Detecting secondary follicles in the spleen and lymph nodes: an exploratory study case to compare image analysis on IHC slides versus AI-based detection on H&E-stained slides

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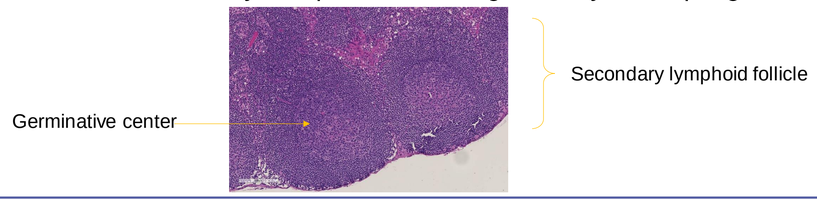
Background:

- Histopathological evaluation of the immune organs plays a major role in the efficacy and safety assessment of new chemical entities and biotherapeutics.
- In the context of the development of a new drug candidate for autoimmune diseases, a 48-day study in the cynomolgus monkey including a T-cell Dependent Antibody Response assessment (KLH immunization by subcutaneous route on Day 3 and Day 24) was conducted. Samples of lymphoid organs were obtained for some animals (one mid-dose and four high dose animals) and processed for microscopic examination.
- The main microscopic change was a decrease in the secondary follicle area and further characterization of the findings was performed. Slides from stock animals and KLH control animals from another study (identical study design with two KLH immunizations) were used for comparison purposes.

Hypothesis and objectives of the study:

- In this exploratory study case, we wanted to evaluate whether a deep learning AI-based algorithm could be developed in order to accurately detect secondary lymphoid follicles on H&E-stained slides.
- The detection of secondary lymphoid follicles on H&E-stained slides is challenging as it relies on subtle morphological criteria. Secondary follicles are round to ovoid structures consisting mainly of lymphocytes; their main characteristic is the presence of a 'germinative (or germinal) center' which forms upon antigenic stimulation. Germinative centers stain less intensely than the surrounding lymphoid tissue due to a lower cell density and presence of tingible body macrophages and apoptotic B-cells.



Immunohistochemistry and image analysis

Methods:

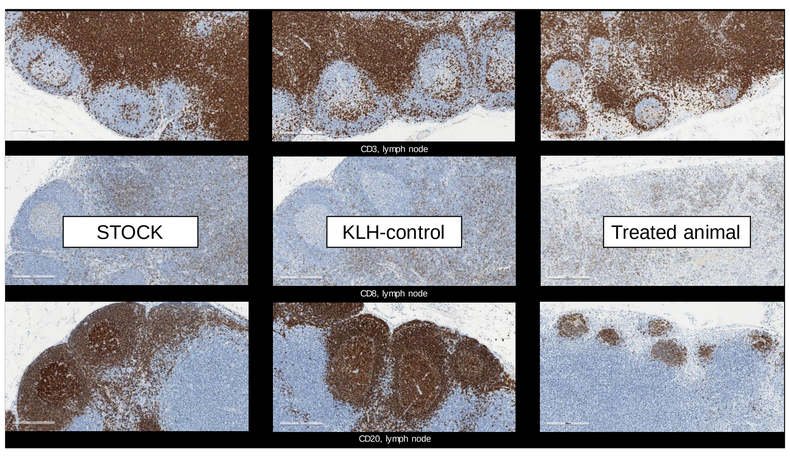
- The immunohistochemistry for T lymphocytes (pan T cell and cytotoxic T cell) and B lymphocytes was performed on the spleen and mesenteric lymph nodes from treated animals (one mid-dose and four high dose animals), three stock monkeys and four control monkeys having received 2 KLH administration using a Discovery Ultra automate from Roche Ventana. All slides were counter-stained with hematoxylin and mounted in resin. Isotypic negative controls were added for each run. Shortly, all primary antibodies used were incubated for 60 minutes at 37°C and epitope retrieval was performed using HIER in a CC1 buffer:
  - Anti-CD3 Rabbit Monoclonal (2GV6), ROCHE, ref: 760-4341 / 05278422001, 0.4 µg/mL,
  - Anti-CD8 (SP57) Rabbit Monoclonal Primary Antibody, ROCHE, ref: 790-4460/ 05937248001, 0.35 µg/mL,
  - Anti-CD20 Mouse monoclonal (L26) Primary Antibody, ROCHE, ref: 760.2531 / 05267099001, 0.3 µg/mL.
- All slides were scanned at 20X with a Leica whole slide scanner AT2® and image analysis was conducted on the CD20-stained slides using Qupath 0.2.3 (an open source, freely available software for whole slide image analysis) as followed:

Automatic detection of the tissue → Automatic detection of the lymphoid follicles (i.e. structures showing high CD20-stained cell density) → Export of the results to excel → Data visualization using Qliksense

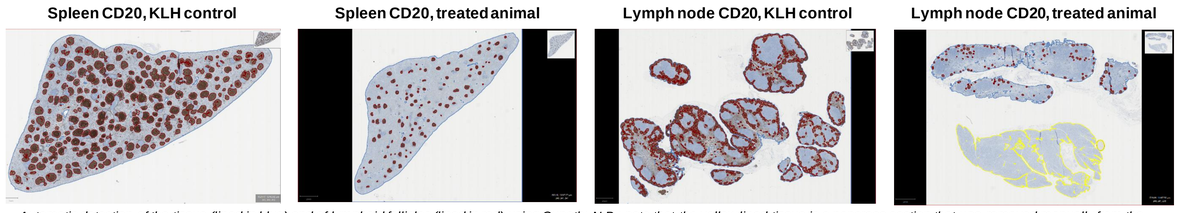
- Data:
  - Total tissue area
  - Number of lymphoid follicle (absolute and per µm²)
  - Total lymphoid follicle area
  - Total lymphoid follicle area per µm² of tissue (i.e. total lymphoid follicle divided by the total tissue area)
  - Mean lymphoid follicle area (i.e. total lymphoid follicle area divided by the number of lymphoid follicles)

Results:

- When compared to the stock animals and KLH-treated animals, the spleen and mesenteric lymph node from treated animals showed:
  - Almost no effect on the T cell population,
  - A marked decrease in the B cell population characterized by a decrease in the lymphoid follicle area in both the spleen and lymph node with almost complete absence of germinal centers.
- The image analysis performed on CD20-immunostained sections showed that both the surface and number of lymphoid follicles were decreased in the spleen and that the main effect observed in the mesenteric lymph node was a decreased surface of lymphoid follicle with no or only limited effect on the number. No differences were observed between the stock animals and the KLH control monkeys.

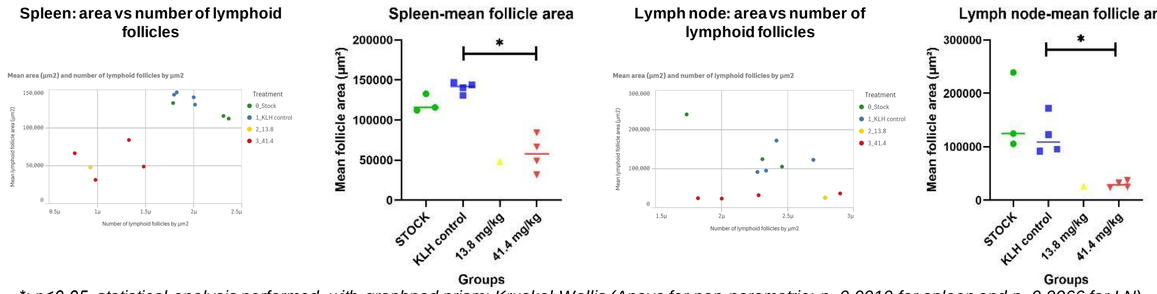


Spleen CD20, KLH control | **Spleen CD20, treated animal** | **Lymph node CD20, KLH control** | **Lymph node CD20, treated animal**



Automatic detection of the tissue (lined in blue) and of lymphoid follicles (lined in red) using Qupath; N.B.: note that the yellow-lined tissue is a pancreas section that was removed manually from the analysis when present on the slide

Spleen: area vs number of lymphoid follicles | **Spleen-mean follicle area** | **Lymph node: area vs number of lymphoid follicles** | **Lymph node-mean follicle area**



**: p≤0,05. statistical analysis performed with graphpad prism: Kruskal-Wallis (Anova for non-parametric: p=0.0010 for spleen and p=0.0066 for LN) followed by Mann-Whitney (t-test for non-parametric: p=0.0286 for the comparison KLH control versus 41.4 mg/kg for both spleen and LN)*

N.B.: the total tissue area was relatively homogenous for spleen sections but a very high inter-individual variability was noted for mesenteric lymph node (data not shown)

AI-based algorithm for secondary follicle detection

Methods:

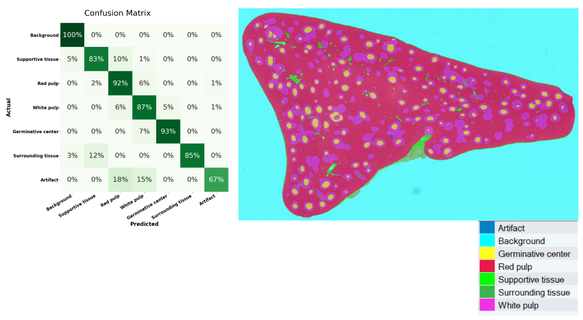
- H&E-stained slides of monkey spleen and mesenteric lymph node were scanned at 20X on a Leica AT2® whole slide scanner and uploaded to Patholytix Preclinical Study Browser.
- Two distinct classifiers were created: one for the spleen and one for the mesenteric lymph node. The training annotations were done at 5X by a qualified pathologist. Few tiles and/or structures were annotated for each slide (i.e. 12 slides for spleen and 11 slides for mesenteric lymph node). Convolutional neural network (CNN) models were developed to create AI generated masks. The models were then tested comparing the algorithm performance to the pathologist and, when necessary, annotations were added. Qualification at the pixel level using confusion matrices and F1 scores were issued.
- After the first version of the mask has been issued, the main challenges encountered were:
  - Presence of artifacts like dusts: an additional class called 'artifact' was added,
  - Margins of the normal tissue class called 'supportive tissue' were not always well defined in the first version probably because only few annotations had been done initially for this class: annotations were added for this class.
- Validation of the two classifiers was performed on 15% of unseen annotated data. Confusion matrices showed that the CNN models were able to identify the different classes with good consistency with actual annotations. In particular, the sensitivity for germinative center were 93% (spleen) and 79% (lymph node) respectively.
- After validation of the two classifiers, the following parameters were assessed:
  - Total area of the germinative center class (µm²)
  - Tissue area (µm²) corresponding to the tissue of interest area:

Spleen: Germinative center + White pulp + Red pulp + Supportive tissue

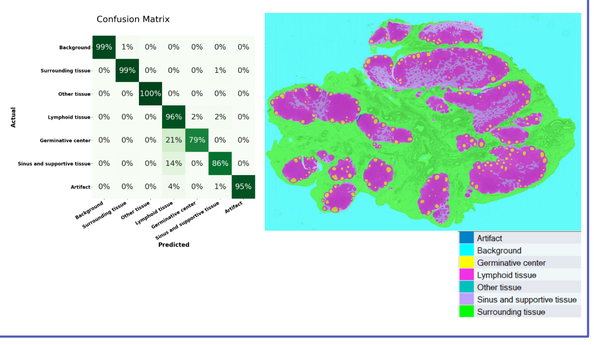
Mesenteric lymph node: Germinative center + Lymphoid tissue + Sinus and supportive tissue

  - Germinative area expressed as % of tissue

Spleen classifier



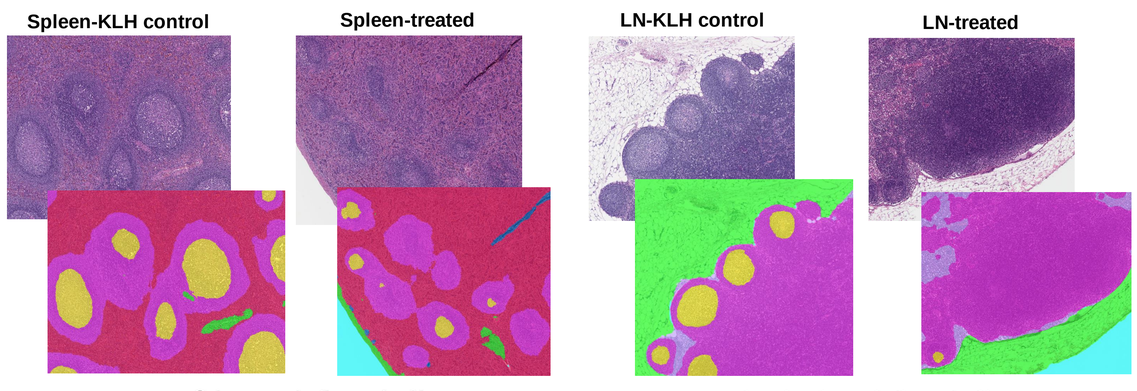
Mesenteric lymph node classifier



Results:

- The germinative center areas (relative to the total tissue area) for both spleen and mesenteric lymph node were higher in 3 out of 4 KLH control animals when compared to the stock animal results. This increase in germinative center might be related to the antigenic stimulation (KLH immunization) and was considered as biologically relevant.
- When compared to the KLH controls, the treated animals showed a moderate or marked decrease in the germinative center area (relative to the total tissue area) confirming the qualitative assessment done on H&E slides.

Spleen-KLH control | **Spleen-treated** | **LN-KLH control** | **LN-treated**



Spleen-germinative center AI | **Lymph node-germinative center AI**

**: p≤0,05. statistical analysis performed with graphpad prism: Kruskal-Wallis (Anova for non-parametric: p= 0.0054 for spleen and p=0.0211 for LN) followed by Mann-Whitney (t-test for non-parametric: p=0.0286 for the comparison KLH control versus 41.4 mg/kg for both spleen and LN)*
H&E slides and IHC slides were from the same animal/same block (except the lymph node H&E slide missing for a stock animal)

Conclusion:

1. The CNN models accurately identified secondary lymphoid follicles on H&E-stained sections despite the low number of control and treated samples used in the training sets.
2. The methodologies used in this study case provided complementary information:
   - Image analysis on IHC slides: provided quantitative data directly related to the CD20-positive B lymphocytes. The script used in Qupath gave 'object-based' information (the 'object' being the entire lymphoid follicle with no distinction between primary or secondary follicles). The limitations of the method were mainly related to the additional wet lab work (IHC staining).
   - AI-based analysis on H&E slides: provided quantitative data on the surface of germinative centers. The classification mask allowed to quickly visualize a potential decrease in the secondary follicle area. Interestingly, this method allowed to discriminate between the KLH-control and the stock animals (without KLH immunization) and to visualize the decrease in secondary follicle area after treatment with an immunosuppressive compound.
3. Use case : These exploratory classification AI masks could be used by the study pathologist as decision support tools to 1) quickly visualize compound-related effects on secondary follicles, and 2) simplify and improve quantification of the scoring of such effects. Further characterization of the cell type involved may be achieved with IHC.

Literature:

(1) Willard-Mack CL. Normal Structure, Function, and Histology of Lymph Nodes. Toxicologic Pathology, 2006;34:409-24
(2) Cesta MF. Normal Structure, Function, and Histology of the Spleen. Toxicologic Pathology, 2006;34:455-465