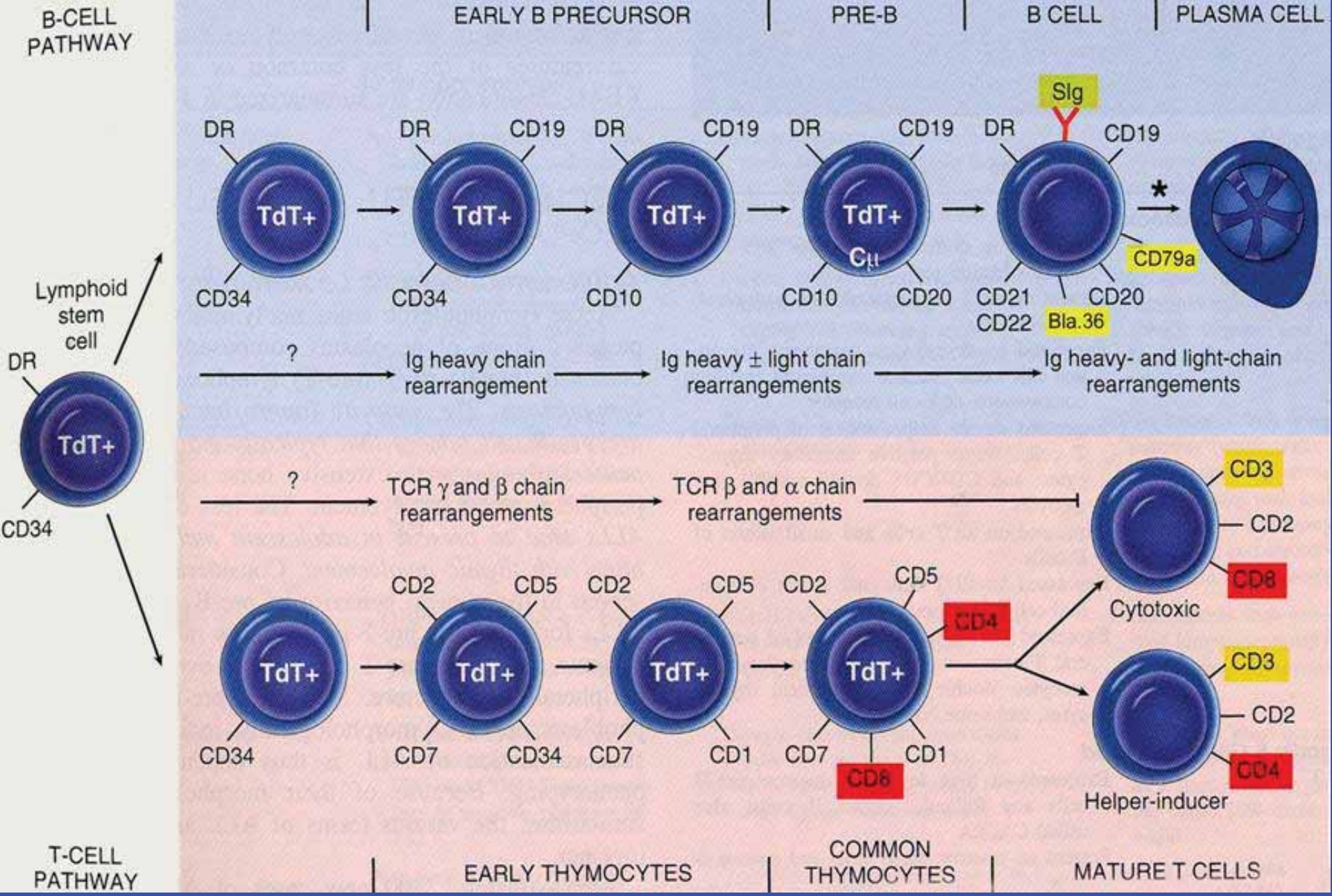
CD45RA: leukocyte common antigen restricted isoform (not histiocytes)

- **Macrophages**

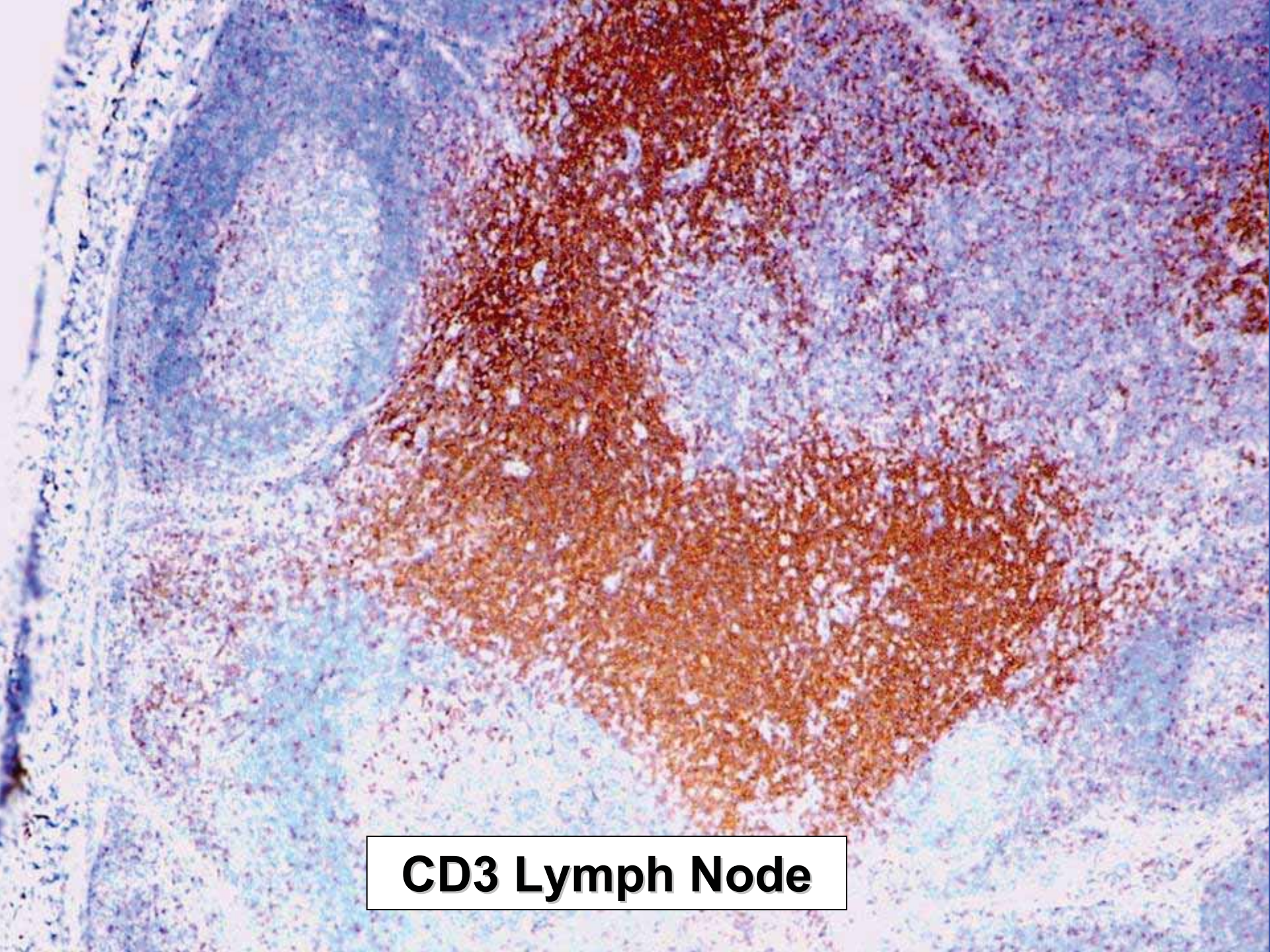
CD11d

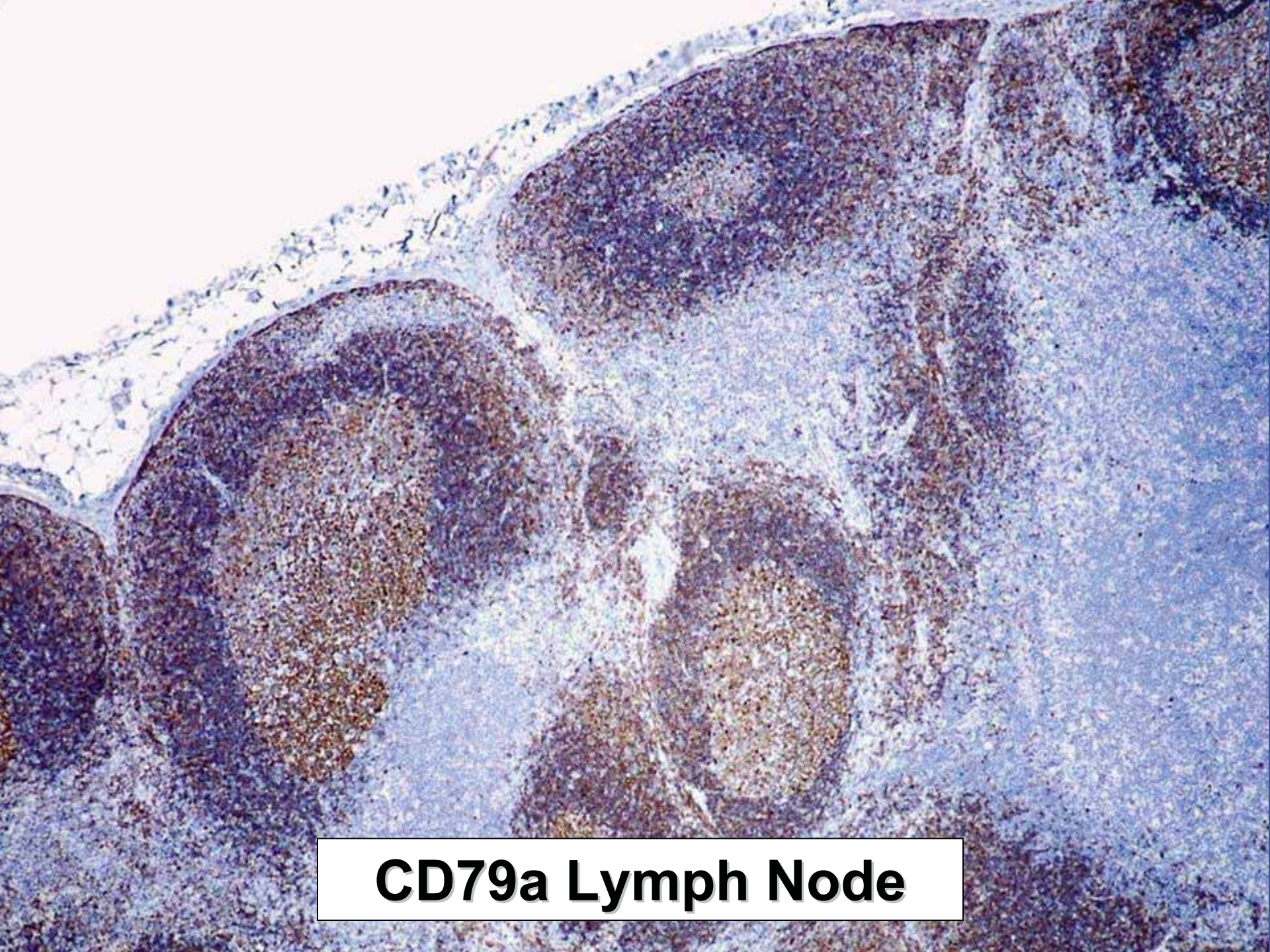
- **Megakaryocytes**

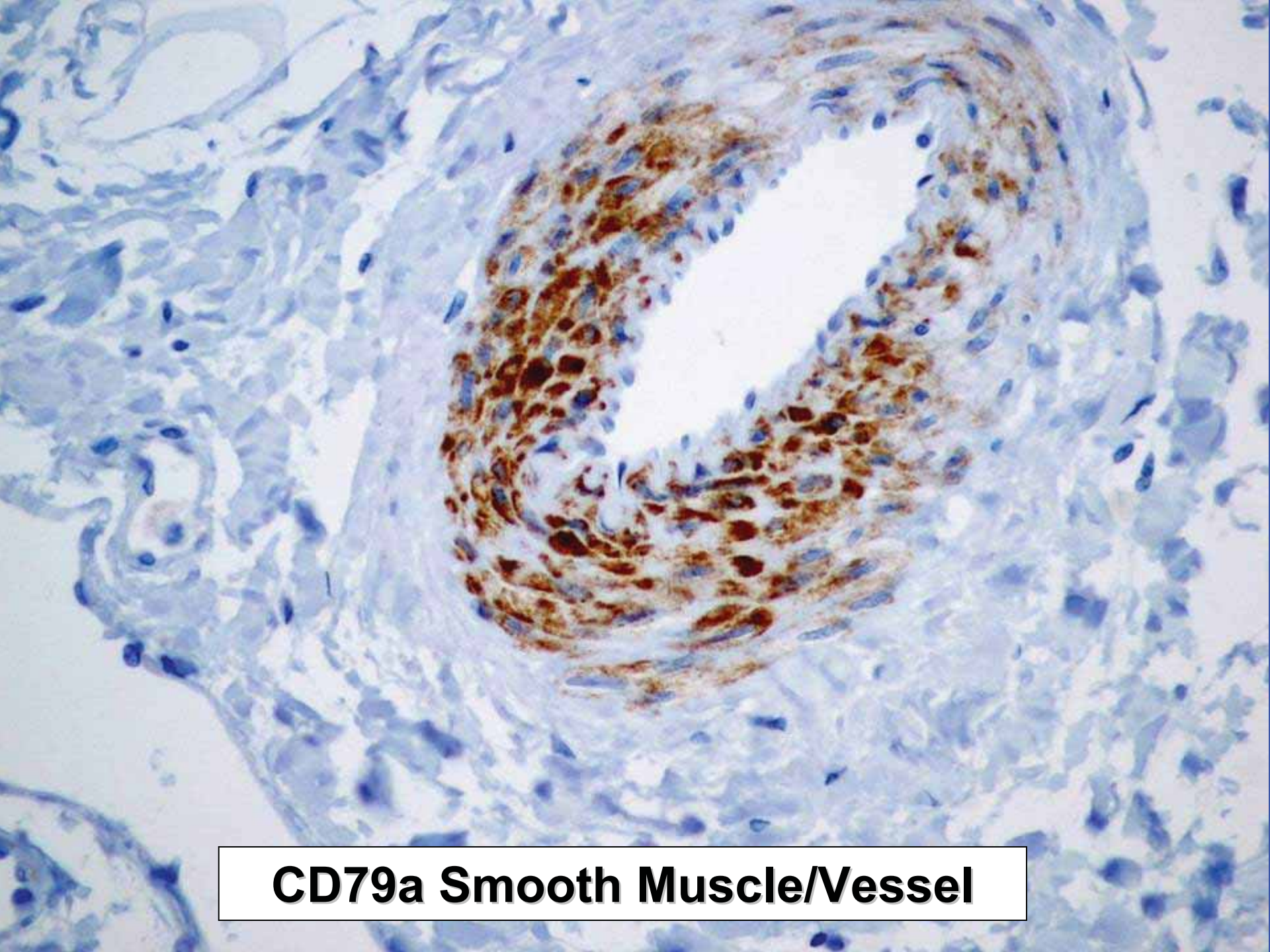
CD61

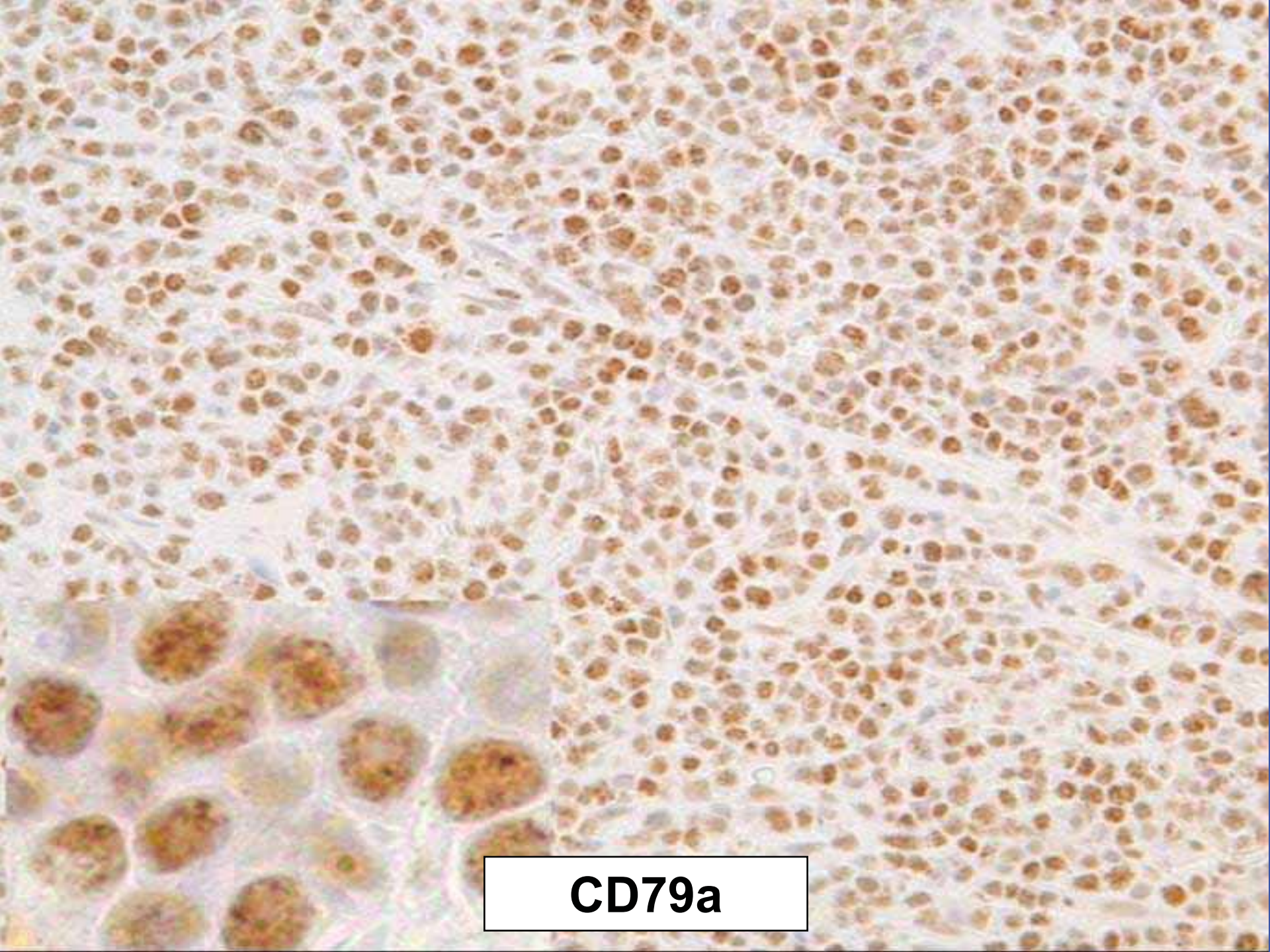


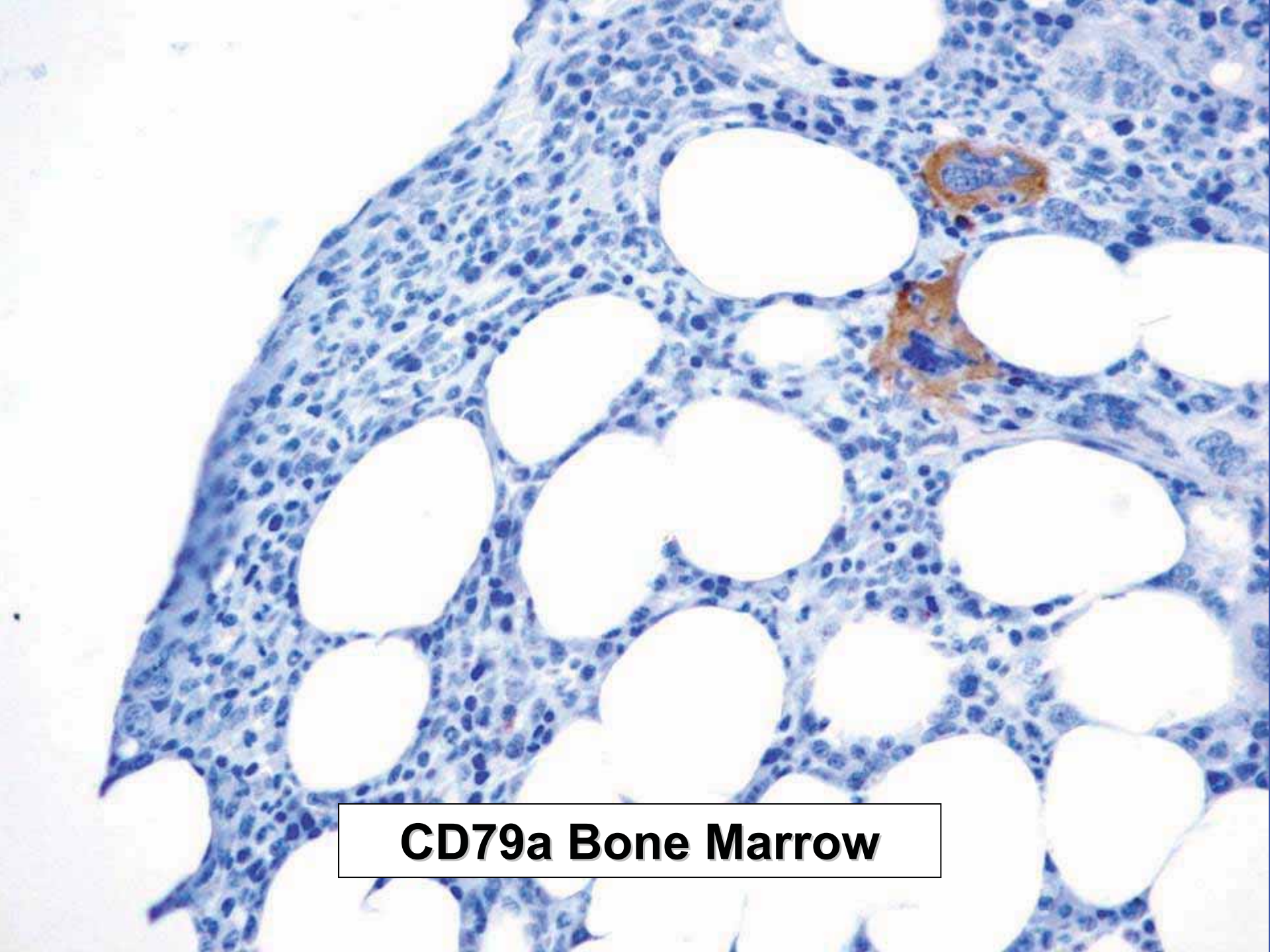
Phenotypic and genotypic changes associated with differentiation of B- and T-cells (Robbins et al., 1999)

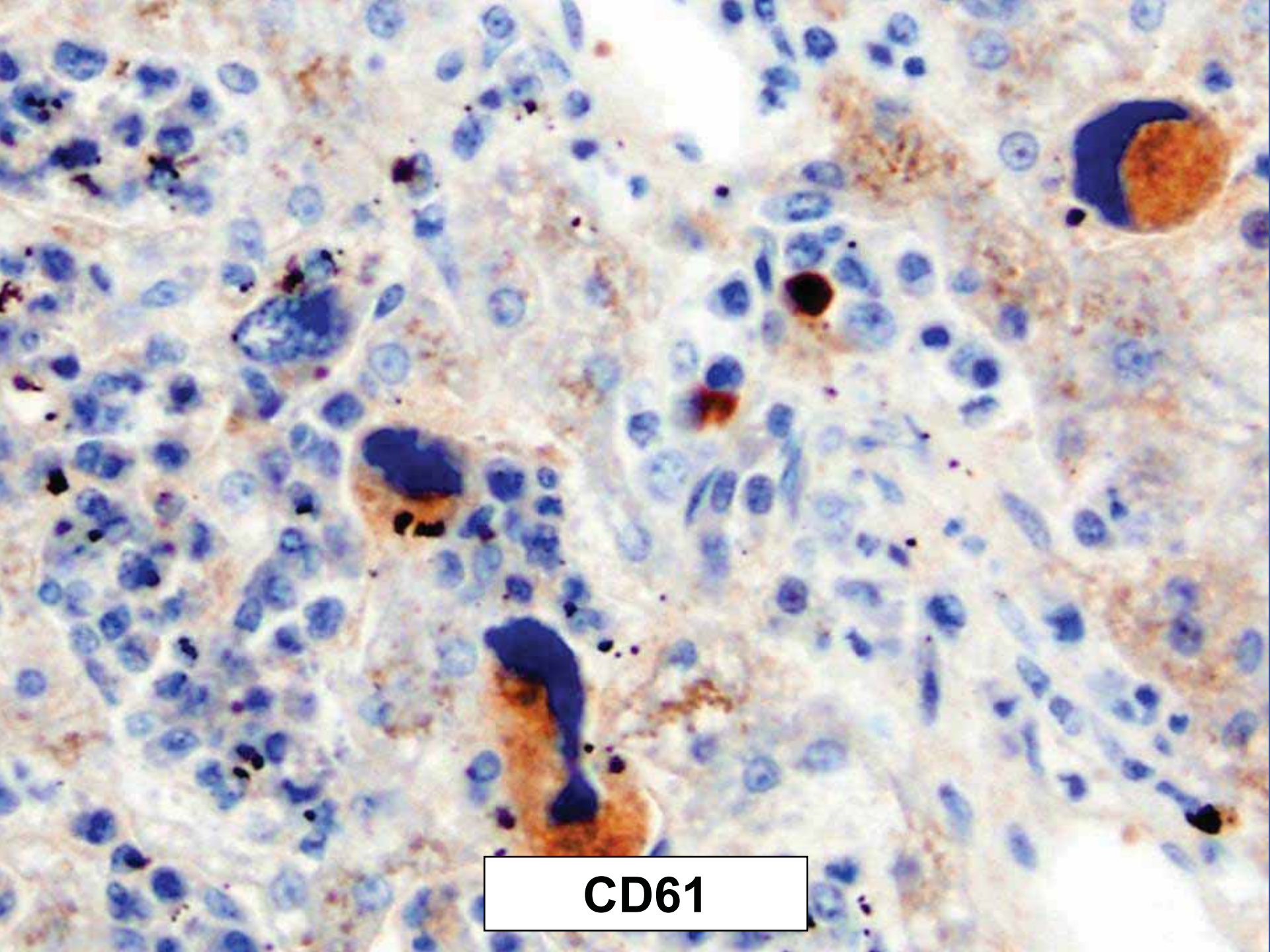

CD3 Lymph Node

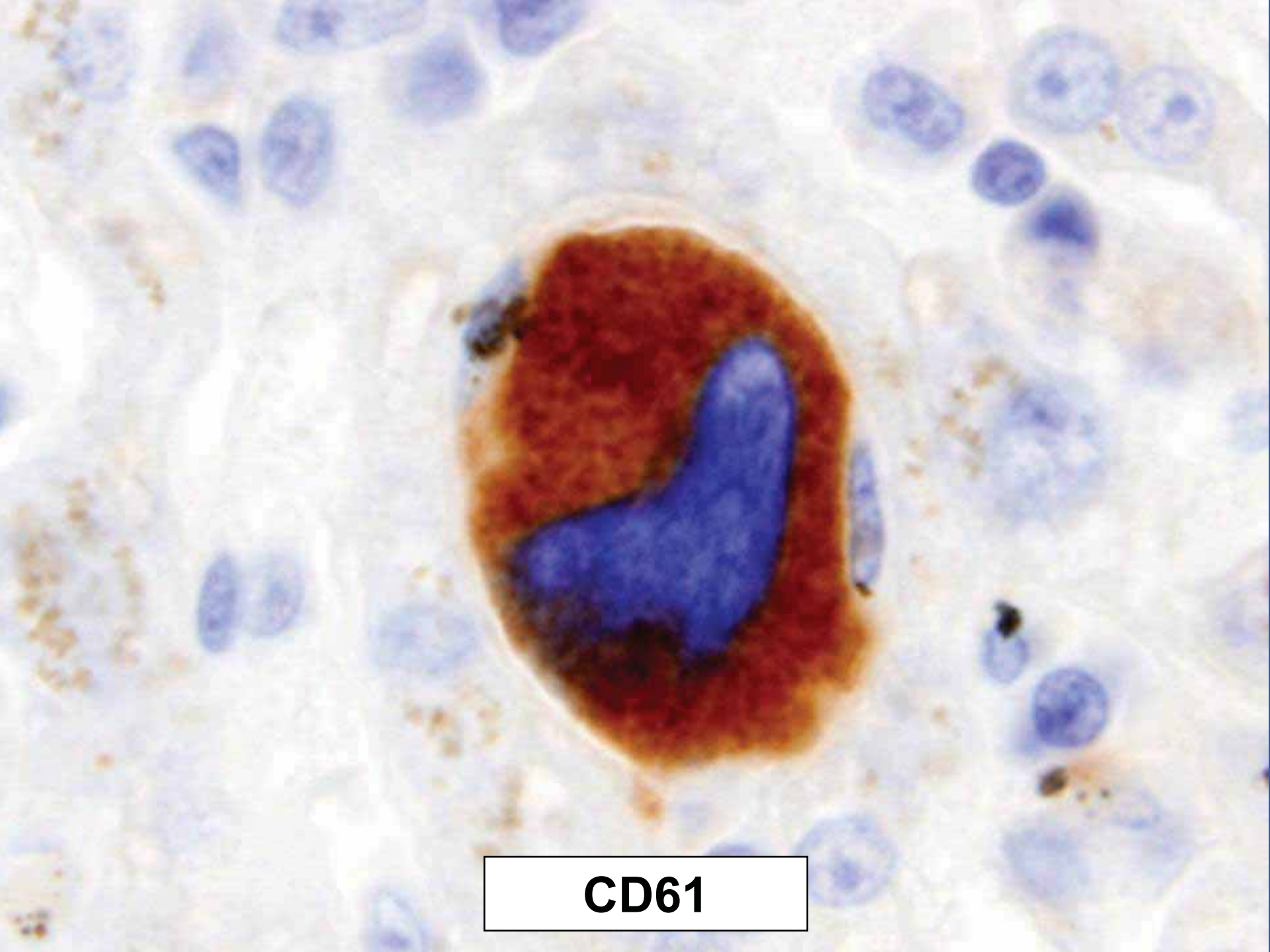

CD79a Lymph Node

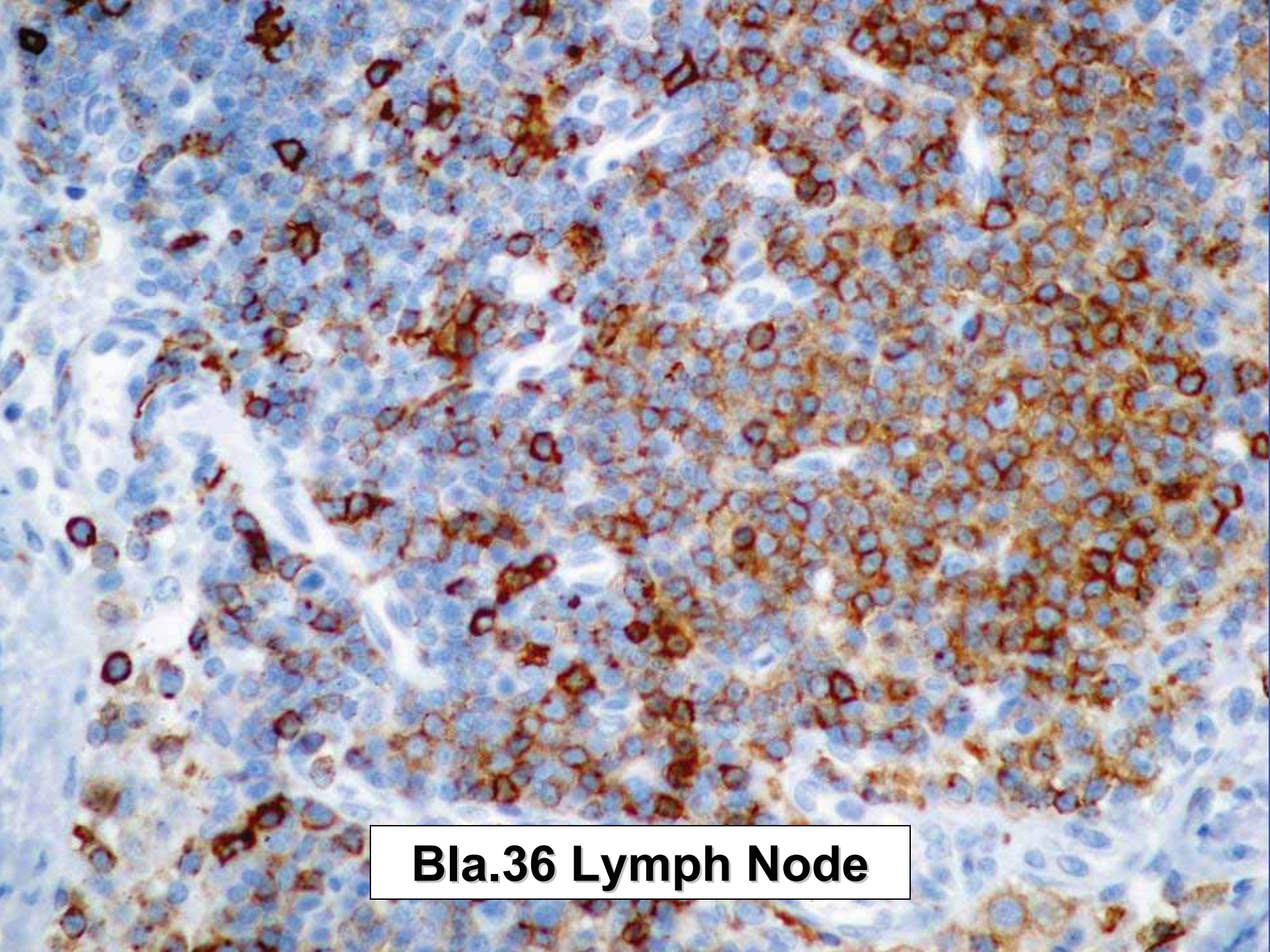

CD79a Smooth Muscle/Vessel

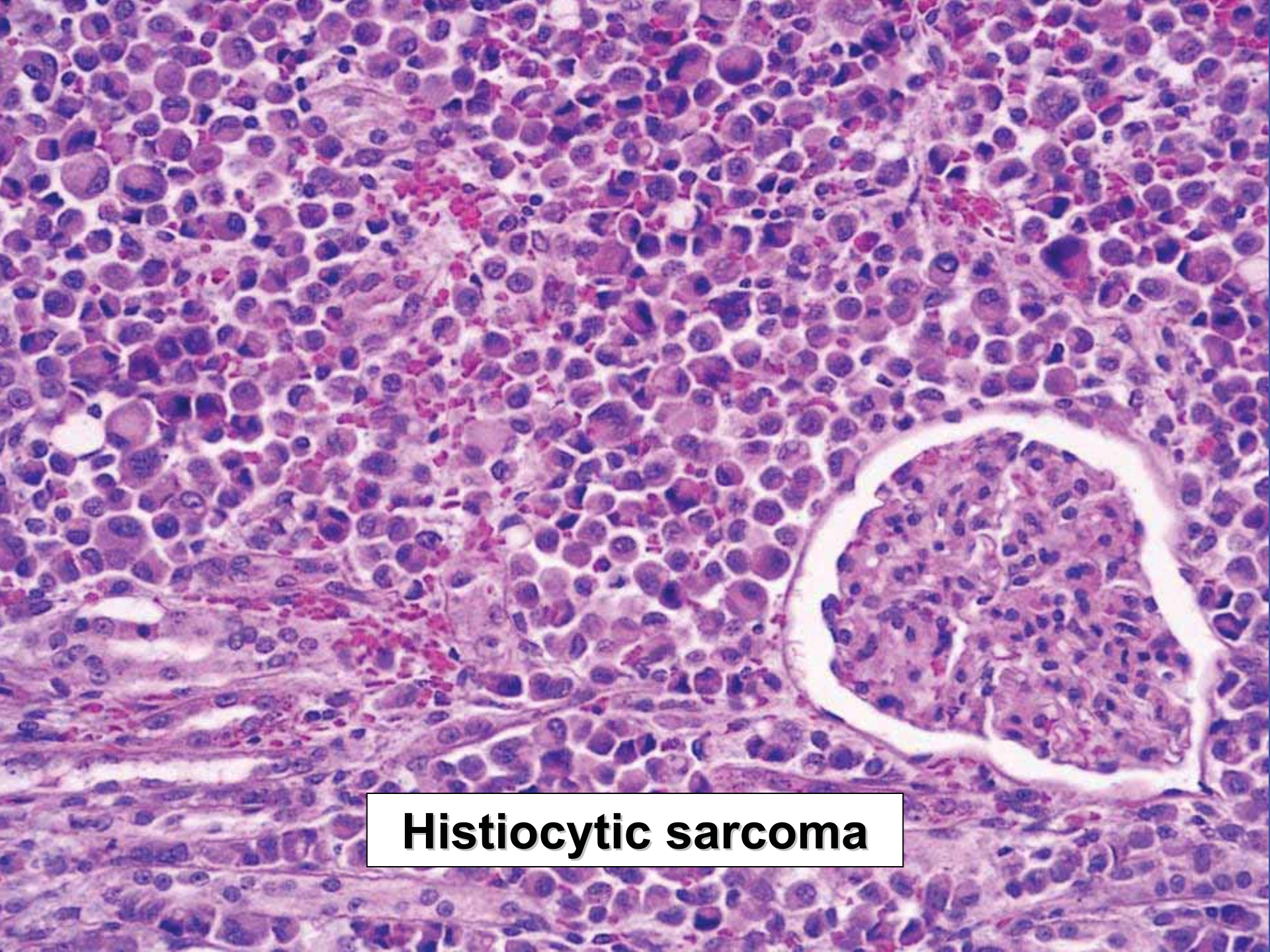

CD79a

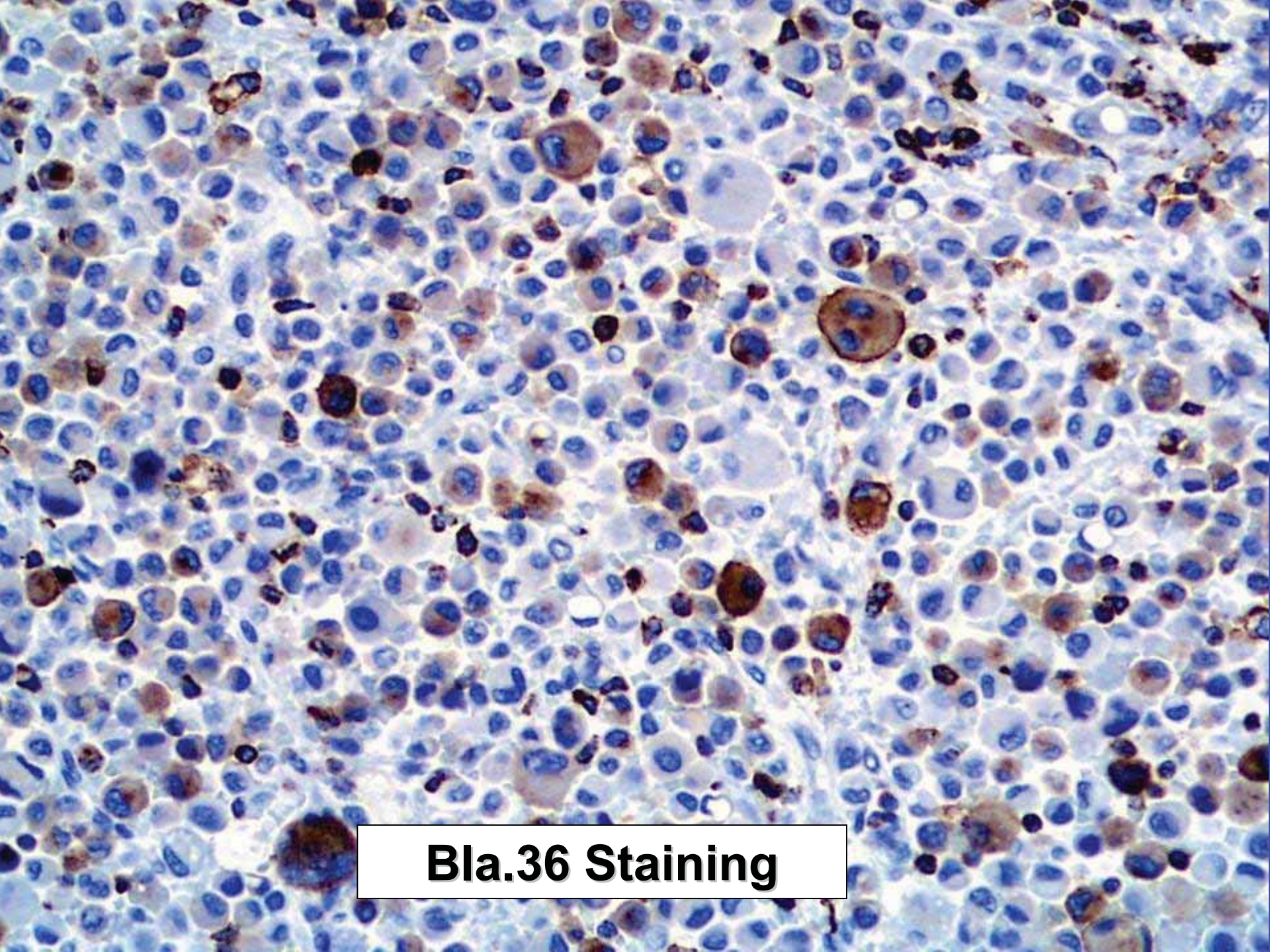

CD79a Bone Marrow


CD61


CD61

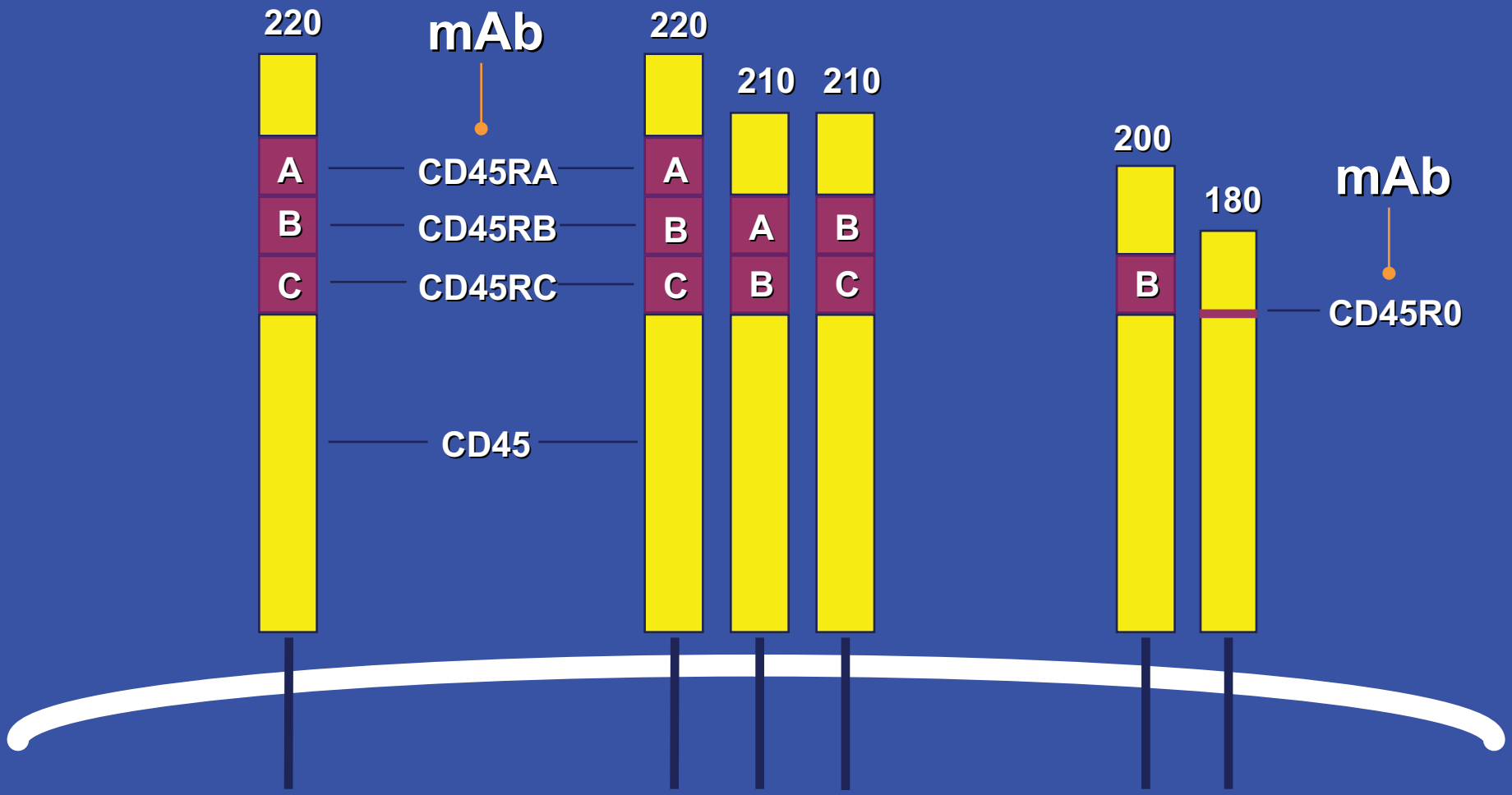

Bla.36 Lymph Node


Histiocytic sarcoma


Bla.36 Staining

CD45

- 180 - 220 kD integral membrane glycoprotein; formerly Leukocyte Common Antigen
- 7 (8) isoforms result from alternative mRNA splicing of 3 exons (A, B, C)
- Cellular expression - leukocytes express at least one CD45 isoform
- B cells, “naïve” T cells express 220 kD form
- “Memory” T cells express 180 kD form



B cells
Plasma cells

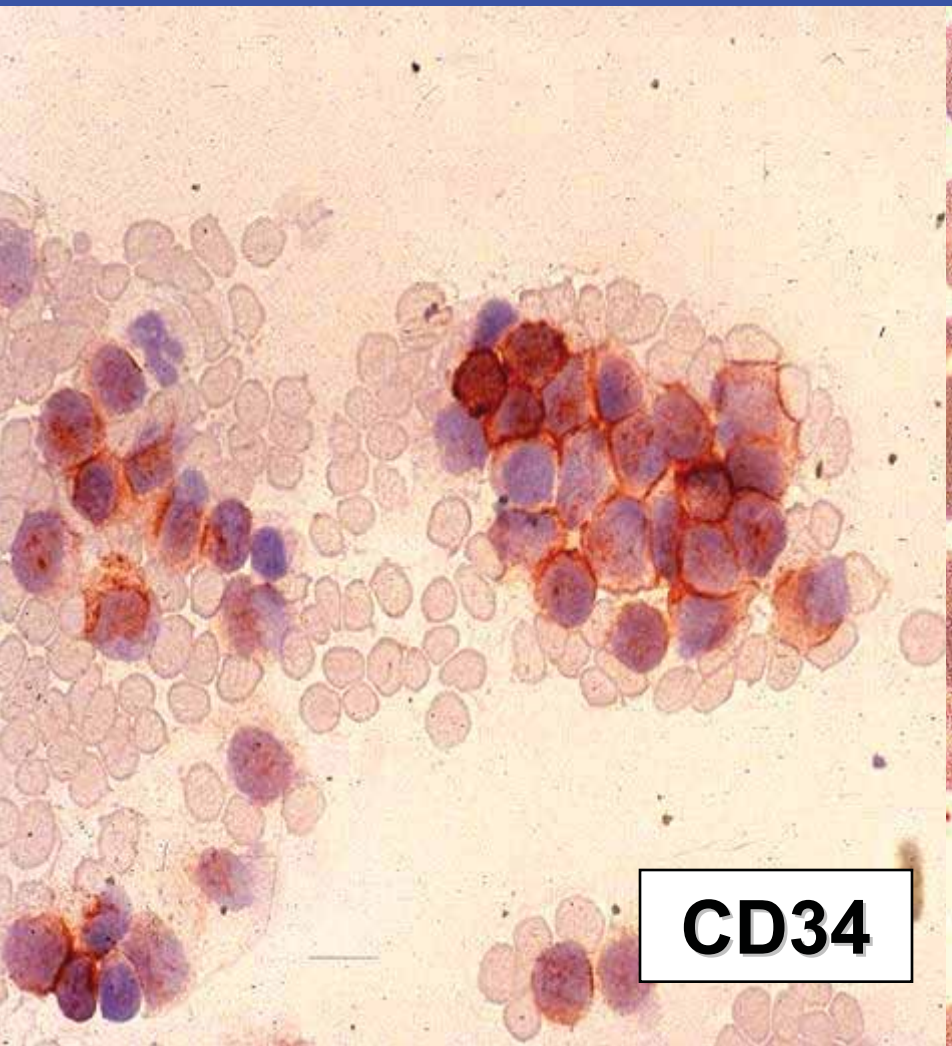
Naive T cells → Activated T cells
Memory T cells (old) ← Memory T cells (recent)
NK cells
Monocytes
Granulocytes
Dendritic cells

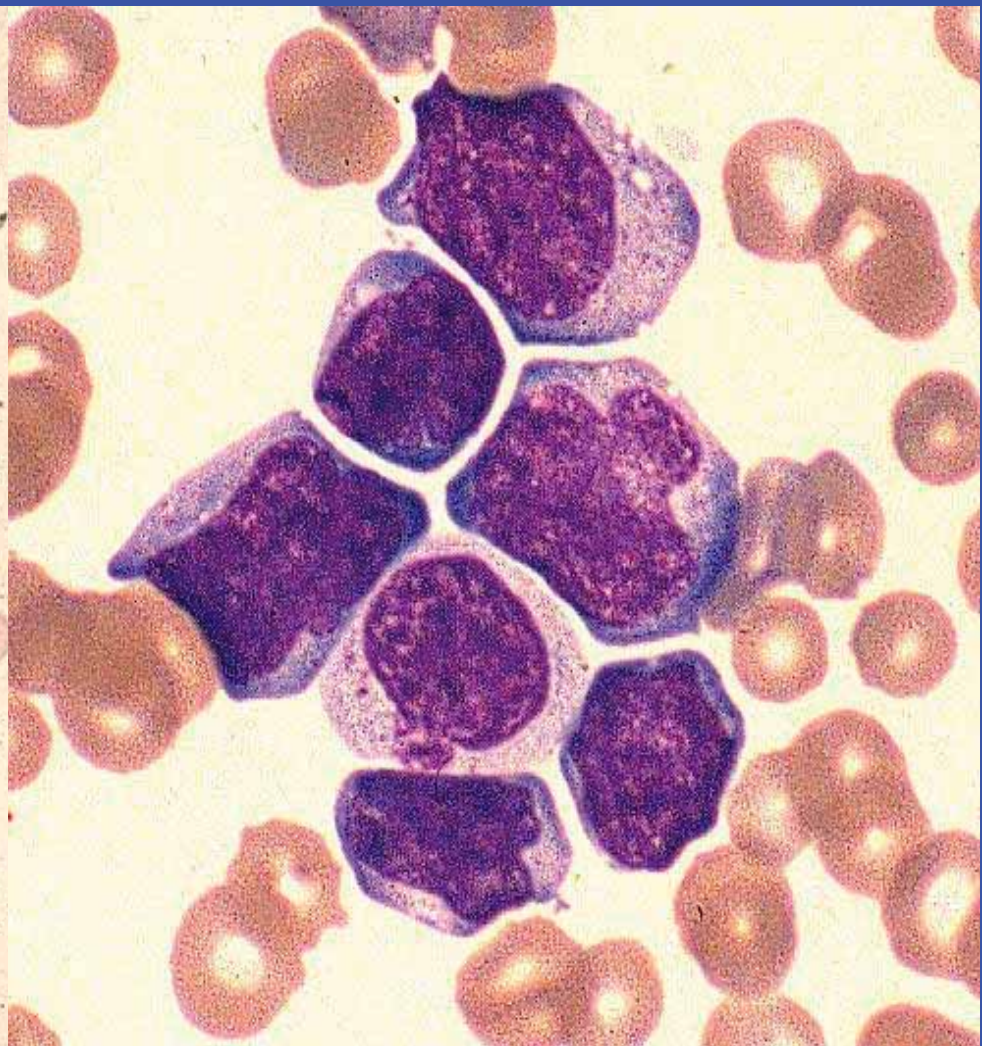
CD45

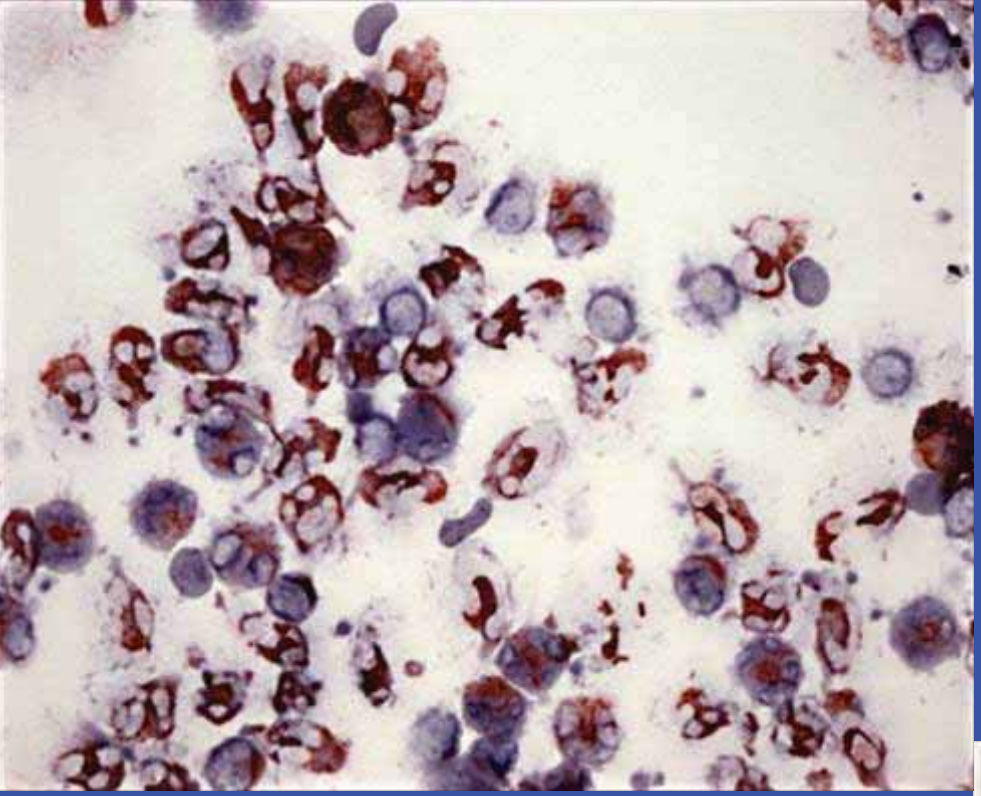
CD34

- 115 kD integral membrane protein; heavily glycosylated
- **Cellular expression** - early lympho-hematopoietic stem cells; endothelial cells
- **Ligands** - L-selectin (CD62L), E-selectin (CD62E); possible role in leukocyte-endothelial interactions
- **Expressed in acute immature cell leukemia** - both lymphoid (ALL) and myeloid (AML); not expressed in lymphoma or CLL

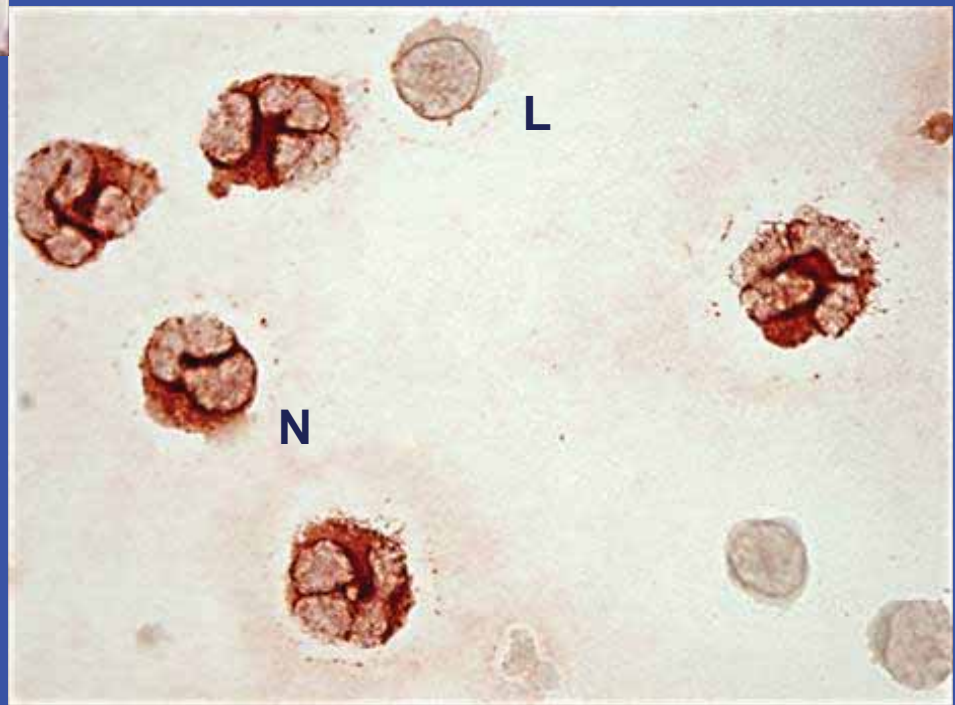
Acute Leukemia





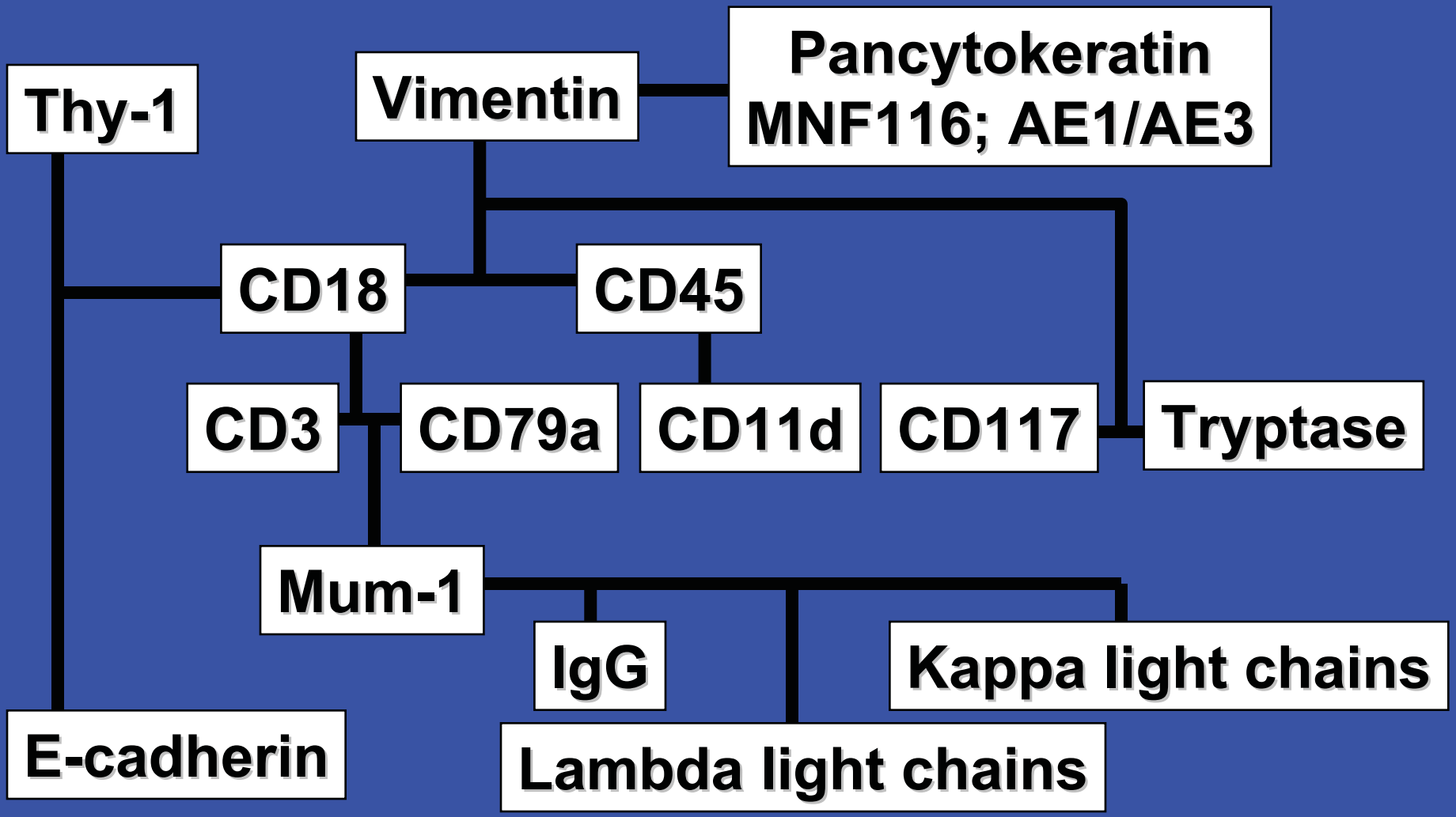


Myeloperoxidase (MPO)

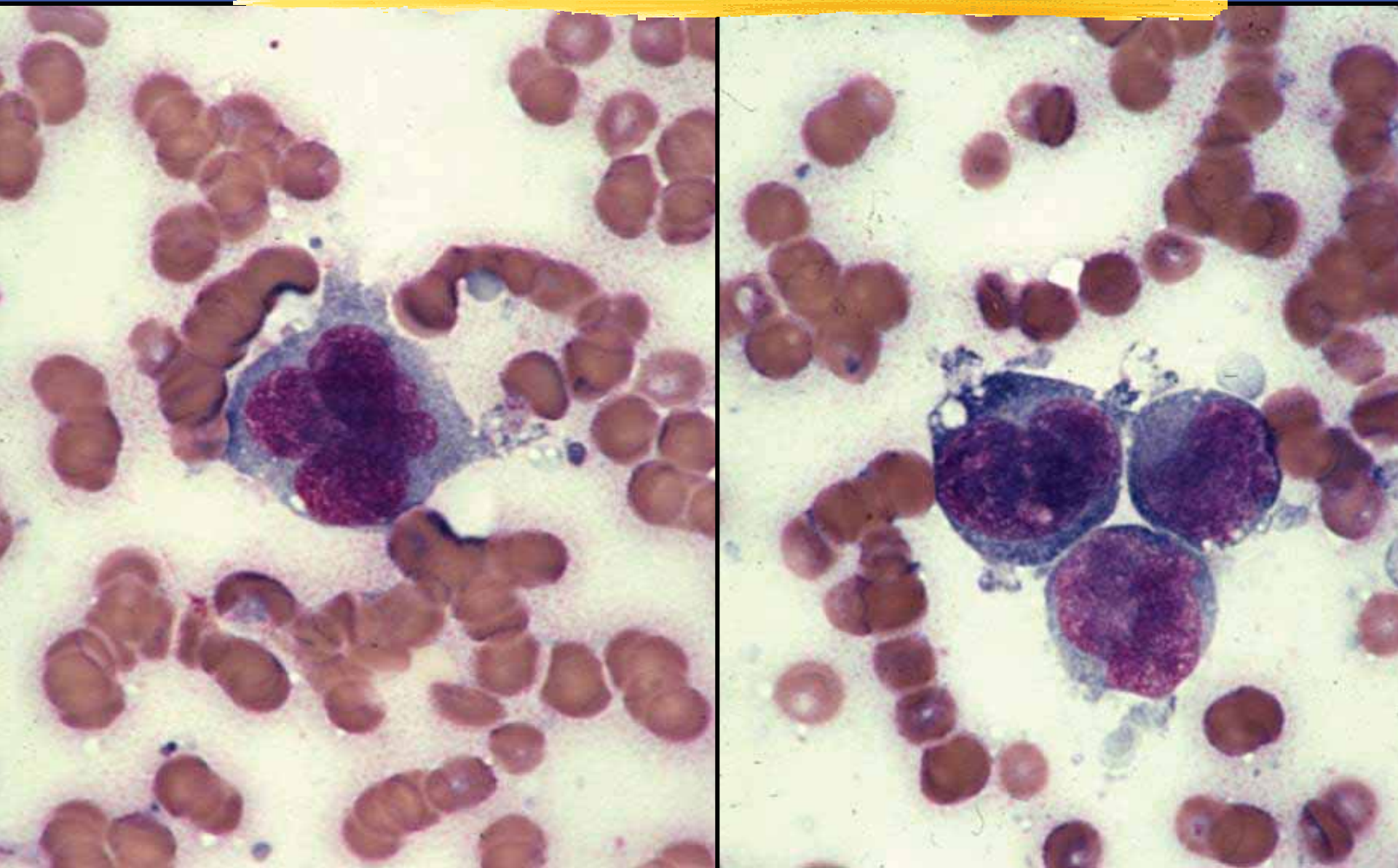


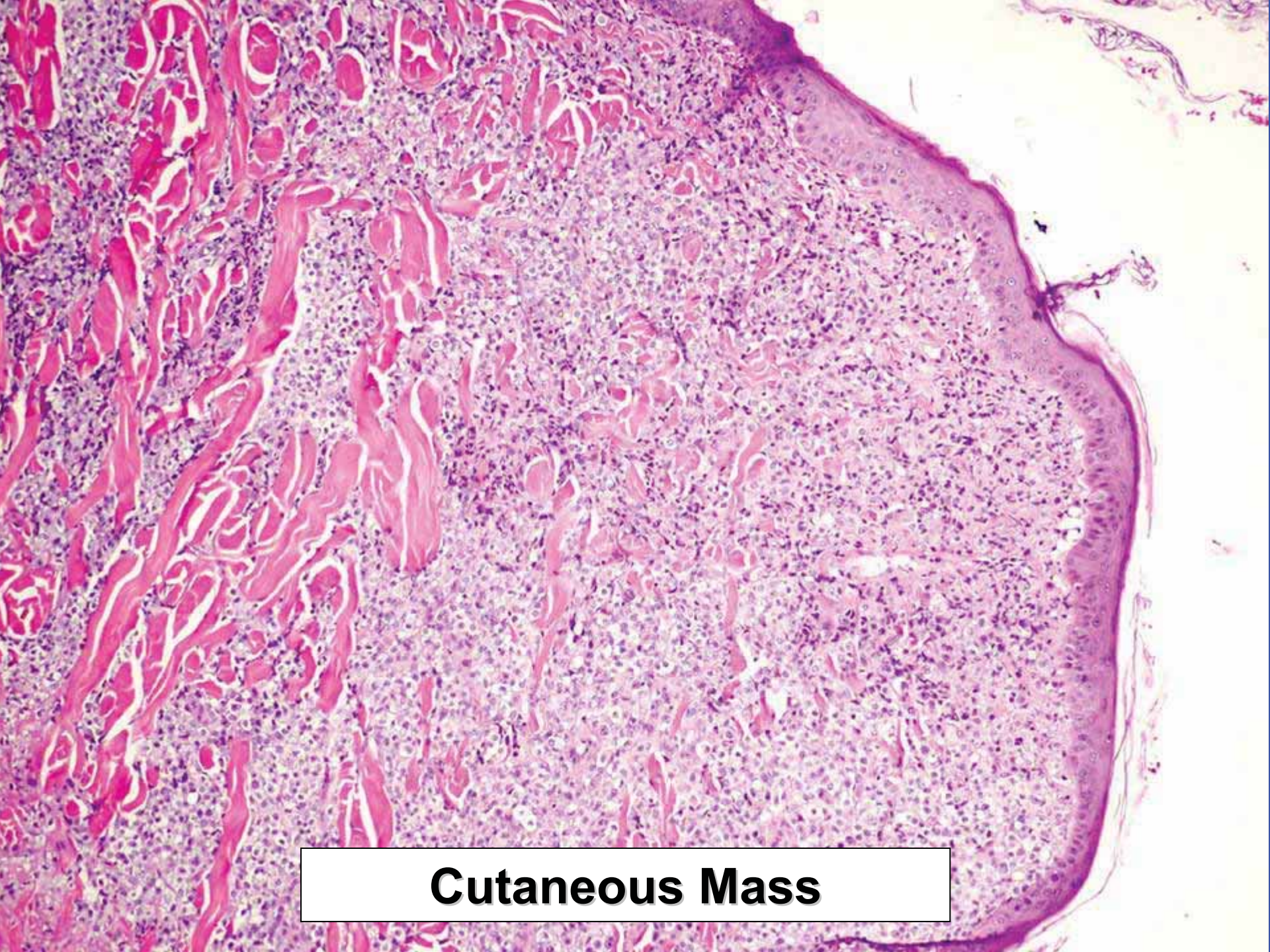
Definitive myeloid marker for acute myeloid leukemia

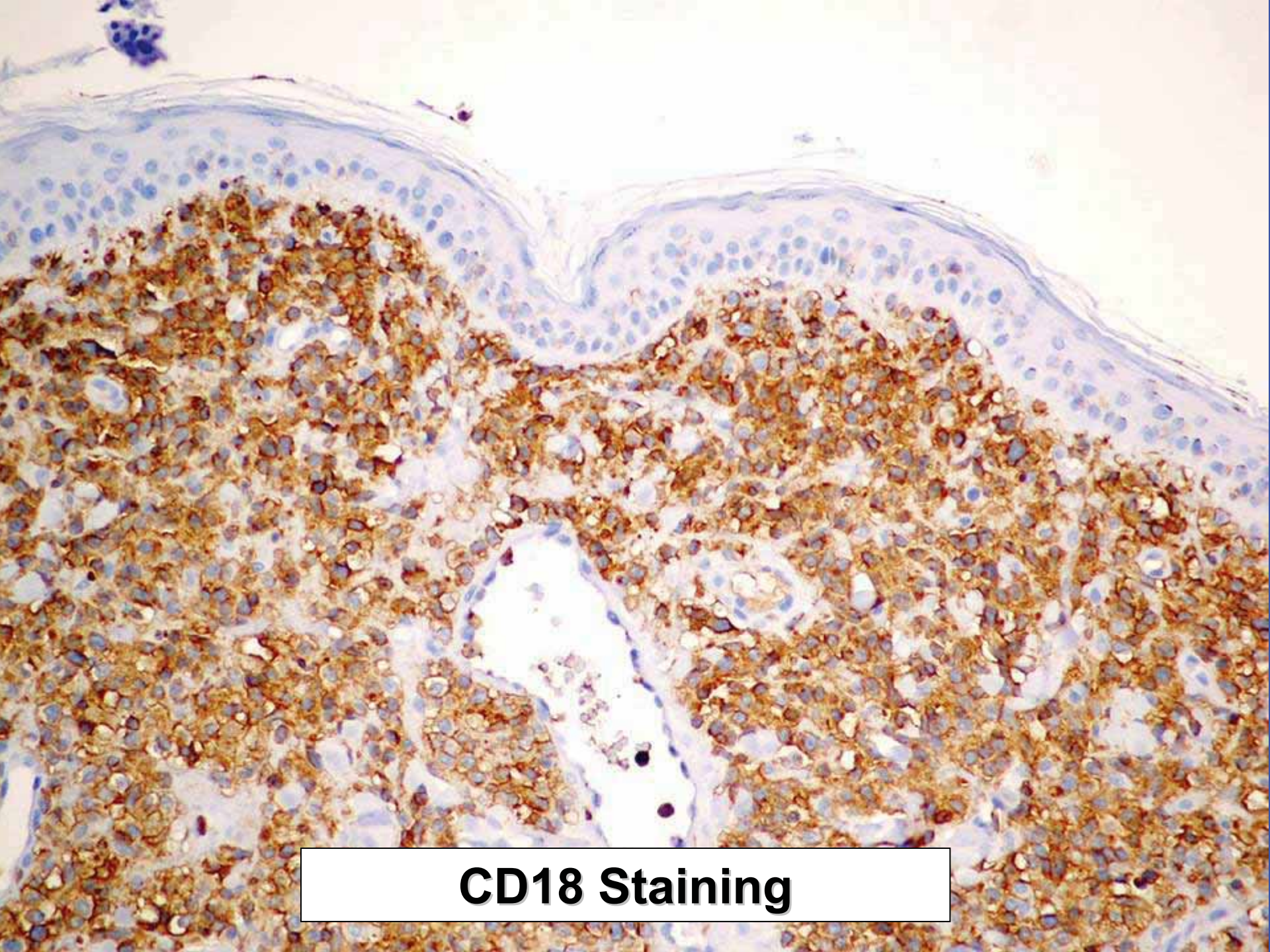
Cutaneous Round Cell Tumors

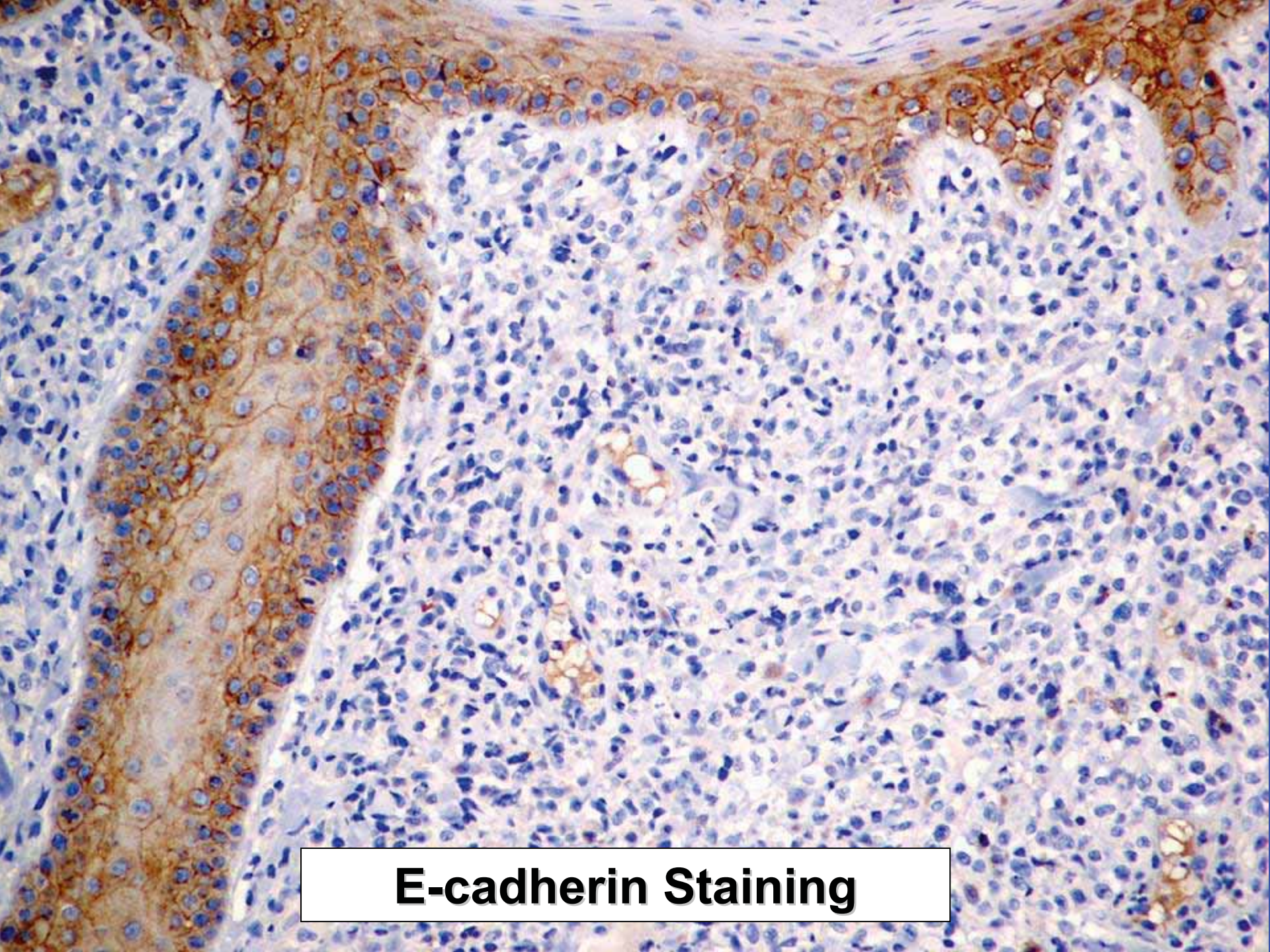


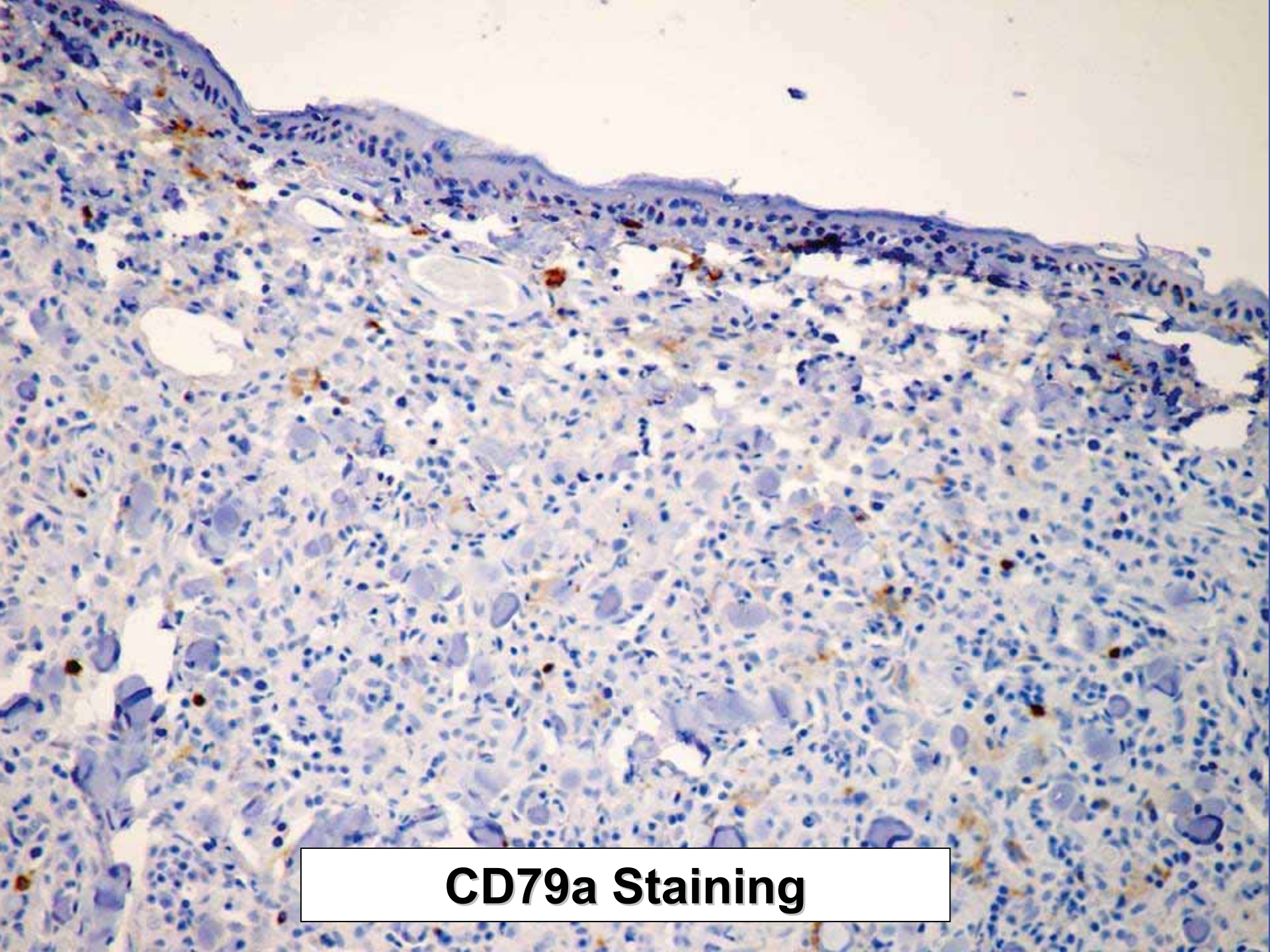
Cutaneous Mass

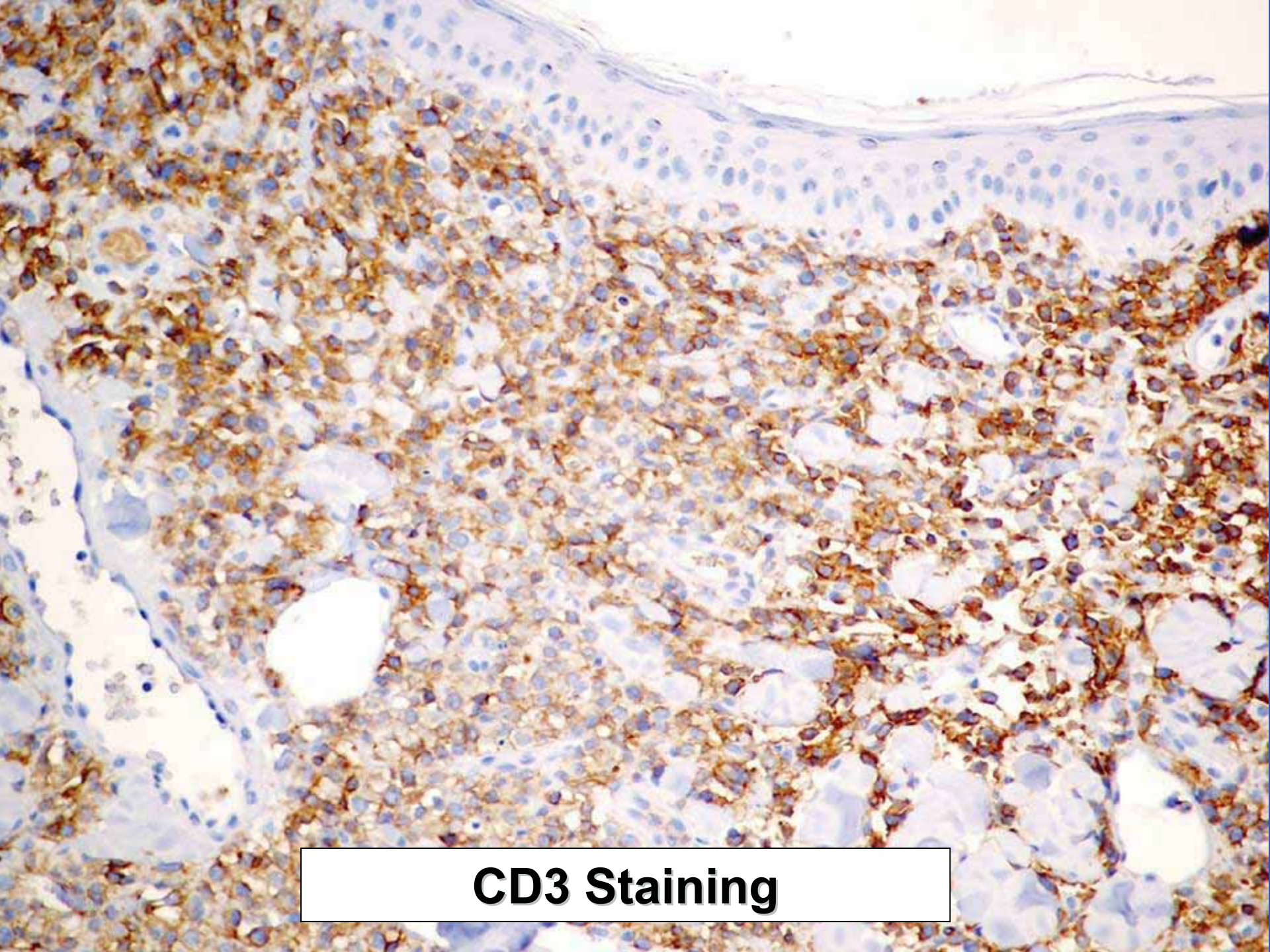



Cutaneous Mass


CD18 Staining

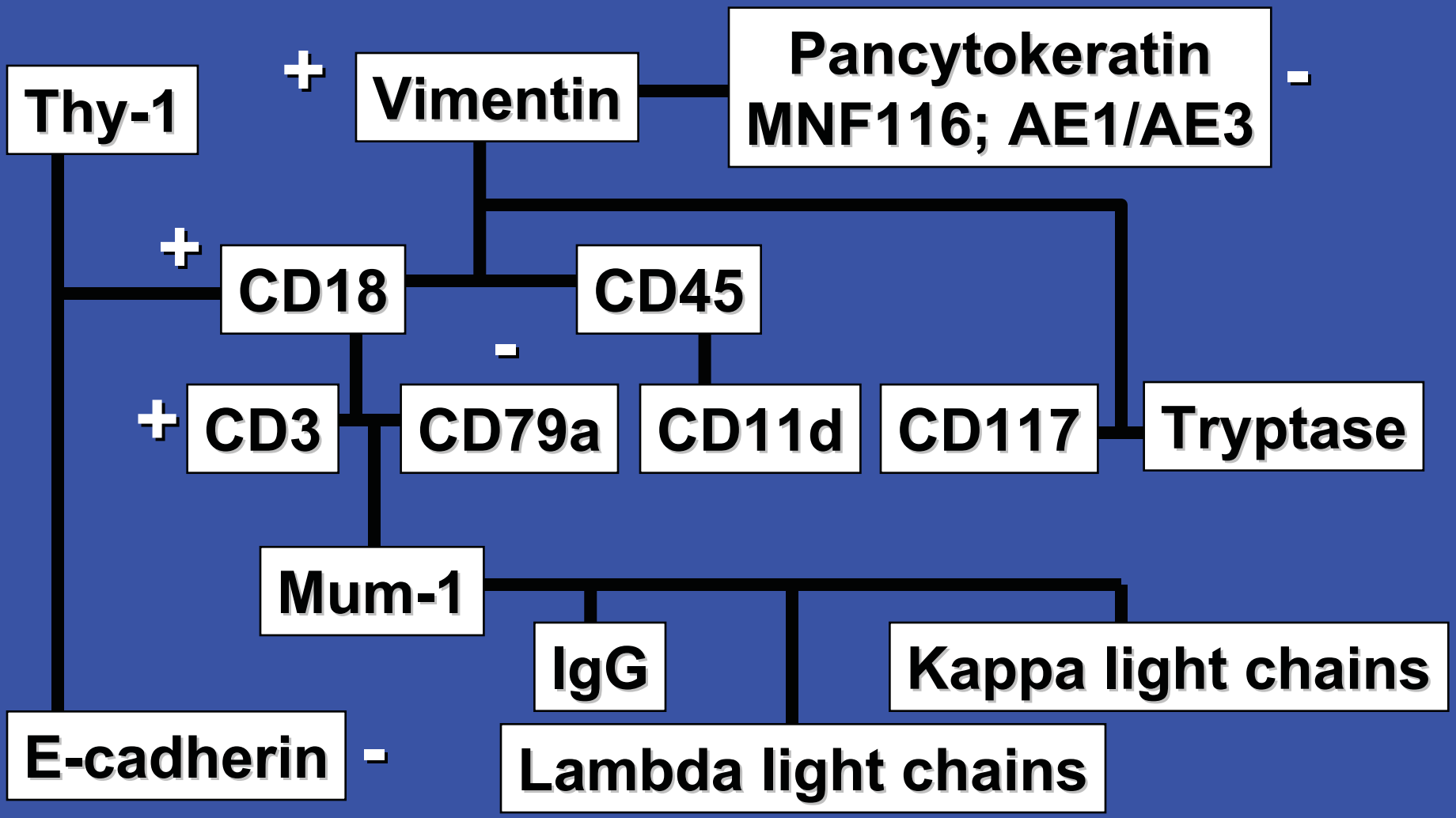

E-cadherin Staining


CD79a Staining


CD3 Staining

What's your Diagnosis

Non-epitheliotrophic T-cell Lymphoma

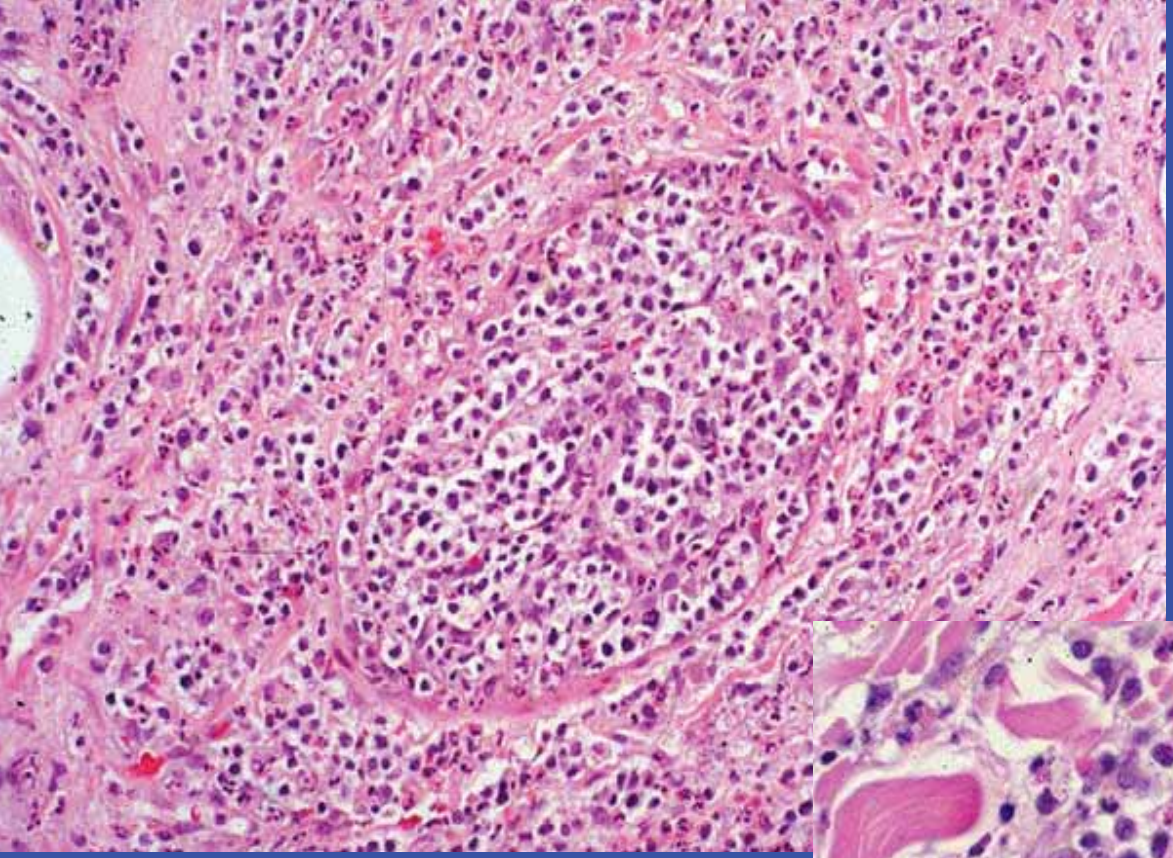


Cutaneous Lymphoma

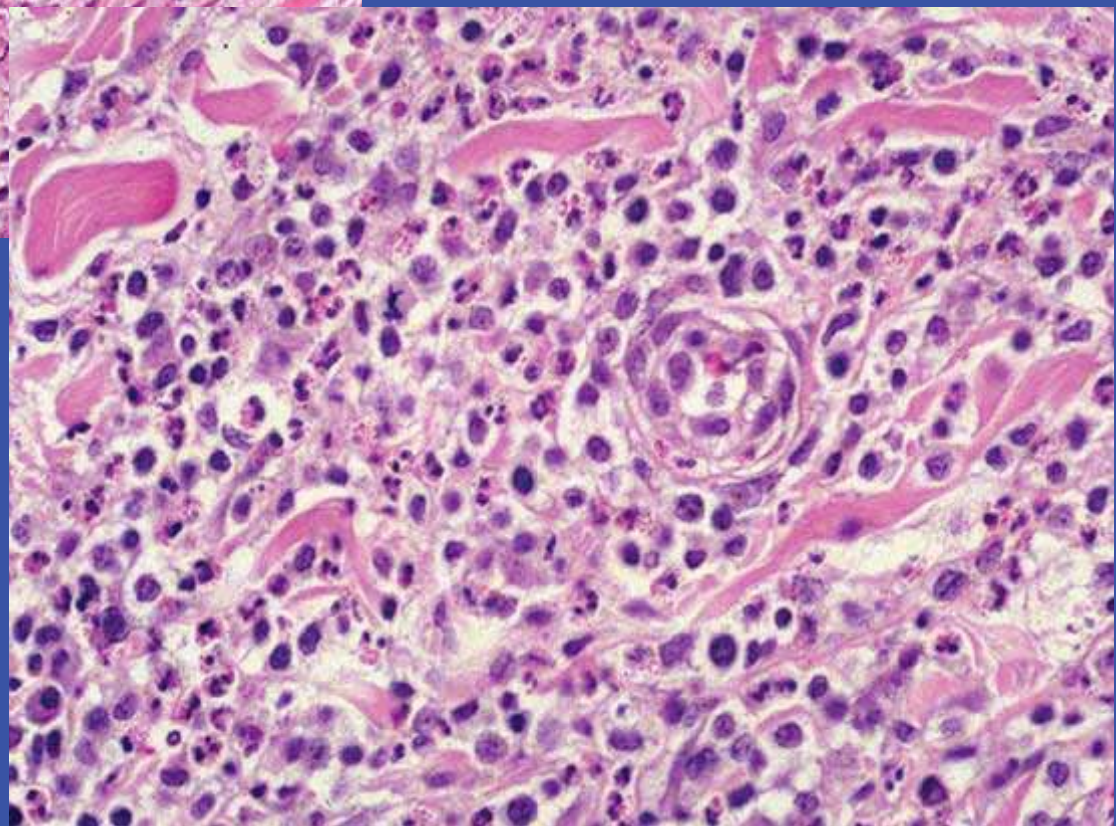
- Cutaneous T cell lymphoma (CTCL)
  - Epitheliotropic CTCL:
    - classical mycosis fungoides (MF)
    - pagetoid mycosis fungoides (PD)
  - Non-epitheliotropic CTCL
- Cutaneous B cell lymphoma
- Cutaneous plasmacytoma (mainly B cell lineage)

Non-epitheliotropic Canine CTCL

- Solitary or multiple nodules in any region of skin or mucocutaneous junctions
- Nodular aggregates oriented around vessels, may infiltrate vessel walls ("lymphomatoid granulomatosis")
- Large cell ("histiocytic") lymphomas
- In older dogs (mean 10+ years)
- Eosinophil infiltration in 50% cases
- Rapidly progressive, poor prognosis despite therapy
- Differentials: histiocytic proliferative disorders and grade 3 mast cell tumors
- Need at least paraffin immunohistology: CD3, CD79a, CD18, CD45RA, c-KIT, tryptase



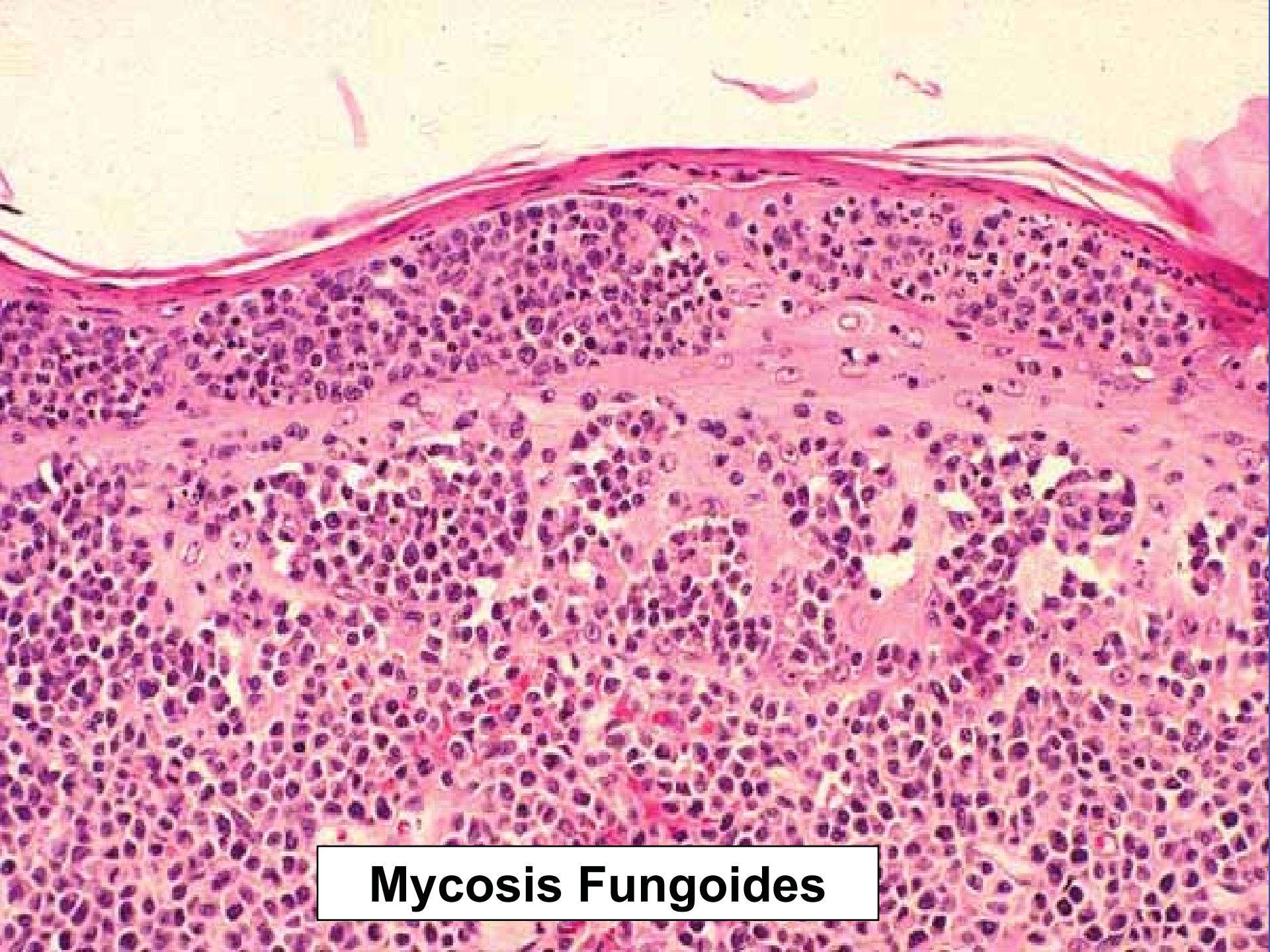
vascular obliteration

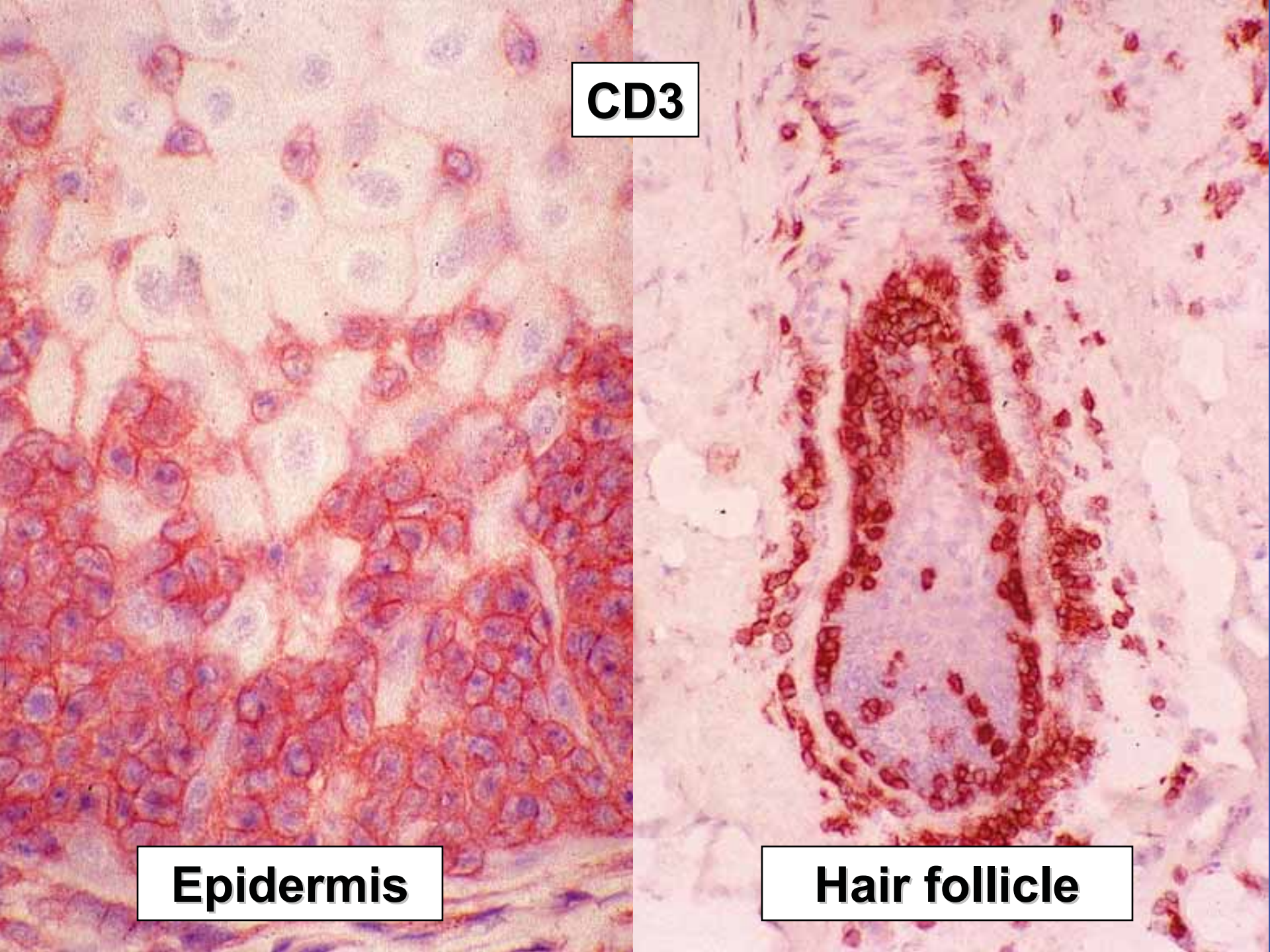


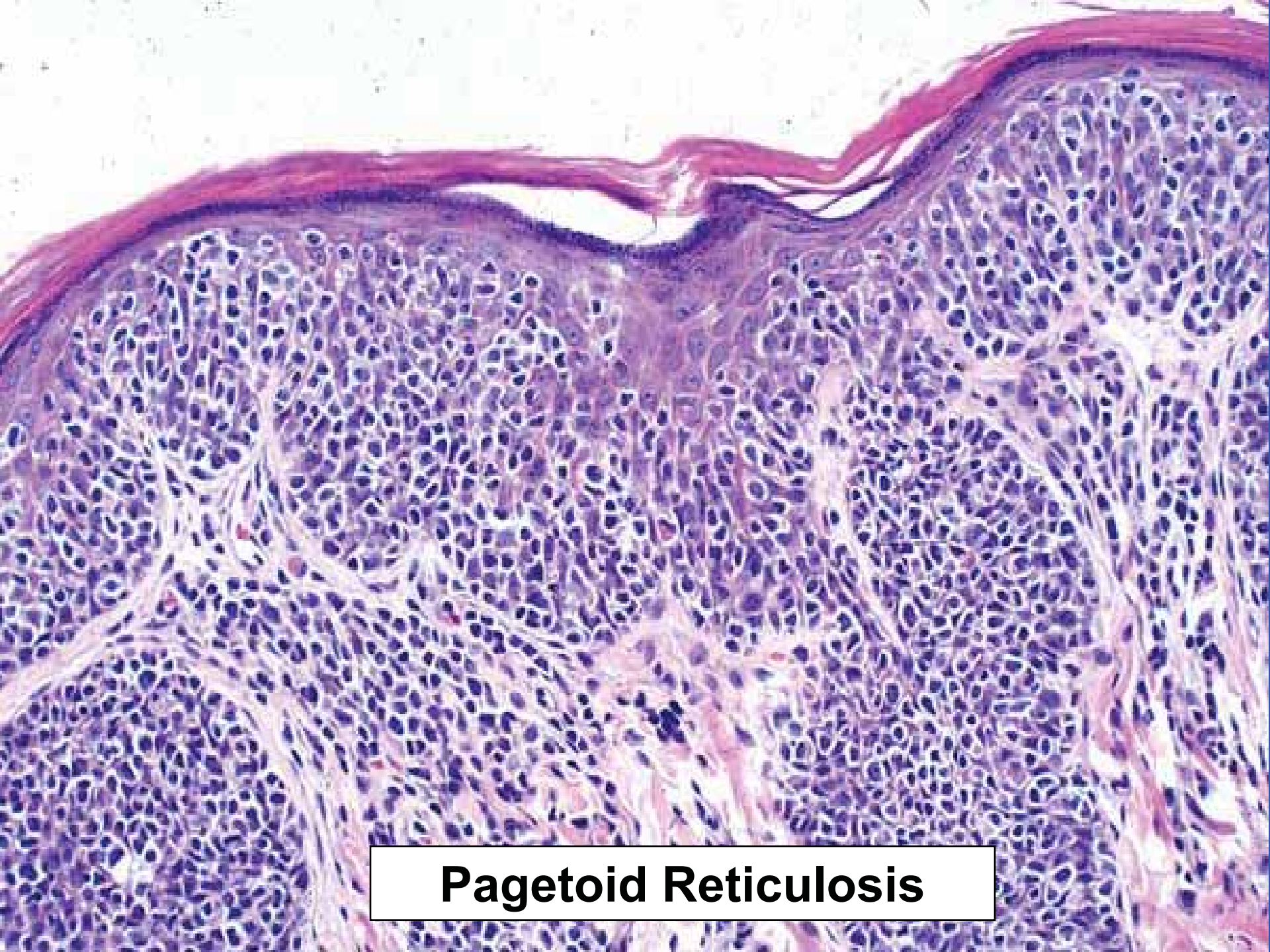
eosinophils

Canine Epitheliotropic CTCL

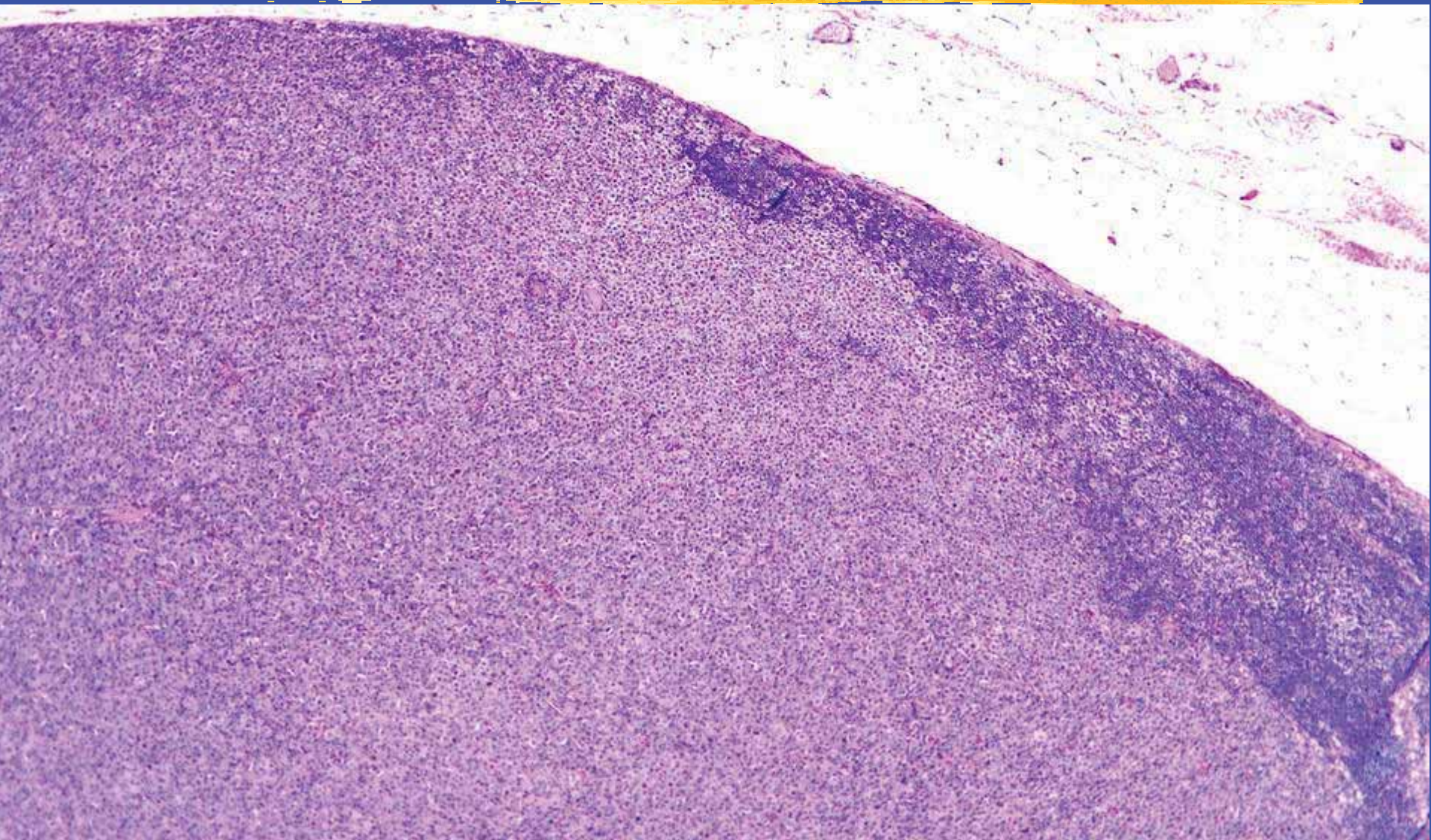
- Mycosis fungoides
  - Locally extensive, cutaneous and mucocutaneous
  - Epitheliotropism and marked adnexal tropism
  - Most common form of CTCL; occurs in old dogs (mean 11 yrs)
  - Lesions occur throughout the skin - preferential involvement of mucocutaneous junctions and oral cavity
  - Lesions occur in 3 principal stages: patch, plaque and tumor stages (leukemia > Sezary syndrome)
  - Progression is more rapid in dogs than humans

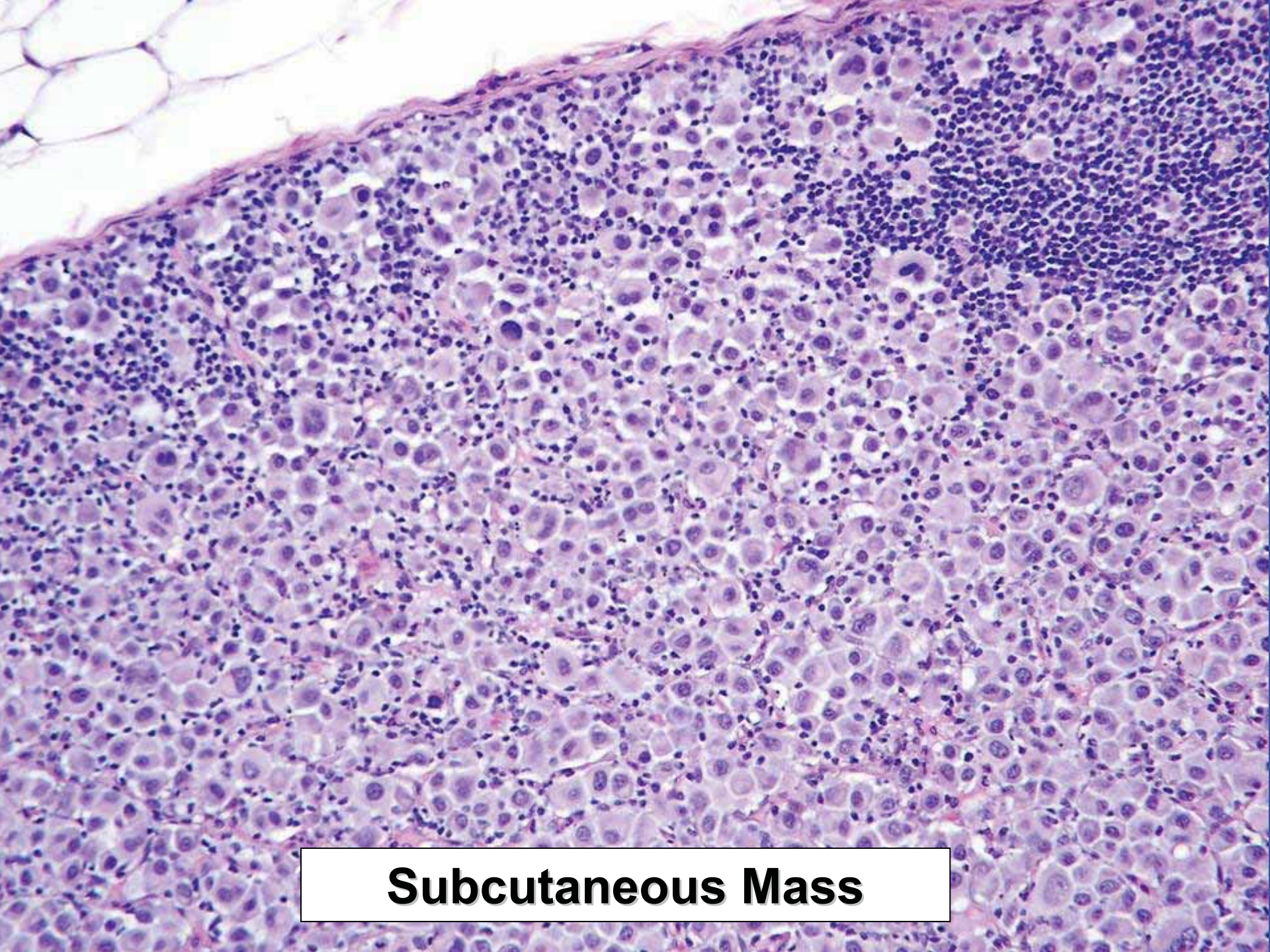

Mycosis Fungoides

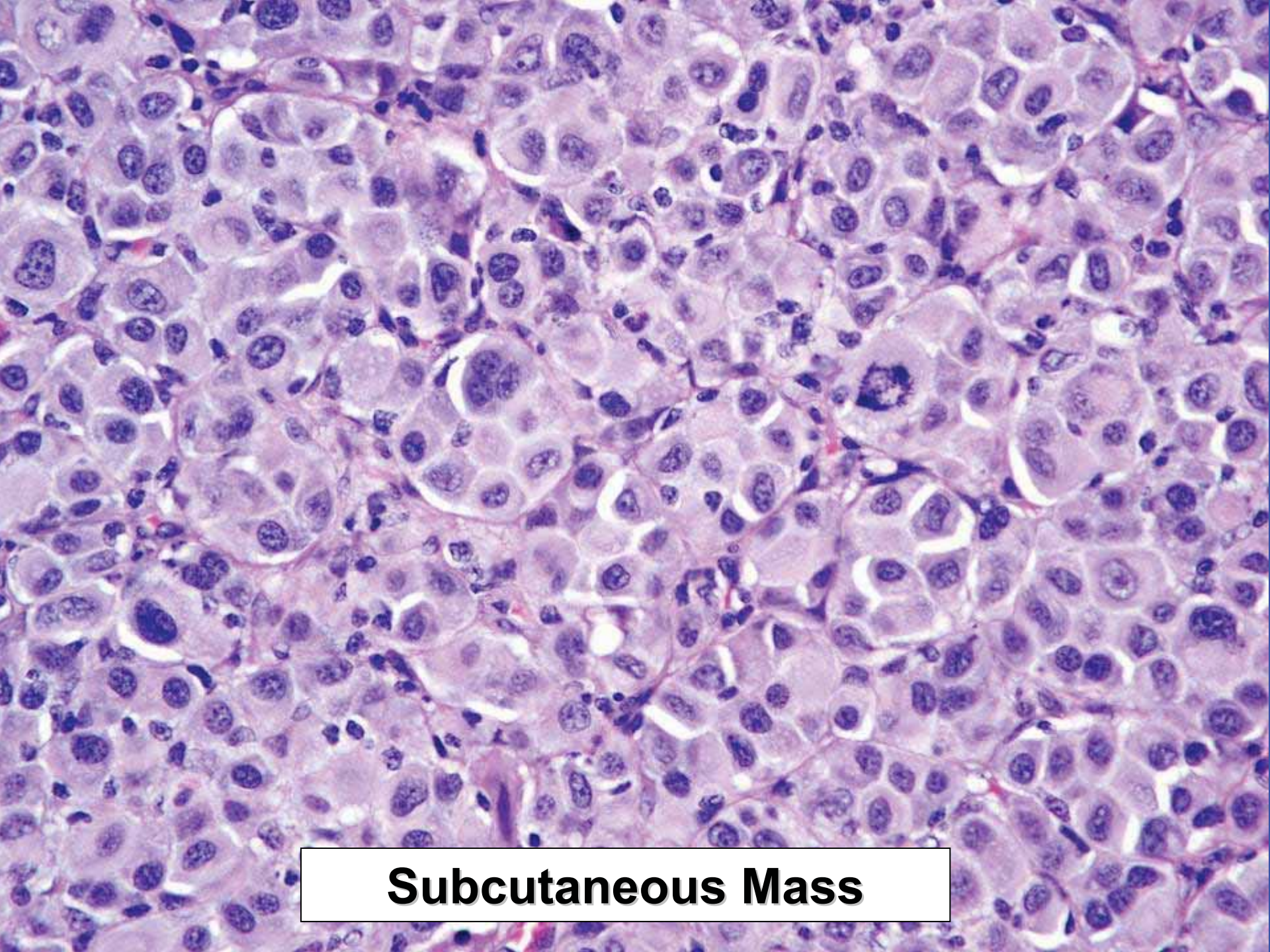

CD3
Epidermis
Hair follicle

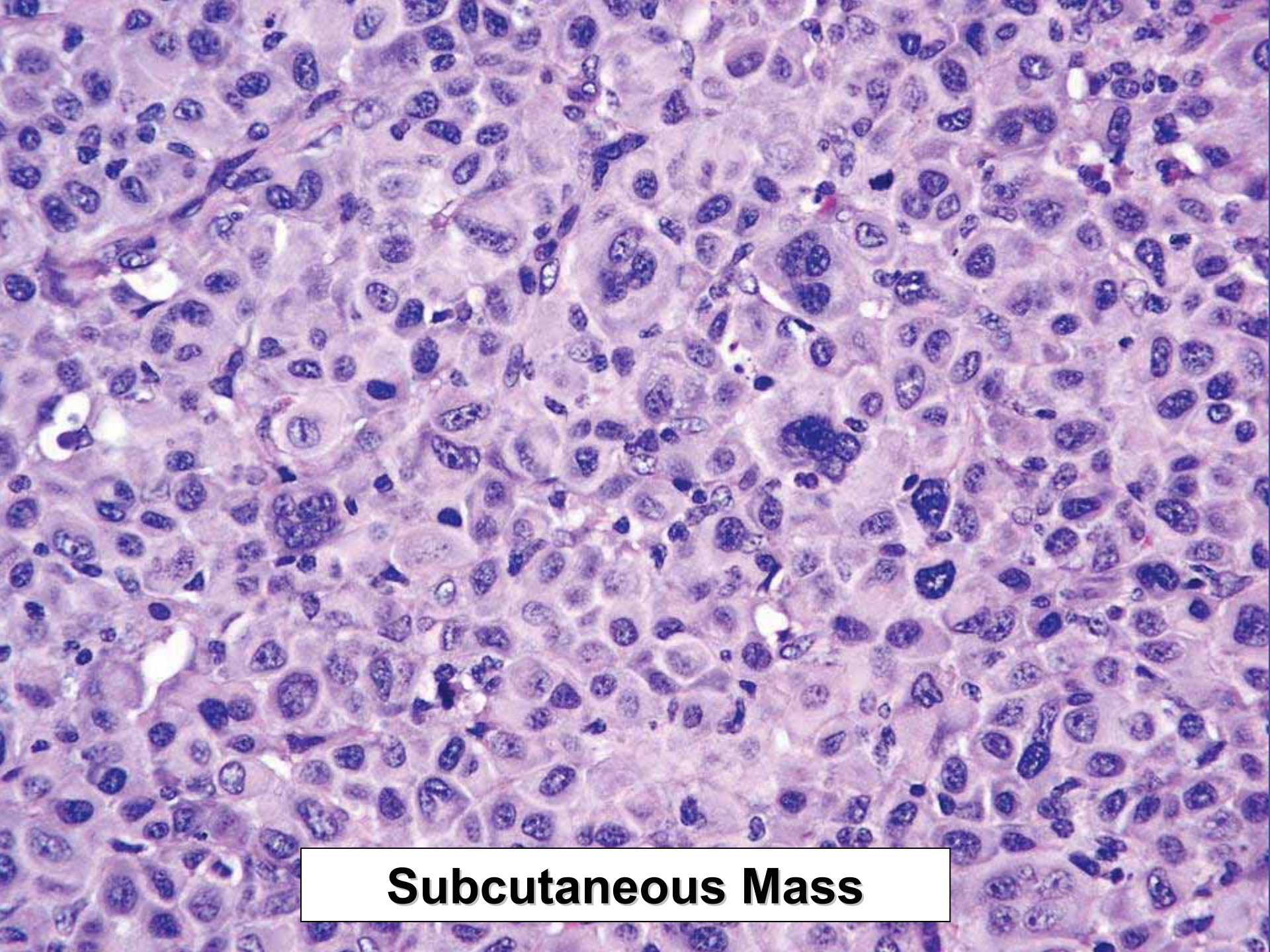

Pagetoid Reticulosis

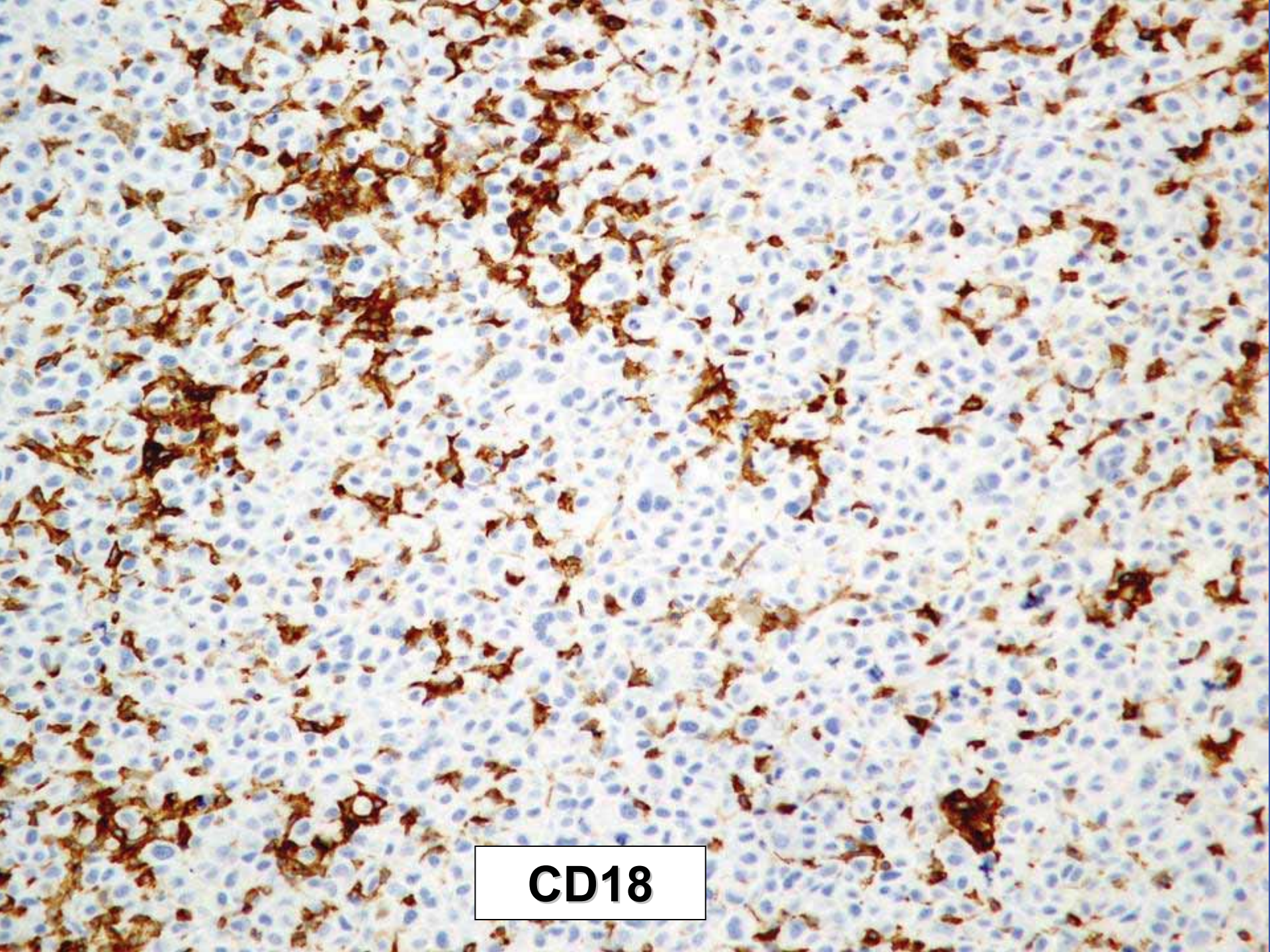
Subcutaneous Mass

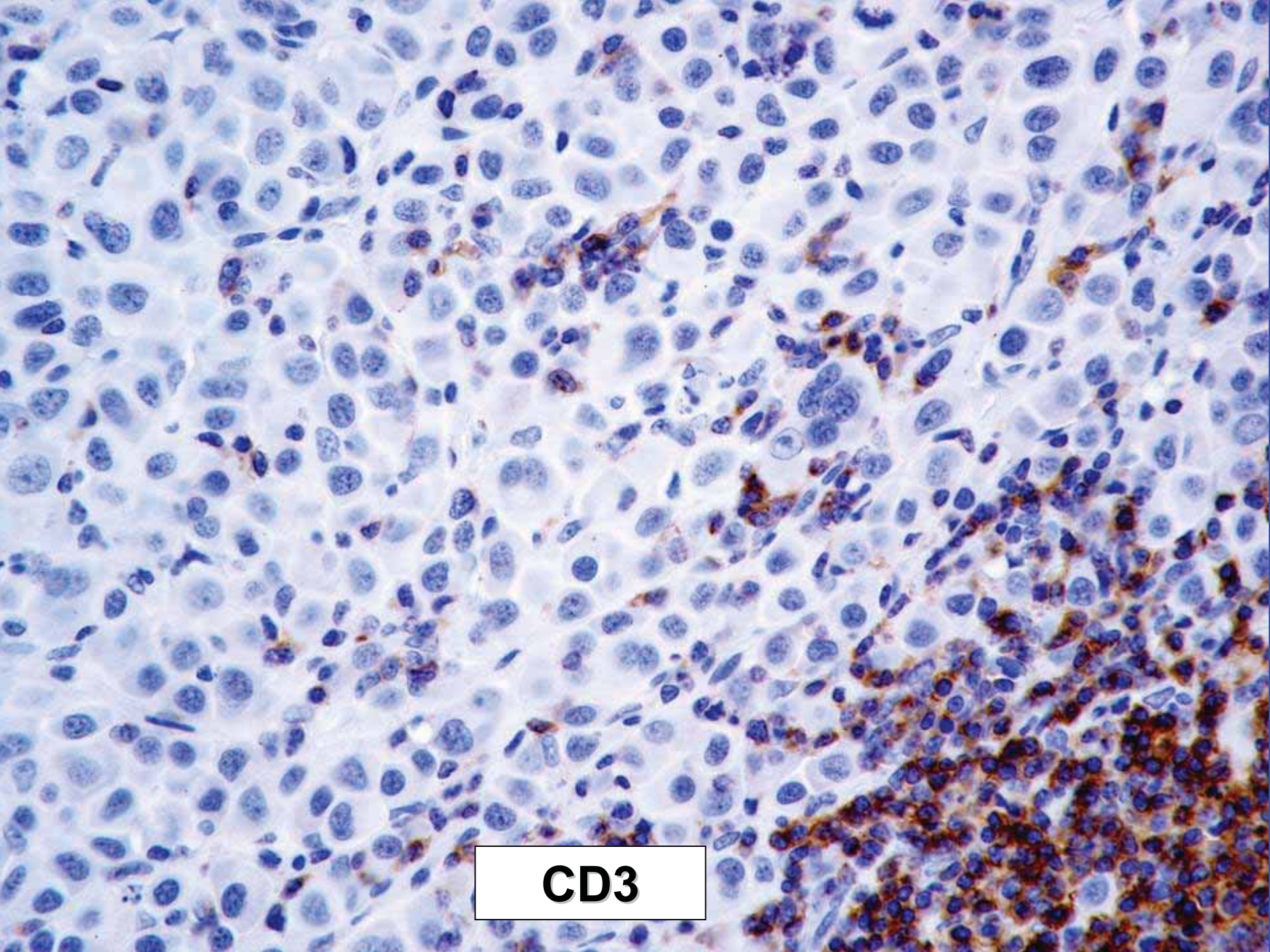


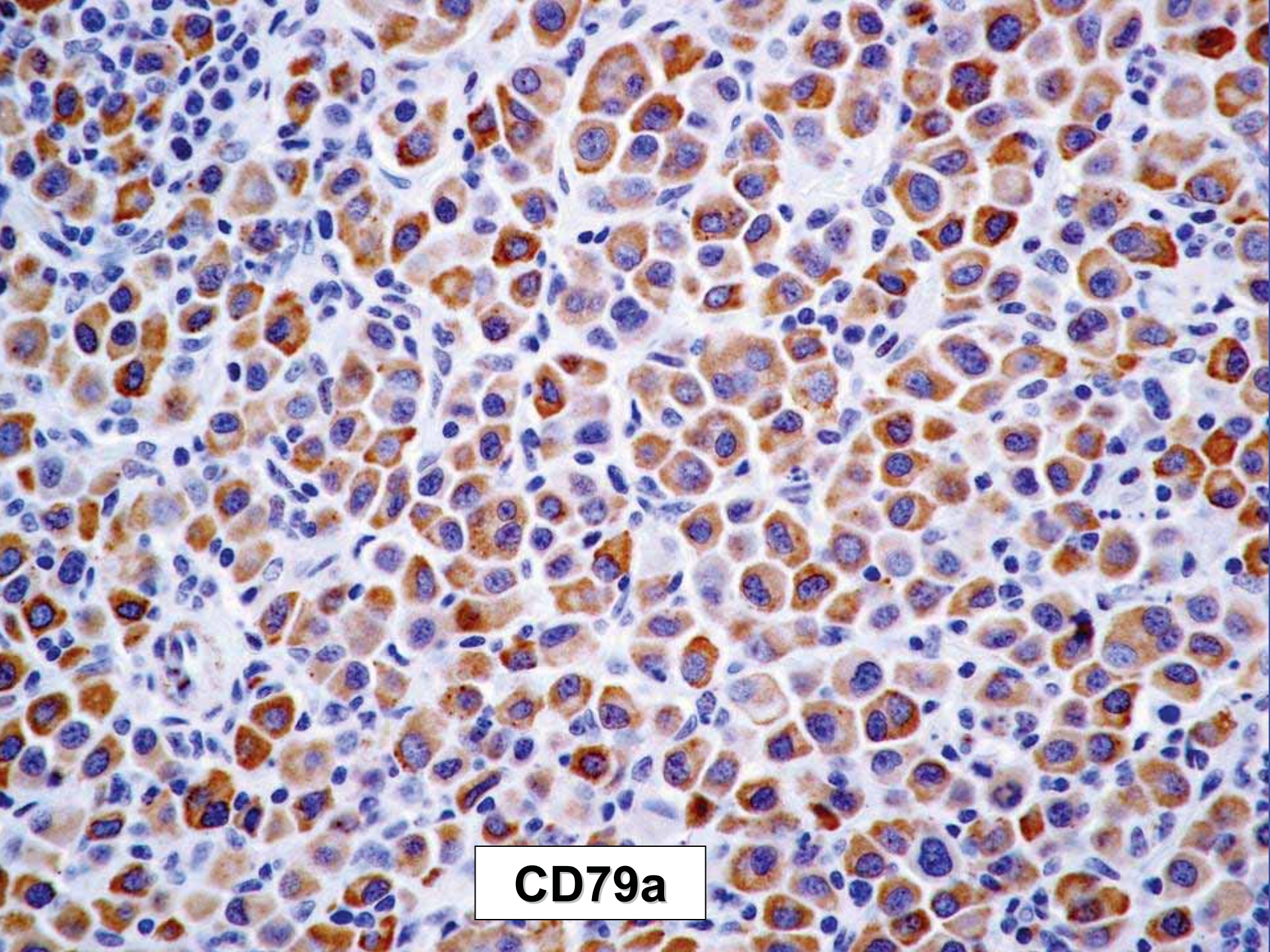

Subcutaneous Mass


Subcutaneous Mass


Subcutaneous Mass

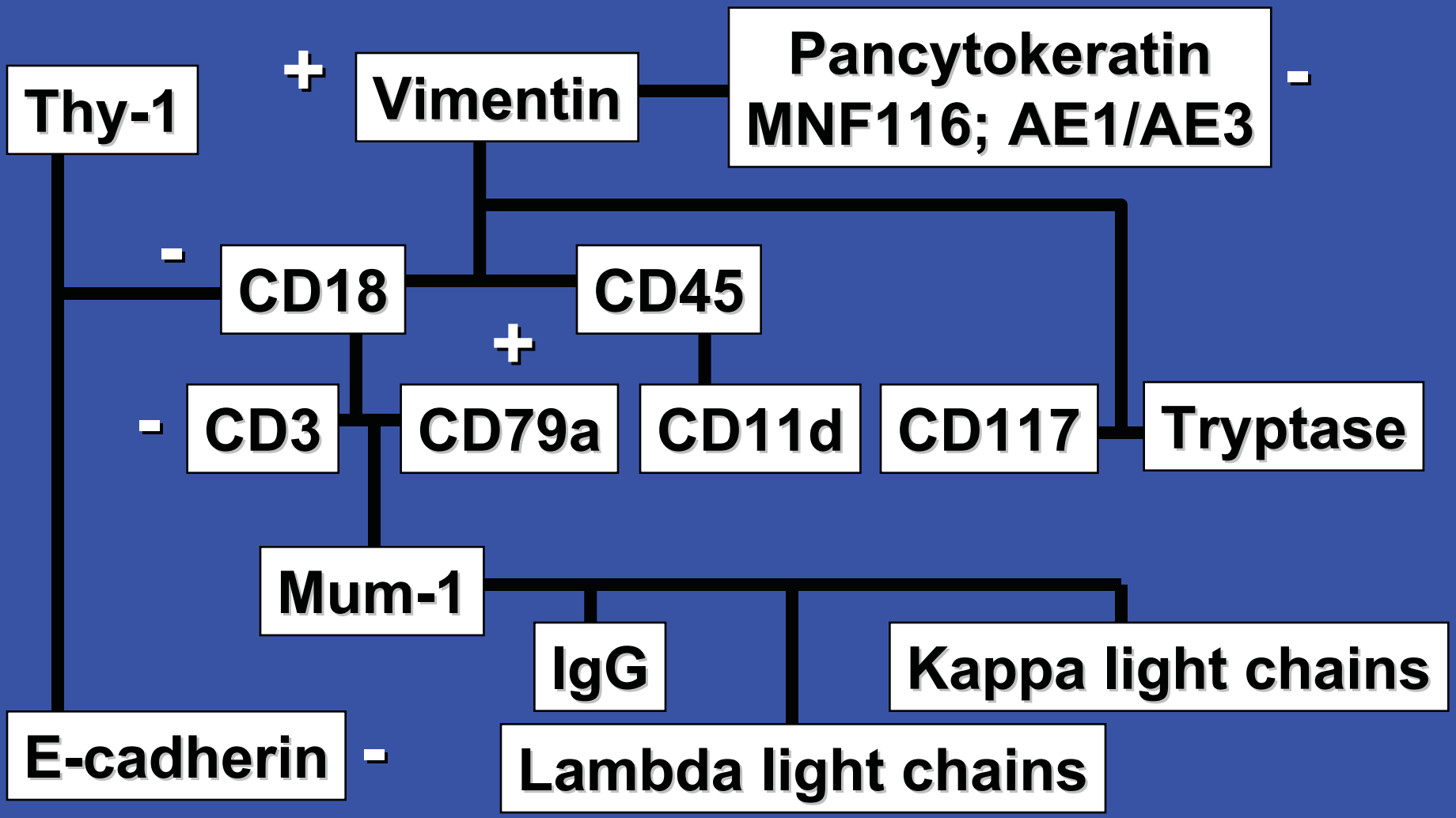

CD18


CD3


CD79a


What's your Diagnosis
now?

Anaplastic Large Cell B-cell Lymphoma



Anaplastic Large Cell Lymphoma

- rarely described in dogs
- require differentiation from histiocytic sarcomas
- Characteristics of ALCL:
  - primary cutaneous or nodal masses
  - B-or T-cell origin
  - marked cellular anaplasia
  - abundant cytoplasm
  - large numbers of multinucleated giant cells

Anaplastic Large Cell Lymphoma

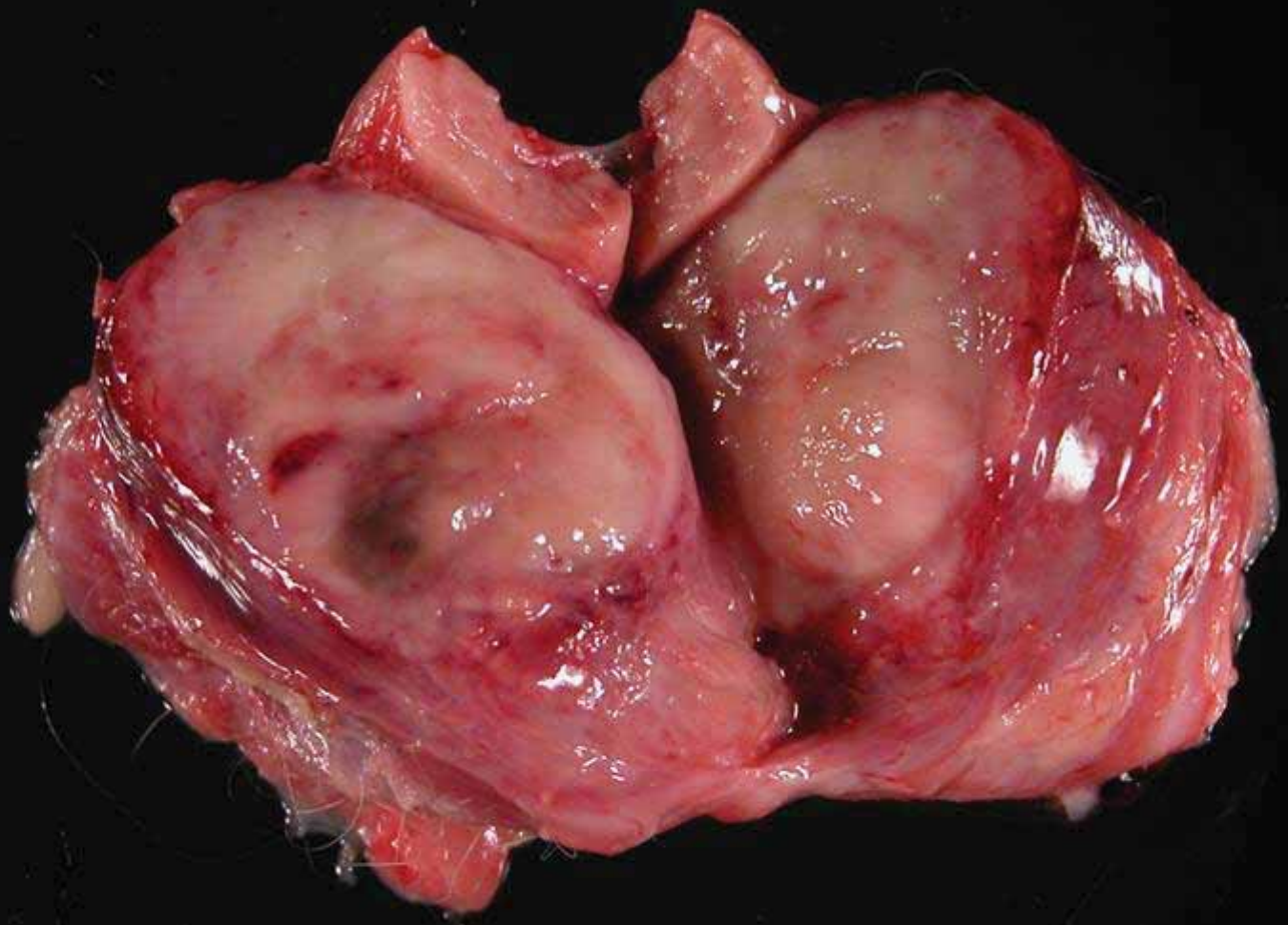
- CD30 and anaplastic lymphoma kinase (ALK, CD246) are essential for the diagnosis of human ALCLs
- vast majority of ALCLs are CD30+ and ALK+
- CD30: - transmembrane receptor
  - member of tumor necrosis factor superfamily
  - normally expressed by activated T cells
  - not specific for ALCL
  - used for Reed-Sternberg and Hodgkin cells
  - staining pattern in ALCL is distinctive (target-like appearance)
- ALK: - tyrosine kinase (insulin receptor superfamily)
  - small subset of cells in the central and peripheral nervous systems of adults

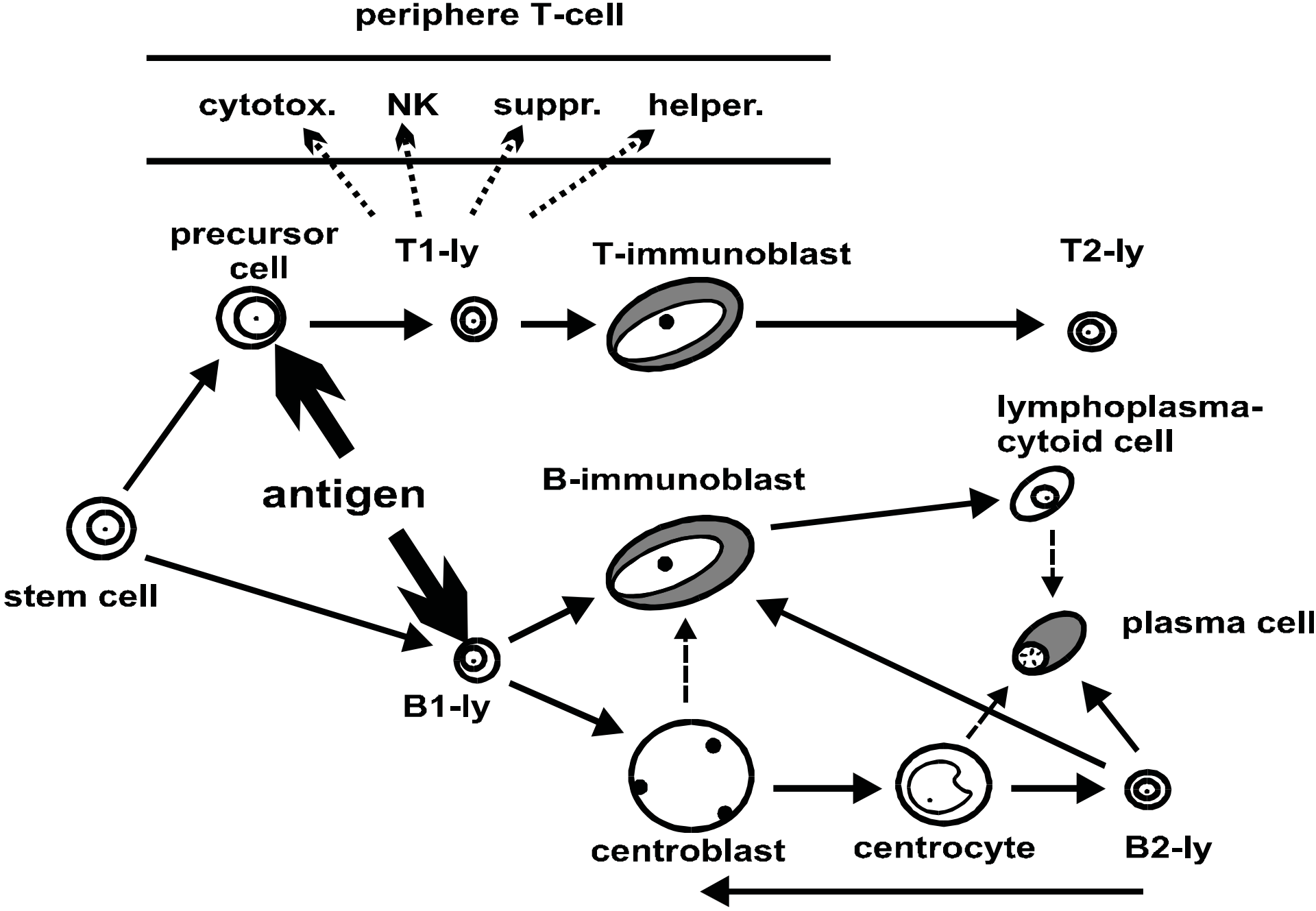
ALCL controversy

- Is ALCL of B-cell phenotype related to the T- or null cell lymphoma of the same morphology?

- WHO excludes CD30+ B-cell lymphomas from ALCL
- grouped with diffuse large cell lymphomas (DLCL)
- these B-cell ALCLs are mainly ALK-, rarely ALK+
- based on clinical behavior and general phenotype there is a current consensus in the literature that ALK- B-cell ALCLs represents a morphological pattern occasionally encountered among diffuse large B-cell lymphomas

Classification of Canine Nodal Malignant Lymphomas





Development of B- and T-lymphocytes (Lennert & Feller, 1990)

KIEL classification	Working Formulation
Low grade malignancy	**Low grade malignancy**
lymphoplasmacytic/immunocytoma	
hairy cell leukemia	not included
Mycosis fungoides	low grade, Mycosis fungoides
centrocytic	follicular, small cleaved
	Intermediate grade malignancy
centroblastic/centrocytic	diffuse, mixed small and large
High grade malignancy	
centroblastic/monomorph	follicular, mainly large
centroblastic/polymorph	diffuse, large
centroblastic/centrocytoid	diffuse, large
	High grade malignancy
immunoblastic	immunoblastic
lymphoblastic	lymphoblastic
Burkitt lymphoma	small non cleaved

Another Lymphoma Classification?

Get REAL!

A Revised European-American Classification of Lymphoid Neoplasms: A Proposal from the International Lymphoma Study Group.

Blood 1994, 84:1361-1392

REAL Classification

1. Precursor B-cell Lymphoma
    - Precursor B lymphoblastic leukemia/lymphoma
2. Mature or peripheral B-cell Lymphoma
    - Chronic lymphocytic leukemia/small lymphocytic lymphoma
    - Lymphoplasmacytoid lymphoma/immunocytoma
    - Mantle cell lymphoma
    - Follicle center lymphoma
        - cytologic grade 1
        - cytologic grade 2
        - cytologic grade 3
    - Marginal zone lymphoma
        - Extranodal (MALT type)
        - Nodal
        - Splenic
    - Hairy cell leukemia
    - Plasmacytoma/plasma cell myeloma
    - Diffuse large B-cell
    - Burkitt lymphoma

REAL Classification

3. Precursor T-cell Lymphoma

- Precursor T lymphoblastic leukemia/lymphoma

4. Peripheral T- and NK-cell Lymphoma

- Chronic lymphocytic leukemia/prolymphocytic lymphoma
- Large granular lymphoproliferative disorder
  - T-cell type
  - NK-cell type
- Mycosis fungoides/Sezary syndrome
- Peripheral T-cell lymphoma, unspecified
- Angioimmunoblastic T-cell lymphoma
- Angiocentric lymphoma (NK/T-cell)
- Intestinal T-cell lymphoma
- Adult T-cell lymphoma/leukemia
- Anaplastic large cell lymphoma

REAL versus KIEL versus Working Formulation

Kiel Classification (updated):

- used mainly in Europe, morphologic and immunologic
- excludes primary extranodal lymphomas
- does not address heterogeneity of follicular lymphomas
- morphologic subclassification of entities is difficult

Working Formulation:

- most widely used in US, relative simple
- no free-standing classification, “Lymphoma Esperanto”
- categories defined by survival data (treatment!)
- categories based on H&E only

REAL classification:

- practical feasibility and scientific validity
- build on existing classifications

Shortcomings of the REAL Classification for Dogs

- Grading of follicular lymphomas (relevance of associated diffuse areas)
- Grading of MALT-type lymphomas
- Classification of cutaneous lymphomas
- Diffuse large B-cell lymphoma are predominant, what do we do with centrocytic/centroblastic lymphomas
- Do Burkitt-like lymphomas exist in dogs?
- No standard for diagnosis of anaplastic large cell lymphoma

Another Lymphoma Classification?

Says WHO?

The World Health Organization Classification of Neoplastic Diseases of the Hematopoitic and Lymphoid Tissues: Report of the Clinical Advisory Committee Meeting - Airlie House, Virginia, November 1997.

J. Clin. Oncol. 1999, 17: 3835-3849.

Classification of Canine Lymphomas

Nodal T zone lymphoma: Same as intermediate form below but with dense chromatin without nuclear detail. (cf hyperplasia also has DCs, Mq)

CLL & small lymphocytic lymphoma: rare; small nuclei, few mitotic figures.

Lympho-plasmacytic: rare, nodal; small nuclei, few mitoses, eccentric nuclei.

Anaplastic large cell lymphoma: Uncommon. Always some multinucleate cells, very irregularly shaped nuclei (horseshoe, elongated); often vacuolated cytoplasm. Mostly T cell, can be B; r/o histiocytic sarc

Small (1-1.25 x RBC)

Large (≥2 x RBC)

Intermediate (1.5 x RBC)

Mitotic rate per hpf

1. Nodular or diffuse? **

2. Nuclear size? (vs. RBC)

LOW: 0-1/hpf

MED: 0-4/hpf

HI: >10/hpf

DLBCL very common; PTCL less common
Grade on mitoses per hpf: LO 0-6, MED 6-10, HI ≥11

Peripheral T cell lymphoma: aniso- & poikilo-karyosis

Diffuse large B cell lymphoma: uniform round nuclei.
- **Centroblastic**: uniformly shaped nuclei, multiple nucleoli impinging on nuclear membrane, scant cytoplasm.
- **Immunoblastic**: >90% of cells have uniform round nuclei with single central nucleolus. (r/o MZL—intermediate nuclei).
- **T cell rich large B cell lymphoma.** Few large B cells amid many small non-neoplastic T cells. Abundant fine stroma—no breaks in tissue. Diffuse. Mainly in horse & cat, rare in dogs.

Nodal T zone lymphoma of intermediate nuclear size. Filling of paracortex & cords, doesn't efface medullary architecture or subcapsular sinus, characteristic prominence of small venules, B follicles are pushed toward stromal trabeculae, ± sinus ectasia. Dense chromatin with faint nucleoli (cf small T zone lymphoma), abundant light cytoplasm, few mitotic figures.
(cf hyperplasia also has DCs, Mq). Transitions to PTCL.

Marginal zone lymphoma. Nodal or splenic, nodular, B cell. Often surrounds arteriole & collapsed mantle ± faded follicles. Mixed cellular appearance cf MCL. Peripheral chromatin (thick nuclear membrane) & central nucleolus are characteristic, abundant cytoplasm.
-cf. MZ hyperplasia retains mantle layer.
-cf. follicular lymphoma has no mantle, no central vessel/GC, uniform across follicle.
-Transitions to low grade immunoblastic.

Splenic mantle cell lymphoma: nodular dark cell proliferations around eosinophilic bare dendritic cells, no nucleoli (MCL) or small nucleoli (MCL blastoid). B cell.
-Always monomorphic cf MZL.
-A (usually) diffuse variant has central infarction & sharply defined hemorrhage, more cellular pleomorphism.

Burkitt-like : Uncommon. Diffuse B cell, many tingible body Mq. <1/hpf large nuclei, always round uniform nuclei ("peas in a pod"), multiple prominent nucleoli, aggregated chromatin. Transitions to DLBCL.

Lymphoblastic : Uncommon. Diffuse. Dispersed chromatin clouds the faint nucleoli. Usually intermediate size, most are T cell. Transitions to PTCL.

Centrocytic DIBCL

Follicular proliferations: nodal, not splenic

Benign follicular hyperplasia
- Retention of mantle: ring of dark cells between follicle and marginal zone.
- Cells have antigen-related polarity: deep dark zone (centroblasts) and light superficial zone (centrocytes), polarity best seen in follicles near capsule.
- Follicular tingible body macrophages present

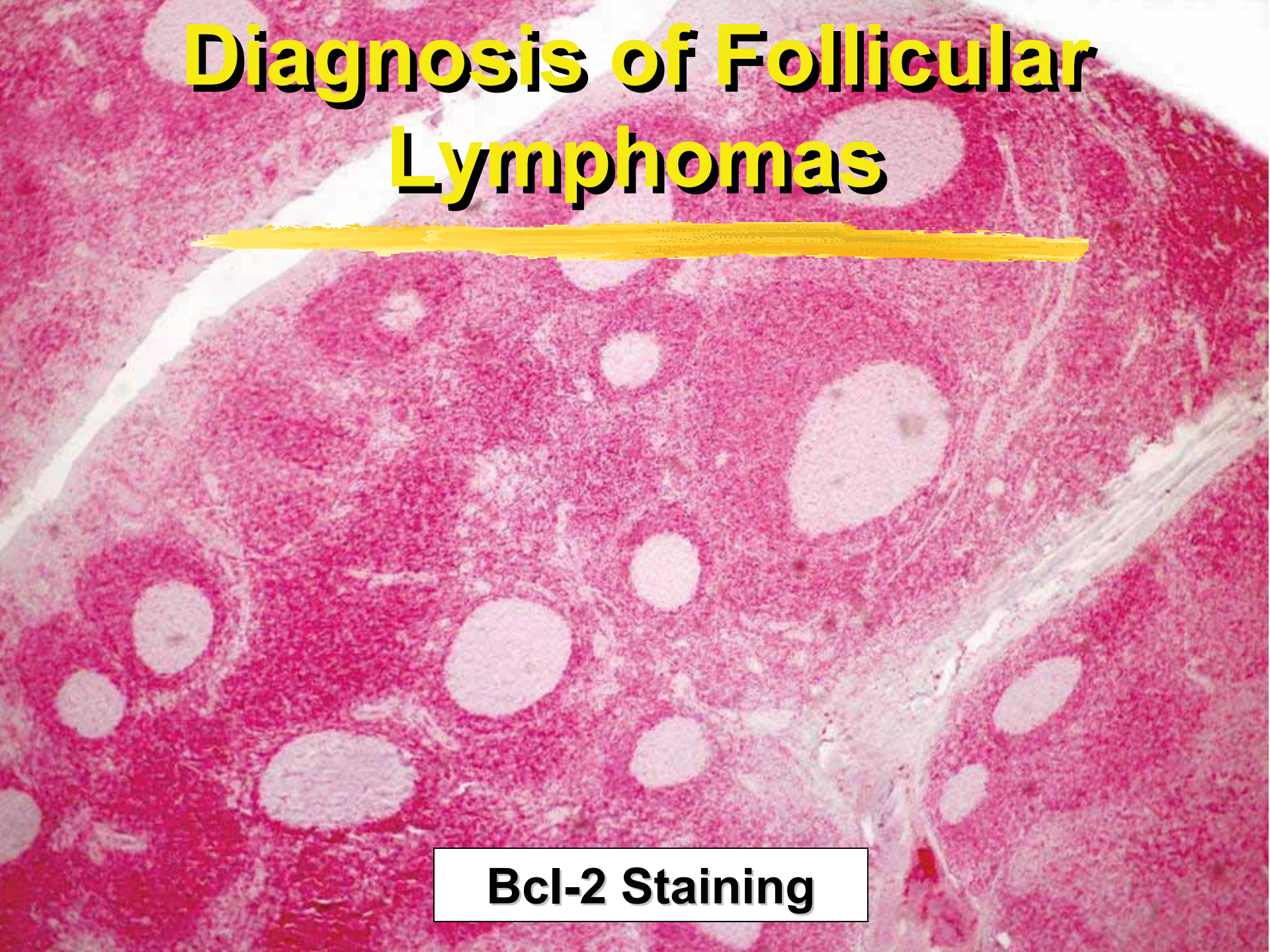
Atypical follicular hyperplasia
- Follicular coalescence, still has polarity and mantle.

Follicular lymphoma
- Never has big vessels in the nodule (cf MZL, MCL)
- Compared to hyperplasia, follicular lymphoma has:
  - No mantle cell cuff
  - Follicular tingible body macrophages absent
  - Loss of polarity; cell types in same proportion in all follicles, lack of normal variation
- Grade based on centroblasts per 400x: FL1= 0-5; FL2= 6-15; FL3= >15 (most are FL3)

Centrocytes: small cleaved nuclei, pale chromatin, 1 or 2 faint nucleoli, no mitoses, scant cytoplasm
Centroblasts: larger vesicular nuclei, multiple nucleoli impinging on nuclear membrane, scant cytoplasm.

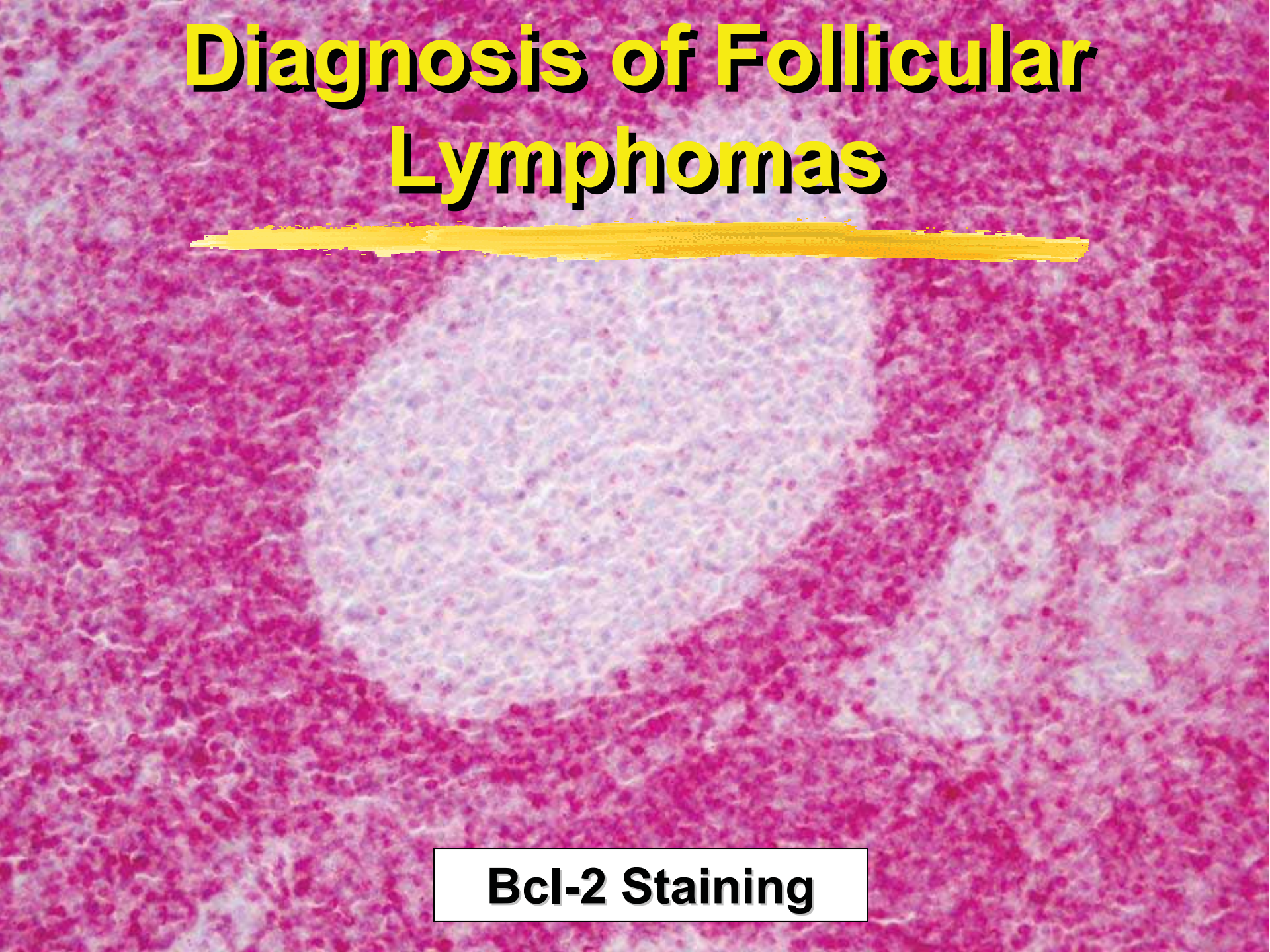
cf: whereas; DC: dendritic cell; GC: germinal centre; IB: immunoblastic; MCL: mantle cell lymphoma; MZL: marginal zone lymphoma; Mq: macrophage; PTCL peripheral T cell lymphoma.

Diagnosis of Follicular Lymphomas



Bcl-2 Staining

Diagnosis of Follicular Lymphomas



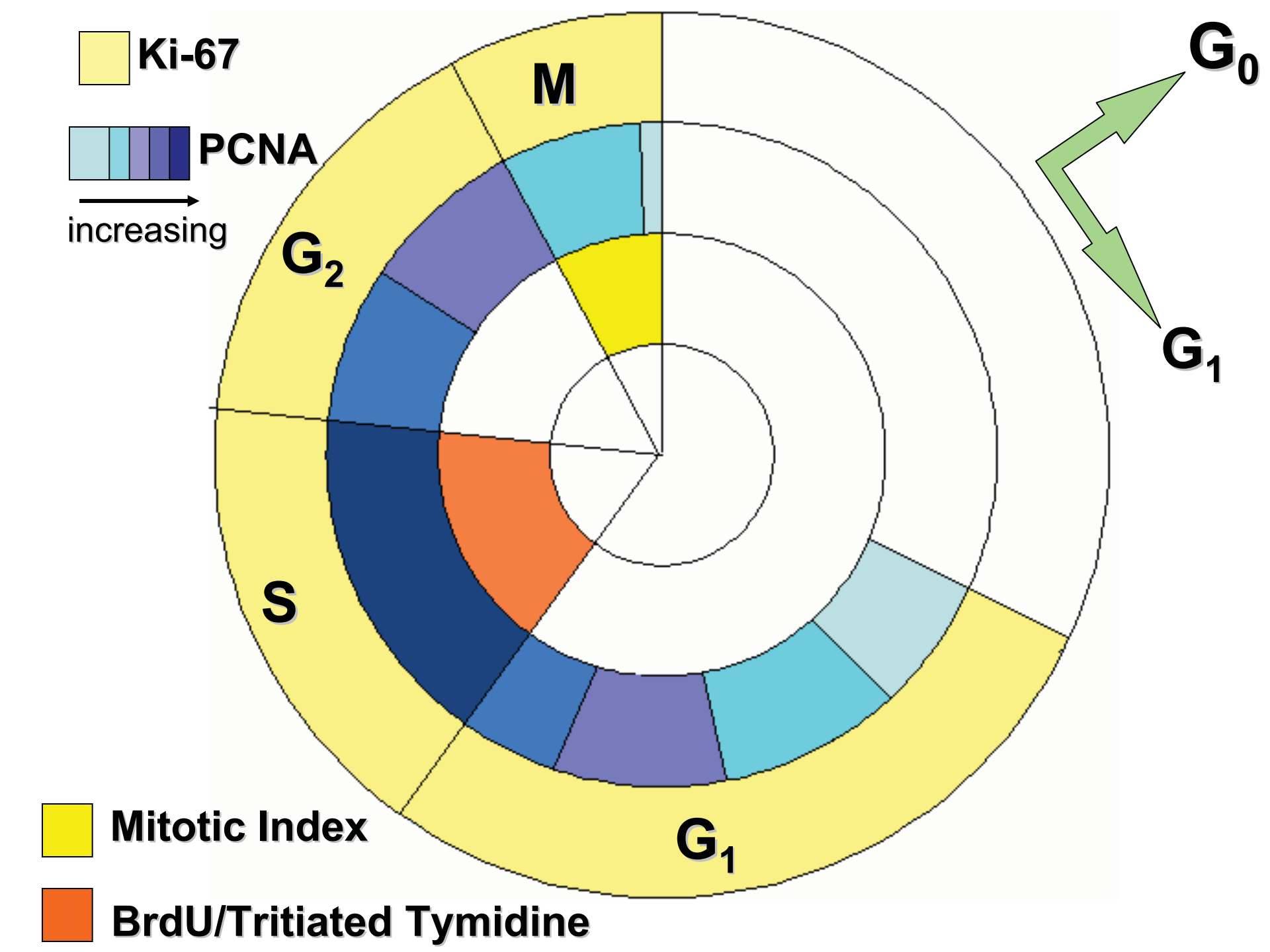
Proliferation of Neoplastic Cells

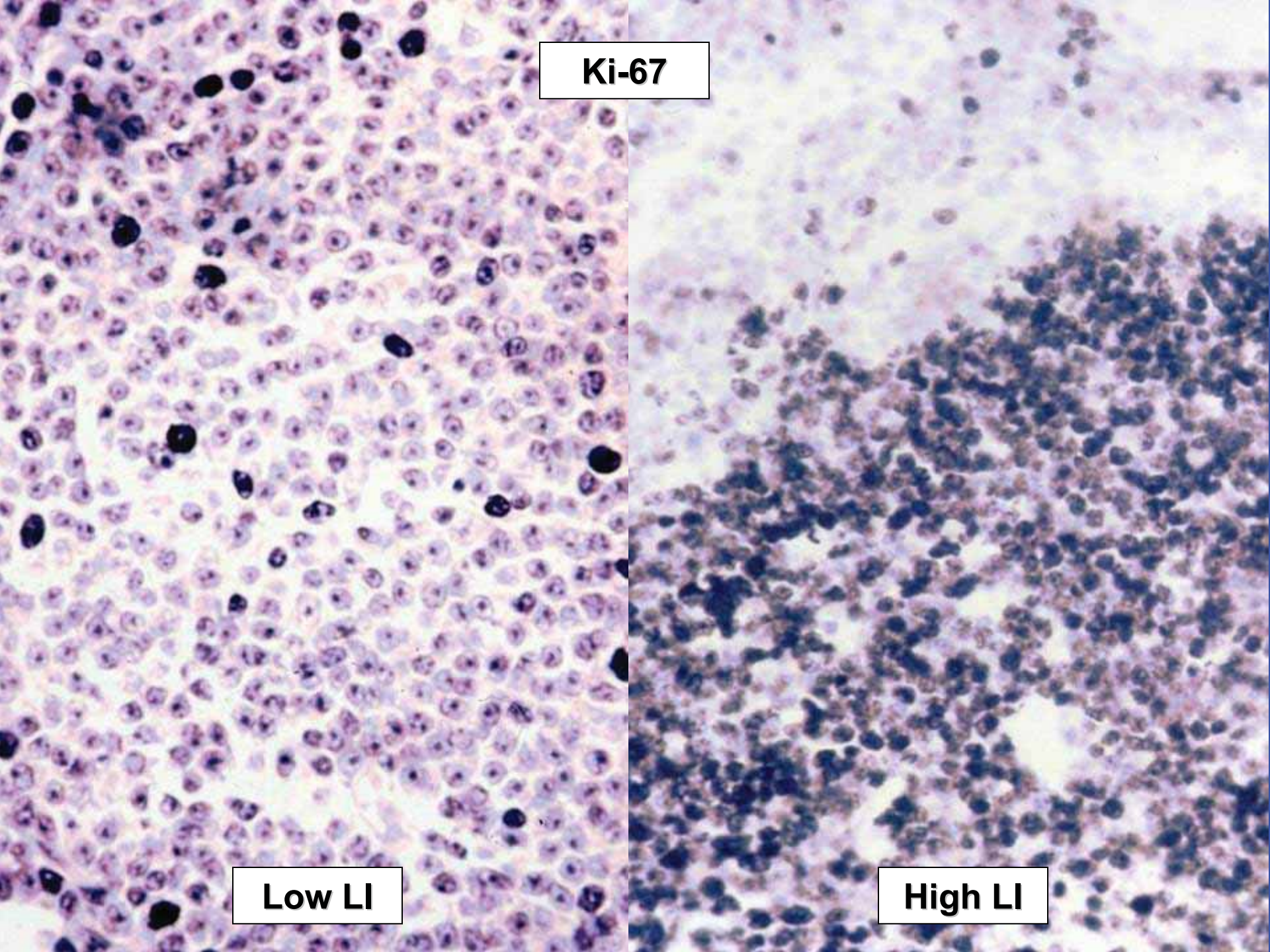
- Cellular proliferation
  - Generation Time (T)
  - Growth Fraction (G)
  - Proliferation = G x 1/T

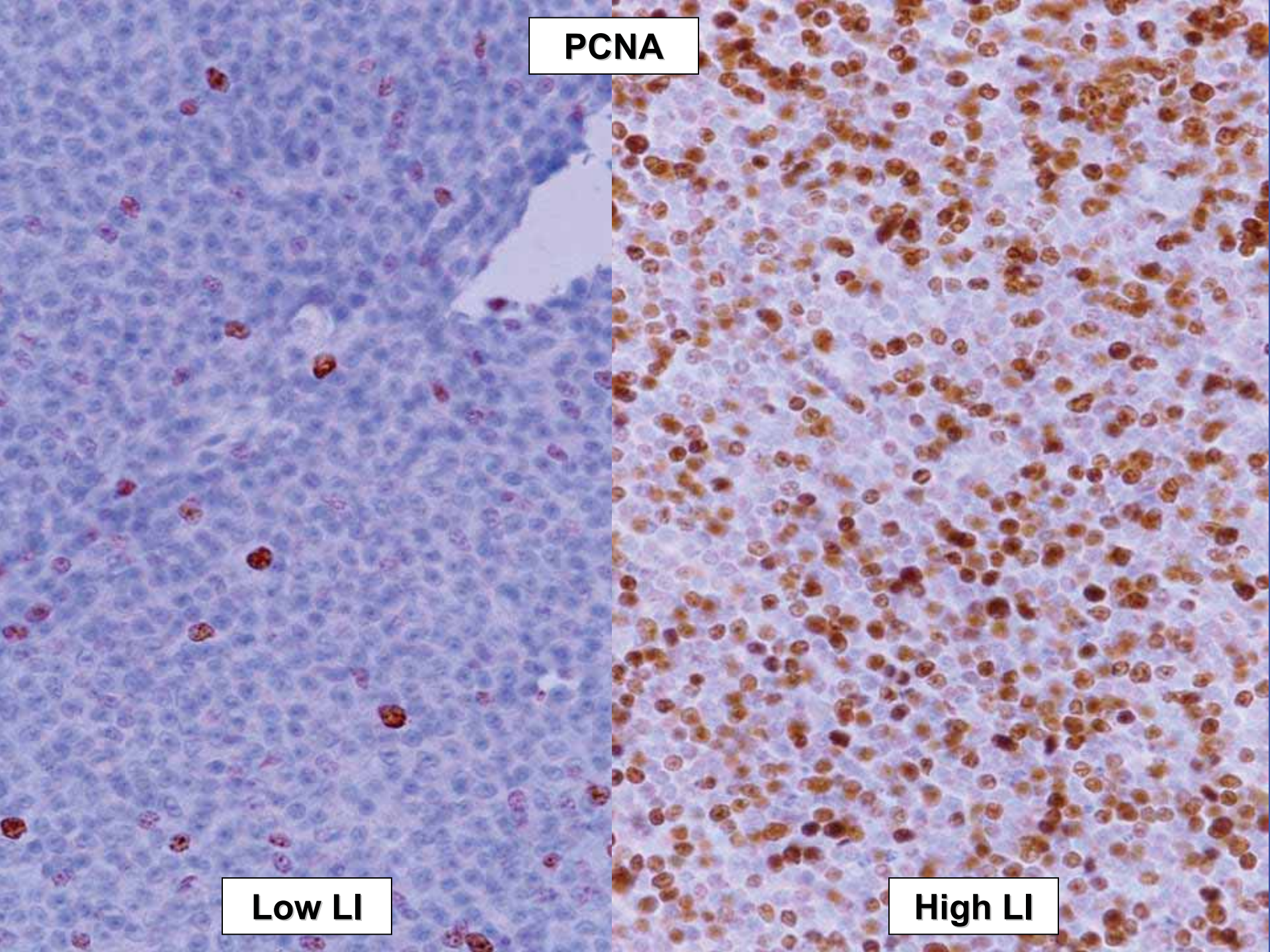
- Cellular loss
  - Apoptosis
  - Necrosis

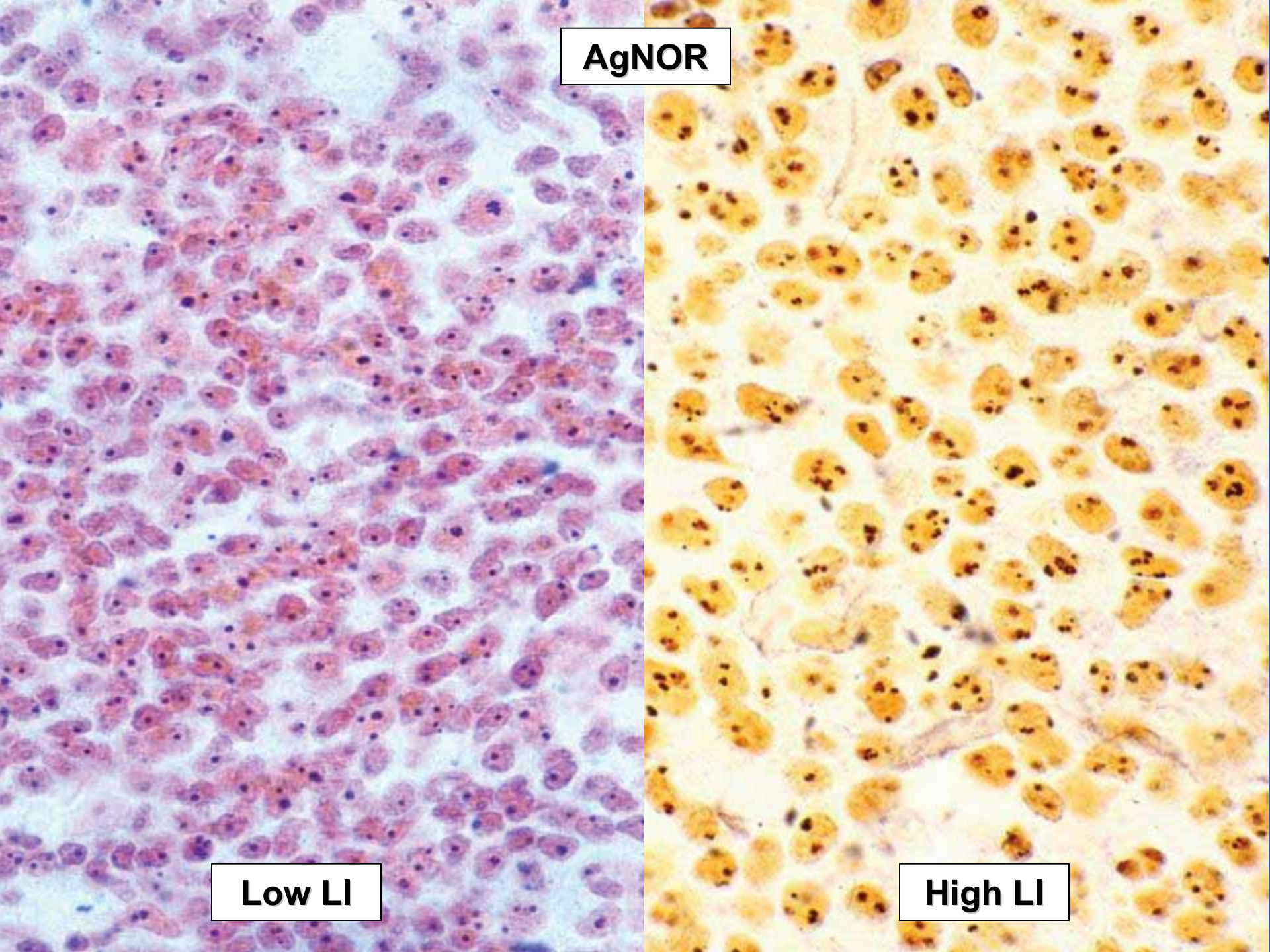
Evaluation of Tumor Kinetics

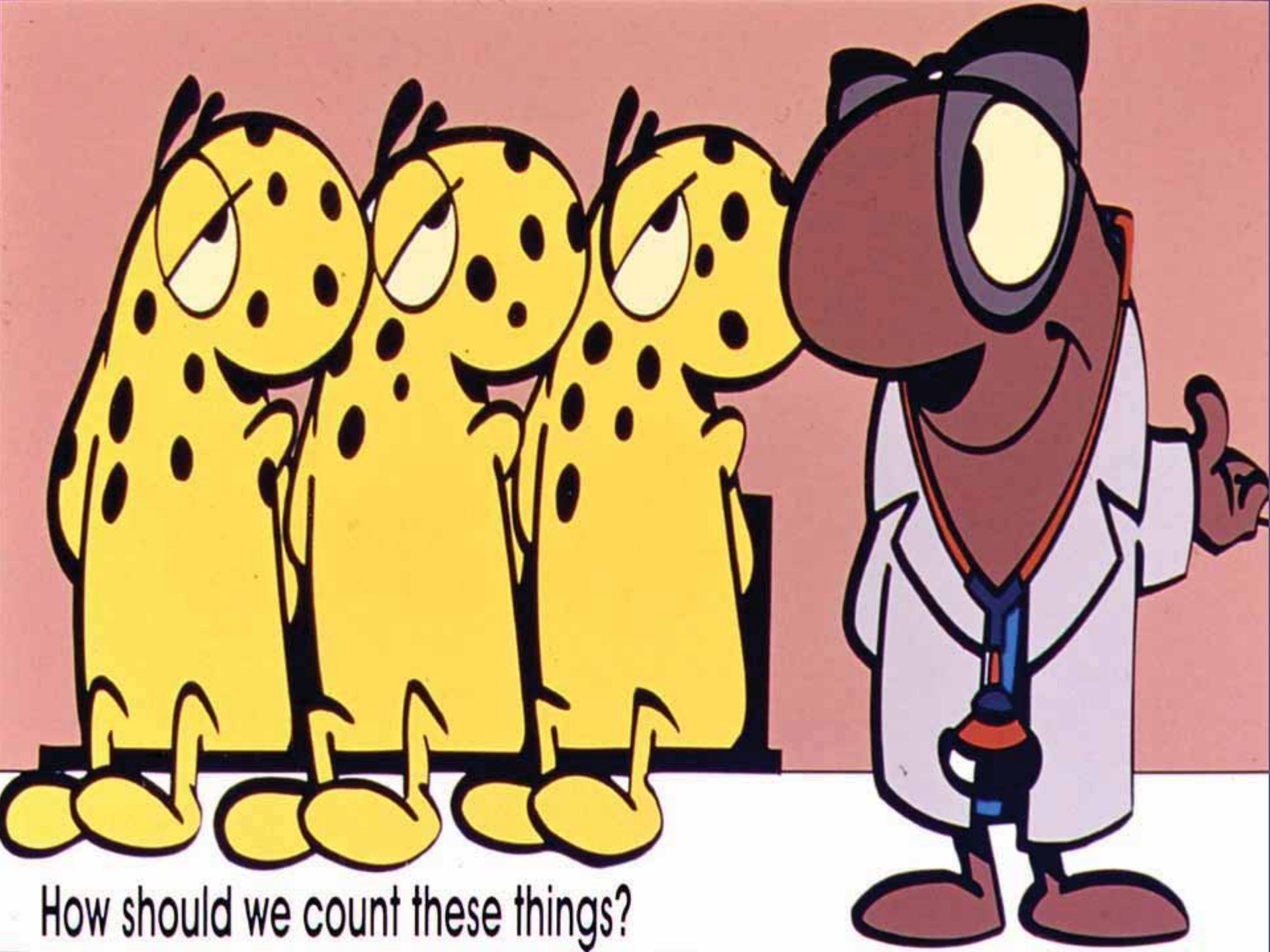
- The proliferation activity (P) is proportional to the growth fraction (G) and inversely proportional to the cell cycle duration (T)
- Assumption:
  - P = G *1/T
  - Ki-67 = G and AgNORs = 1/T
  - P = Ki-67 * AgNORs
- an increased S-phase index correlates to karyologic abnormalities (quality of proliferating cells):
- PCNA LI = Ki-67 LI * AgNORs


Ki-67
PCNA
increasing
M
G2
S
G1
G0
G1
Mitotic Index
BrdU/Tritiated Tymidine

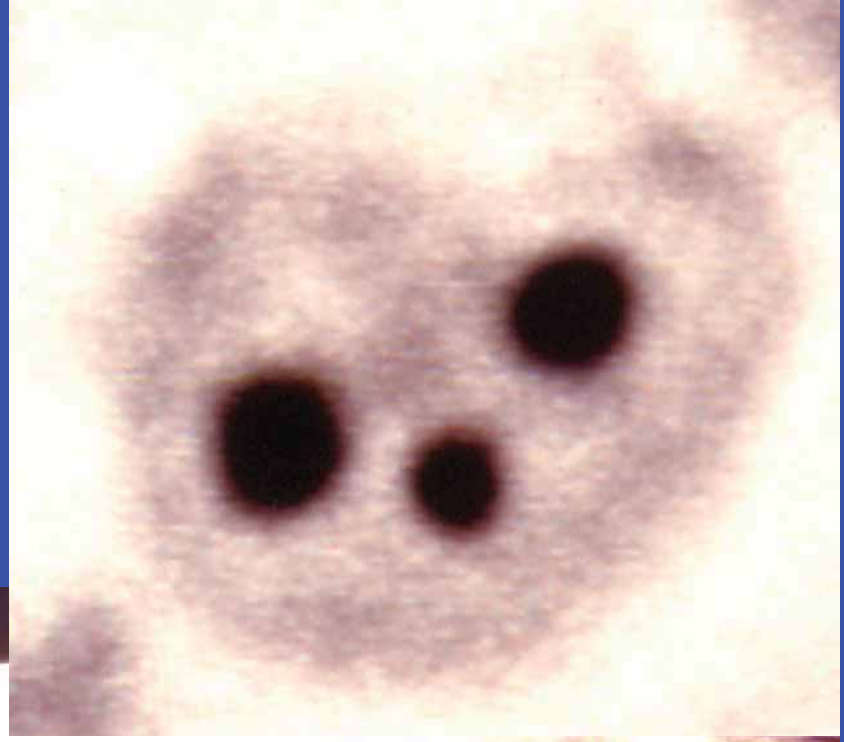

Ki-67
Low LI
High LI


PCNA
Low LI
High LI


AgNOR
Low LI
High LI


How should we count these things?

Quantitative Evaluation of AgNORs



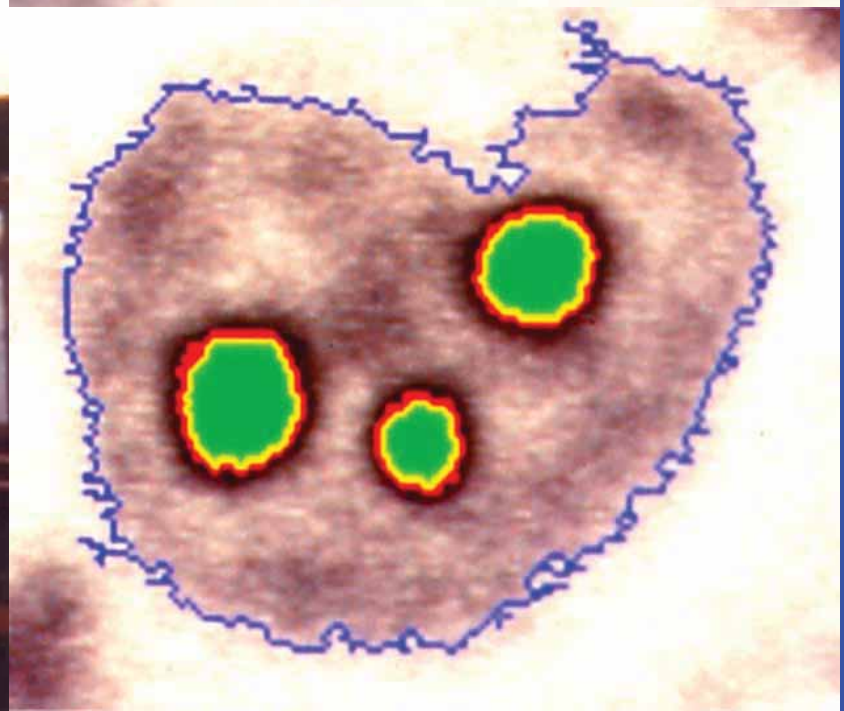
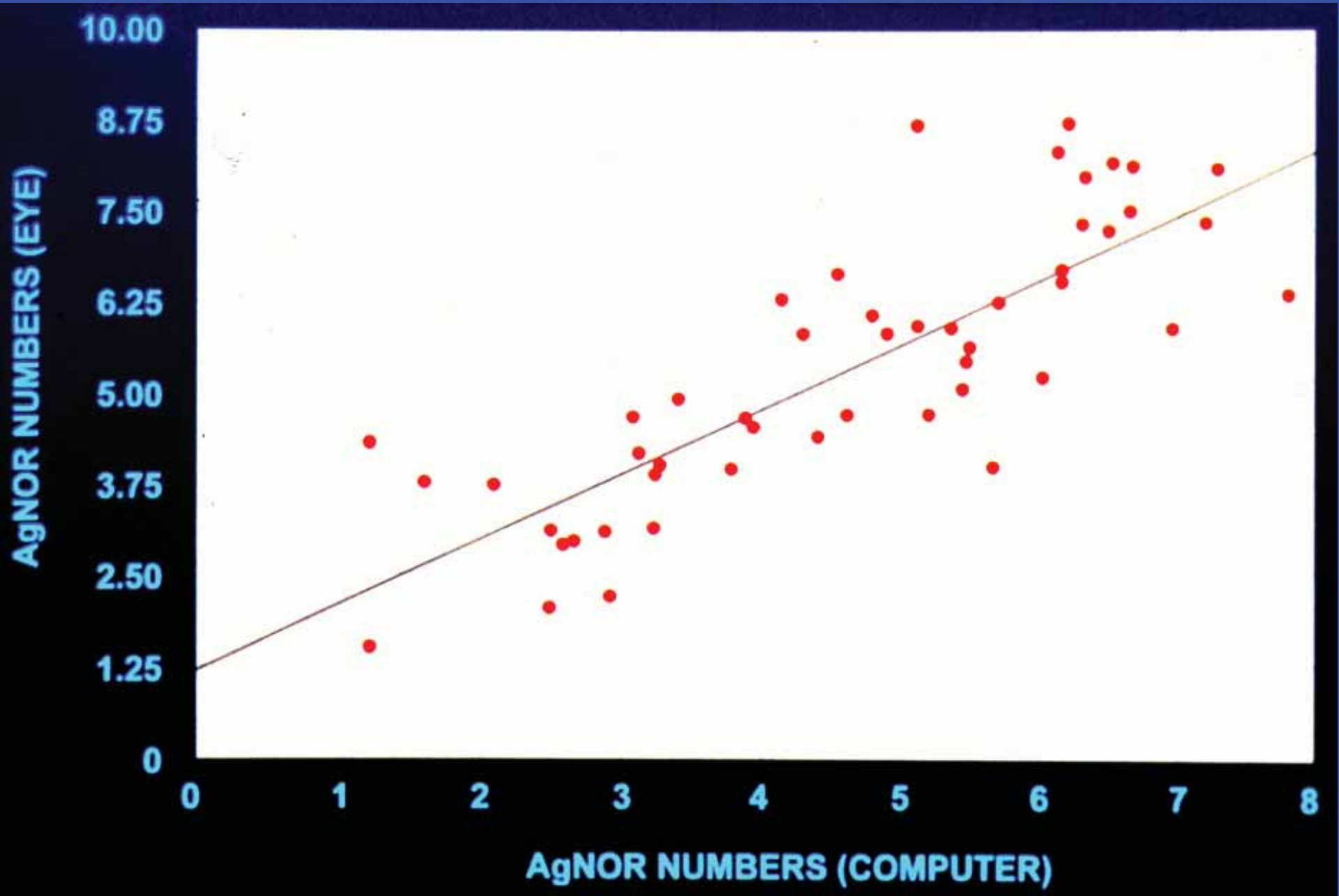


Image-Analysis versus Direct Counting



Study Population

- 74 peripheral lymph nodes from 74 dogs with malignant lymphoma:
  - Ln. mandibular
  - Ln. cervical
  - Ln. popliteal
- Age: - 2… 13 years (6… 8 years)
- Sex: - 36 female, 38 male

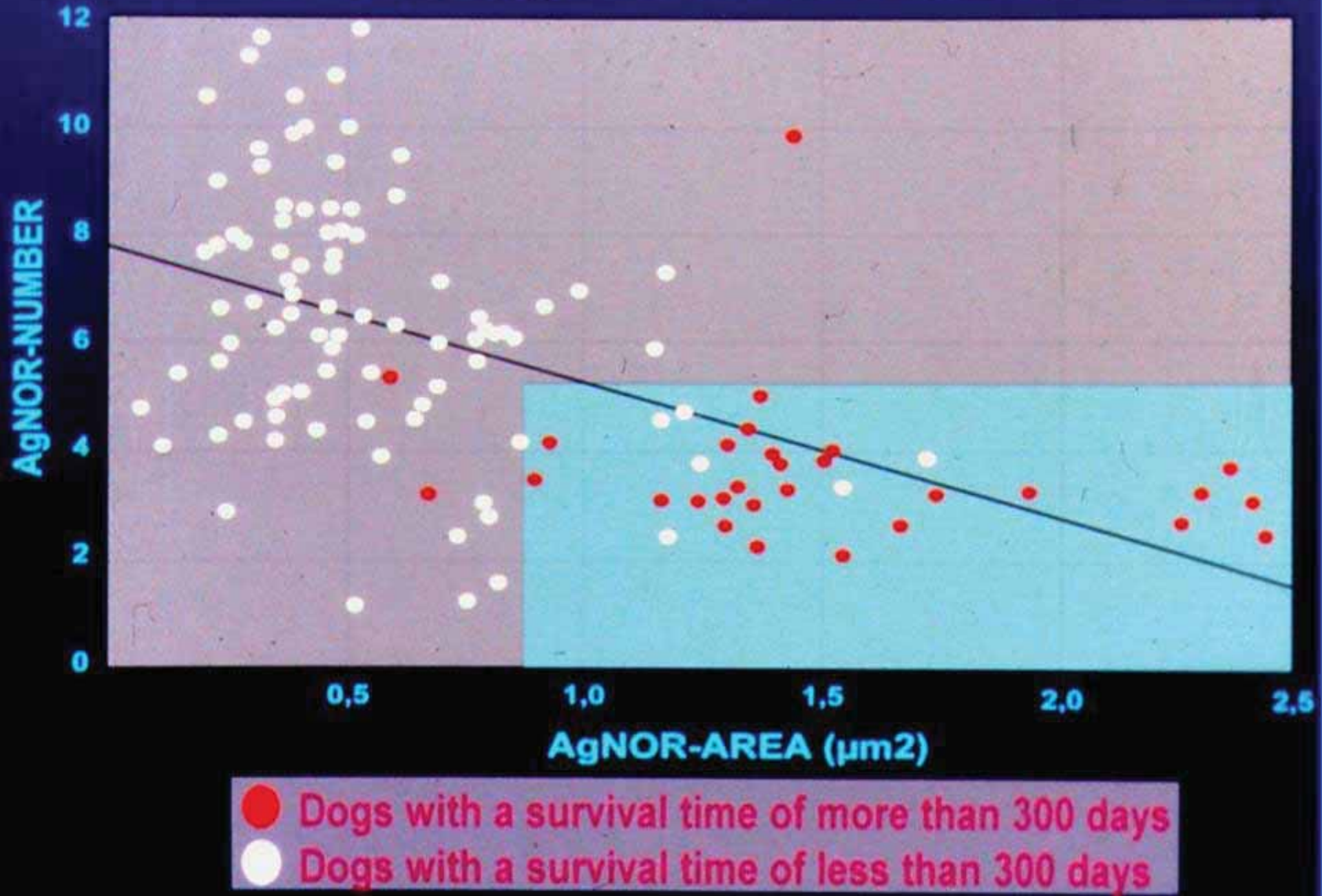
Methods

- Fixation in 10 % formal-saline, paraffin embedded
- Thickness of section: 3 μm
- HE-staining, Giemsa:
  - lymphoma diagnosis
  - Kiel Classification
  - Working Formulation
  - Mitotic Index
- Immunohistochemistry:
  - CD3
  - CD79a
  - PCNA
  - Ki-67
- AgNOR - silverstaining (Ploton et al., 1986):
  - quantitative evaluation:
    1. visual method
    2. image analysis
  - qualitative evaluation: staining- and distribution patterns

Treatment
(Utrecht)

Drug	Dosage	Week 1	2	3	4	5	6	7	8	9	10	11	12	14
Asparaginase	10 IU/kg	x	x		x		x			x		x	x	x
Vincristine	0,6 mg/m^2			x		x			x		x			
Cyclophosphamide	200 mg/m^2				x									
Doxorubicin	30 mg/m^2						x					x		
Chlorambucil	30 mg/m^2									x				
Prednisolone	0,5 mg/kg/daily			xx	xx	xx	xx	xx	xx	xx				

Correlation between AgNOR-number and AgNOR-area



Univariate analysis

Characteristic	Disease free survival		Survival	
	P-value	Hazard	P-value	Hazard
Age	0.079	0.8872	0.165	0.9249
Weight	0.733	0.6856	0.549	0.5316
Sex	0.436	0.7629	0.116	0.6409
Kiel-Classification	0.198	1.767	0.408	1.288
Working-Formulation	0.331	1.99	0.32	1.518
Immunophenotyp	0.932	1.032	0.239	0.6987
PCNA 0-20	0.266	1.2276	0.009	1.2682
21-40	0.436	1.47	0.457	1.368
41-60	0.14	2.124	0.033	2.506
>60	0.059	2.875	0.003	3.89
Ki-67	0.207	1.24	0.078	1.405
Mitotic index 0-9	0.137	1.17	0.028	1.38
10-12	0.39	1.597	0.287	1.67
>12	0.052	17.36	0.005	6.38
Mean AgNOR-number				
0-3	0.001	1.543	0.001	1.227
3-6	0.399	1.772	0.182	1.877
>6	0.008	6.647	0.001	9.34
Mean AgNOR-area				
0-0.5	0.001	0.6546	0.001	0.475
0.5-1.0	0.014	0.2647	0.022	0.406
>1.0	0.001	0.076	0.001	0.0314
Maximal AgNOR-area				
0-1.0	0.001	0.5163	0.001	0.5965
1.0-1.5	0.574	0.7783	0.095	0.5625
1.5-2.0	0.004	0.1797	0.001	0.1784
>2.0	0.001	0.136	0.001	0.1161
AgNOR type 1	0.118	0.865	0.008	0.8822
AgNOR type 2	0.889	1.098	0.668	1.249
AgNOR type 3	0.32	1.762	0.147	1.968
AgNOR type 4	0.012	28.28	0.007	10.68
AgNOR type 5	0.082	3.063	0.007	3.889

Multivariate analysis

Characteristic	Survival time (n=48) P-value	Hazard ratio	95% C-intervall
Immunophenotype			
B-cell lymphoma			
T-cell lymphoma	0.027	2.997	1.134 - 7.919
MEANAR			
0 - 0.5000	< 0.001	0.443	0.0714 -2.748
0.5001 - 1.0000	< 0.001	0.1053	0.0287 - 0.3859
> 1.0000	< 0.001	0.0058	0.0007 - 0.0453
AR_RAT			
0 - 110	0.008	2.513	0.3169 -19.92
> 110	0.008	37.45	2.529 - 554.6
MAXDIST			
0 - 0.425	0.009		
0.425 - 0.500	< 0.001	13.16	2.950 - 58.74
0.500 - 0.575	0.005	10.64	2.012 - 56:32
> 0.575	0.039	6.278	1.100 - 35.82

P = 0.026

Characteristic	Disease free survival time (n=48) P-value	Hazard ratio	95% C-intervall
NORNBC			
0 - 3.00	< 0.001	2.456	0.7095 -8.5
3.01 - 6.00	0.002	26.09	3.285 - 207.2
> 6.00	< 0.001	63.80	5.782 - 704.0

Conclusions

- Dogs with B-cell lymphoma have a better prognosis for the total survival time than dogs with T-cell lymphomas
- Lower total survival time correlated with a larger average area of AgNORs , a shorter distance between two AgNORs and a smaller ratio of AgNOR area/nucleus
- Longer disease free survival time correlated with a smaller number of AgNORs per nucleus
- Extended studies are necessary to analyze other parameters more accurately

Summary

- Staging and immunophenotyping of canine malignant lymphomas (CML) are prognostically significant and should be routinely performed
- Orientation on REAL/WHO classification should be used to microscopically classify CMLs
- However, the prognostic significance of this classification is unknown and modification for dogs will be necessary!!!!!

Summary

- Proliferaration marker seem to be promising for prognostic evaluation
- Molecular marker are the future!
- To determine the prognostic significance of a marker it is necessary to:
    - have sufficient case numbers in a study
    - use uni-/multivariate survival analysis
    - evaluate prognosis in context to treatment

Molecular Based Diagnostic Tests in Canine Hematologic Malignancies

- Detection of individual mutations in oncogenes: c-kit, p53
- Detection of chromosomal translocations, deletions and duplications: bcr-abl
- Detection of clonality in lymphomas

Response to Cytotoxic Drugs