ARTICLE - Hodgkin Lymphoma

PDL1 shapes the classical Hodgkin lymphoma microenvironment without inducing T-cell exhaustion

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Received: September 25, 2021.
Accepted: June 9, 2022.
Early view: July 14, 2022.

https://doi.org/10.3324/haematol.2022.280014

Supplementary Tables

Antibodies for Immunohistochemistry

TARGET	CLONE	VENDOR	CATALOGUE NO.	DILUTION	STRIP VALIDATION
CD68	KP1	Dako	GA609	1:8000	Pass
CD3	LN10	Leica	CD3-565-L-CE	1:500	Pass
CD4	4B12	Leica	368-L-CE	1:500	Pass
CD8	C8/144B	Dako	M7103	1:400	Fail
CD30	BerH2	Dako	M0751	1:200	Pass
EOMES	R poly	Atlas	HPA028896	1:200	Pass
FOXP3	263A/E7	Abcam	ab20034	1:500	Pass
GATA3	D13C9	Cell Signaling	5852	1:50	Unassessed
LAG3	R poly	Atlas	HPA013967	1:200	Pass
LMP1	CS.1-4	Dako	M0897	1:12000	Fail
MHC-II	CR3/43	Dako	M0775	1:500	Pass
PD1	NAT105	Abcam	ab52587	1:25-50	Pass
PD1	EH33	Cell Signaling	43248	1:50	Pass
PD1	D4W2J	Cell Signaling	86163	1:50	Pass
PDL1	E1L3N	Cell Signaling	13684	1:200	Pass
TBET	4B10	Santa Cruz	sc21749	1:500	Pass
TIM3	G poly	R&D systems	AF2365	1:200	Pass
RORγT	6F3.1	Merck	MABF81	1:1000	Pass

R poly = rabbit polyclonal, G poly = goat polyclonal

Antibodies for Imaging Mass Cytometry

TARGET	CLONE	VENDOR	CATALOGUE NO.	DILUTION	METAL TAG
ALPHA SMA	1A4	Fluidigm	3141017D	1:200	141Pr
HLA-DR	LN3	Biolegend	327002	1:50	142Nd
CD34	ICO115	Cell Signaling	3569BF	1:25	143Nd
CD14	EPR3653	Fluidigm	3144025D	1:100	144Nd

TBET	D6N8B	Fluidigm	3145015D	1:50	145Nd
CD16	EPR16784	Fluidigm	3146020D	1:50	146Nd
CD163	EDHu-1	Fluidigm	3147021D	1:100	147Sm
CD30	E4L4I	Cell Signaling	54535BF	1:40	148Nd
CD10	Polyclonal	R&D Systems	AF1182	1:50	150Nd
CD31	EPR3094	Fluidigm	3151025D	1:100	151Eu
CD45	CD45-2B11	Fluidigm	3152018D	1:100	152Sm
LAG3	D2G40	Fluidigm	3153028D	1:50	153Eu
CD11C	D3V1E	Cell Signaling	45581BF	1:50	154Sm
FOXP3	236A/E7	Fluidigm	3155016D	1:50	155Gd
CD4	EPR6855	Fluidigm	3156033D	1:200	156Gd
IRF4	Polyclonal	R&D Systems	AF5525	1:62.5	158Gd
CD68	KP1	Fluidigm	3159035D	1:50	159Tb
CD20	H1	Fluidigm	3161029D	1:400	161Dy
CD8A	C8/144B	Fluidigm	3162034D	1:100	162Dy
TIM3	D5D5R	Cell Signaling	45208BF	1:25	164Dy
PDL1	E1L3N	Cell Signaling	13684BF	1:50	165Ho
B7H4	H74	Fluidigm	3166030D	1:50	166Er
GRB	EPR20129-217	Fluidigm	3167021D	1:100	167Er
KI67	B56	Fluidigm	3168022D	1:50	168Er
COL1	Polyclonal	Fluidigm	3169023D	1:300	169Tm
CD3	Polyclonal	Fluidigm	3170019D	1:100	170Er
CX3CR1	8E10.D9	Biolegend	824001	1:25	171Yb
PD1	EH12.2H7	Cell Signaling	86163BF	1:33	172Yb
CD45RA	HI100	Biolegend	304102	1:66	173Yb
CD206	E2L9N	Cell Signaling	91992BF	1:33	175Lu
HISTONE H3	D1H2	Fluidigm	3176023D	1:300	176Yb
DNA1	DNA Intercalator	Fluidigm	201192B	1:1000	191Ir
DNA2	DNA Intercalator	Fluidigm	201192B	1:1000	193Ir

Reagents for Immunohistochemistry

REAGENT	MANUFACTURER	ITEM CODE	DILUTION
ANTIGEN UNMASKING SOLUTION	Vector	H3300	1:100
ANTIBODY DILUENT	Zytomed	ZUC025-500	1:1
SUPERSENSITIVE™ POLYMER-HRP	Biogenex	QD440-XAKE	neat
DAB SUBSTRATE	Biogenex	QD440-XAKE	As per kit
VIP PEROXIDASE (HRP)	Vector	SK-4600	As per kit
HAEMOTOXYLIN SOLUTION GILL II	Sigma	GH216	
SCOTT'S SOLUTION	Sigma	S5134-6X	1:10
DPX MOUNTANT	Sigma	06522	neat

Antibodies for Flow Cytometry

ANTIGEN	VENDOR	FLUORO	CAT #	CLONE	DIL/TEST
DAPI	BD Bio	*live/dead*	564907	-	1:2000
CFSE	Thermo	*proliferation*	C34554	-	
CD3	Biolegend	BV421	300434	UCHT1	2.5 µl
CD3	BD Bio	FITC	561806	UCHT1	2.5 µl
CD3	Biolegend	PercP-Cy5.5	300430	UCHT1	2.5 µl
CD3	Biolegend	*functional*	300438	UCHT1	10 µg/ml
CD4	Biolegend	APC-Fire 750	344638	SK3	2.5 µl
CD4	Biolegend	APC-Cy7	300518	RPA-T4	1 µl
CD4	Biolegend	PE-Cy7	317414	OKT4	1 µl
CD25	Thermo	APC-efl780	47-0251-82	CD25-4E3	1µl
CD28	Biolegend	*functional*	302934	CD28.2	1µg/ml
CD127	Biolegend	FITC	351312	A019D5	1µl
CD183	BD	PE-Cy5	551128	1C6/CXCR3	2µl
CD194	Biolegend	BV604	359418	L291H4	1µl
CCR10	BD	PercP-Cy5.5	564772	1B5	1µl
FOXP3	Biolegend	PE-Daz 594	320126	206D	5µl
IFNγ	eBioscience	APC	17-7319-41	4S.B3	2.5µl
IL2	BD Bio	PE	554566	MQ1-17H12	2.5µl

KI67	Biolegend	BV711	350516	Ki-67	2.5µl
PD1	BD Bio	BB515	564494	EH12.1	2.5µl
PD1	eBioscience	PE	12-2799-42	eBioJ105	2.5µl
TBET	BD Bio	BV711	563320	O4-46	5µl
ZY	Biolegend	*live/dead*	423104	-	1:1000

Reagents for Flow Cytometry

REAGENT	**DETAILS**
BD BRILLIANT STAIN BUFFER	BD Bio (cat. 563794), 50ul/test when 2 Brilliant™ antibodies used in same cocktail
CELL STIMULATION COCKTAIL	eBioscience (cat. 00-4970-93), 1:500
IC FIX & PERM BUFFER SET	eBioscience (cat. 88-8824-00) (contains IC fix buffer, Perm wash buffer)
PROTEIN TRANSPORT INHIBITORS	eBioscience (cat. 00-4980-03), 1:500
TRANSCRIPTION FACTOR BUFFER SET	BD Bioscience (cat. 562574) (contains Fix/Perm solution, Perm/Wash buffer)
LYMPHOPREP	Stemcell Technologies (Cat# 07801)
RED CELL LYSIS BUFFER	Biolegend (Cat# 420301)
CD14 MICROBEADS HUMAN	Miltenyi (cat. 130-050-201)
NAÏVE CD4 ISOLATION KIT	Stemcell EasySep (cat. 17555).

Full Immunohistochemistry protocol:

Sections were oven-dried at 60°C overnight before incubations in xylene (2x 2 minute), industrial methylated spirits (IMS) (1x 2 minute), IMS with 2% hydrogen peroxide (2x 5 minute), IMS (1x 2 minute) before submerging in running tap water. Heat-induced epitope retrieval was performed for 10 minutes at pressure in a pressure cooker in citric acid based antigen unmasking solution before cooling in running tap water and transfer to tris-buffered saline with tween (TBS-T). Antibody

incubation was for forty minutes at room temperature (RT) before detection with SuperSensitive™ Polymer-HRP Kit (20 minutes in Super-Enhancer, 30 minutes in SS-Label separated with TBS-T washes) before detection with DAB or VIP chromogen for 10 minutes. After detection counterstain was for 5 minutes in Gills II Haematoxylin, transfer to running tap water (2 minutes), 5 dips in acid-alcohol, running tap water (2 minutes) then bluing in Scott's solution (3 minutes), dehydration with changes of IMS (3x 2 minute) and clearing with xylene (3x 2 minute). Finally, DPX mounting and scanning. Coverslip removal post scanning by soaking in xylene.

Multiplex Immunohistochemistry protocol:

Multiplex IHC (mIHC) was performed using the same protocol as above. VIP chromogen and the associated primary antibody is removed by Heat Induced Epitope Retrieval, whereas DAB chromogen is heat stable and cannot be removed. For multiplex staining the standard IHC protocol was completed in full before scanning and repeating in full selecting VIP or DAB chromogen depending on whether signal was to be retained or stripped (Figure S1). For all mIHC panels validation was performed in tonsil with both stripped and fresh stained tonsil sections assessed to monitor for excessive background or signal loss with serial stripping iterations. Most antibodies strip completely. For validation the antibody was stained as per the standard IHC protocol with VIP chromogen, scanned, then the protocol was repeated without adding further antibody but completing all antibody detection steps and without counterstain to assess for residual antibody signal (Figure S2). Results of validation reported in the Antibodies for Immunohistochemistry table (Supplementary methods, above). Antibodies that failed stripping validation were placed last in stripping panels.

Full Imaging Mass Cytometry protocol

In-house metal conjugation of antibodies was with Maxpar X8 labelling kits (Fluidigm) according to manufacturer's instruction. Antibody recovery was assessed with Nanodrop (Thermo Fisher) and conjugated antibodies were stored at 4°C in Candor Antibody stabiliser (Candor Bioscience) with 0.05% sodium azide. Antigen retrieval was performed as per IHC staining protocol up to and including pressure cooker before washing for 10 minutes in distilled water, rinsing twice in TBS-T,

washing twice in PBS and blocking with SuperBlock at room temperature for 45 minutes. Antibody master-mix was prepared in antibody diluent in a 0.1μm spin-filter and centrifuged at 12000 rcf for 1 minute to remove antibody aggregates. Slides were then incubated with 200μl of master-mix in a hydration chamber at 4°C overnight. Slides were washed twice in TBS-T and twice with PBS, before nuclear staining with iridium intercalator in PBS in a hydration chamber at room temperature for 30 minutes. Slides were then washed twice in distilled water and air died overnight. Prior to acquisition, the Hyperion imaging mass cytometry system was tuned with a 3-element tuning slide (Fluidigm) for daily quality control (QC) as per manufacturer's protocol. TMA cores were ablated at 200Hz. Stain quality was compared to IHC (Figure S3). MCD files were converted to tiff image stacks and segmented to extract single cell data for further analysis (https://github.com/BodenmillerGroup/ImcSegmentationPipeline) as previously described.[1,2] Briefly, random image crops were generated, scaled-up x 2 and pixels manually classified as nuclear, membrane/cytoplasmic or background using a combination of CellProfiler (version 4.2.1) and Ilastik (version 1.4.0b15). The trained classifier was used to generate probability masks from which single cell data was extracted in R.

Flow cytometry Protocol:

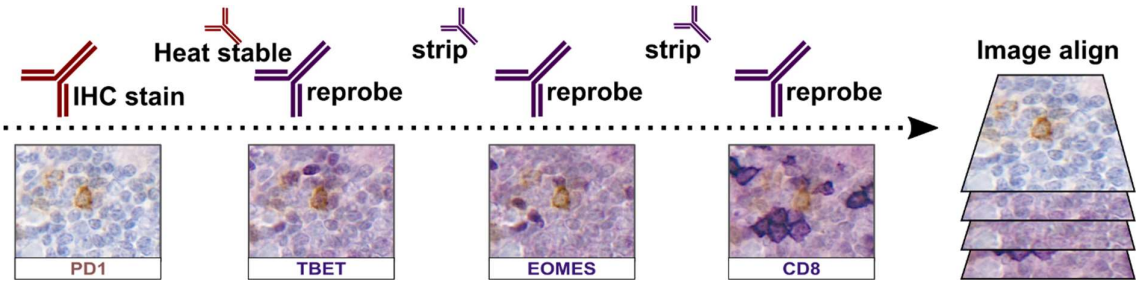
Flow cytometry surface staining was performed for 20 minutes. Cytokine staining used the IC Fix & Perm buffer set with 40-minute fixation and 40-minute intracellular staining steps. Transcription factor staining used the Transcription factor buffer set with 40-minute fixation, 30-minute Perm/Wash incubation and 40-minute intracellular staining steps. All incubations were performed at RT. Viability stains were included in all cocktails.

R Packages:

broom (v0.5.6), conflicted (v1.0.4), cowplot (v1.0.0), corrr (v0.4.2), extrafont (v0.17), formattable (v0.2.1), future (v1.17.0), ggpubr (v0.3.0), ggsignif (v0.6.0), ggthemes (v4.2.0), here (v0.1), pacman (v0.5.1), psych (v2.1.6), readxl (v1.3.1), rstatix (v0.6.0), scales (v1.1.1), spatstat (v1.63-3), tidyverse (v1.3.0), viridis (v0.5.1), wrapr (v2.0.0), XML (v3.99-0.3),

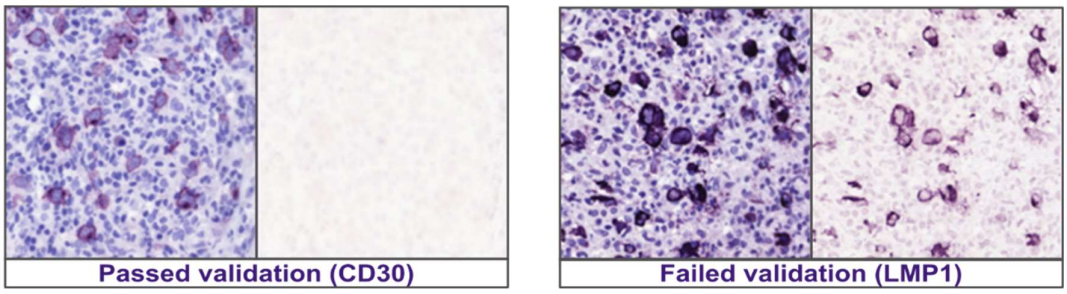
Supplementary Figures

Figure S1: Serial Stripping of Antibodies for Virtual Multiplex IHC



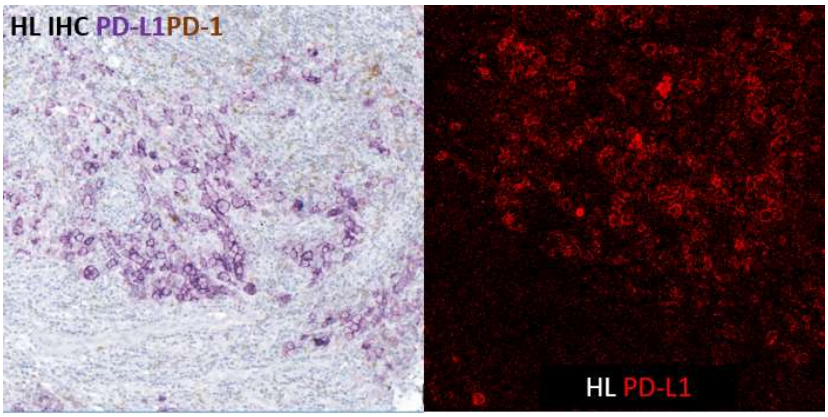
Legend: Antibodies and serially stained, scanned before stripping and restaining. Images are virtually aligned for multiplex analysis. DAB (brown) chromogen is selected when stain is to be retained due to being heat stable so not removed by heat induced epitope retrieval. VIP (purple) chromogen is selected when stain is to be removed due to being heat unstable so both the chromogen and associated primary antibody are stripped by heat induced epitope retrieval.

Figure S2: Antibody Validation for Inclusion in Multiplex IHC Stripping Panels



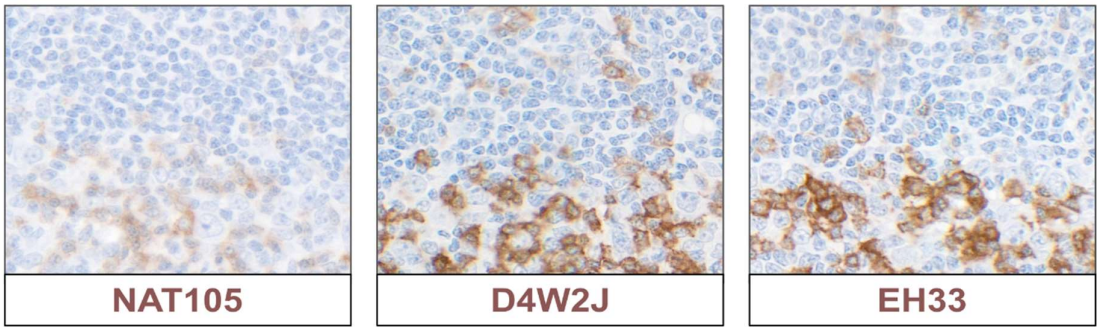
Legend: Antibodies are validated for stripping by assessing residual signal on restaining. Antibodies are stained using the standard IHC protocol (methods) and VIP (purple) chromogen. Staining process is repeated in full but omitting the addition of a second antibody or counterstain. Antibody is removed by heat induced epitope retrieval step. Antibodies are validated for stripping when no residual signal is seen (CD30, left). In some cases, residual signal remains (LMP1, right). These antibodies are placed last in a staining panel to avoid compromising subsequent stains within the panel. Conventional IHC controls were stained in parallel to stripping panels to assess signal loss and background due to the stripping process. Staining performed using DAKO Autostainer.

Figure S3: Immunohistochemistry vs. Imaging Mass Cytometry validation



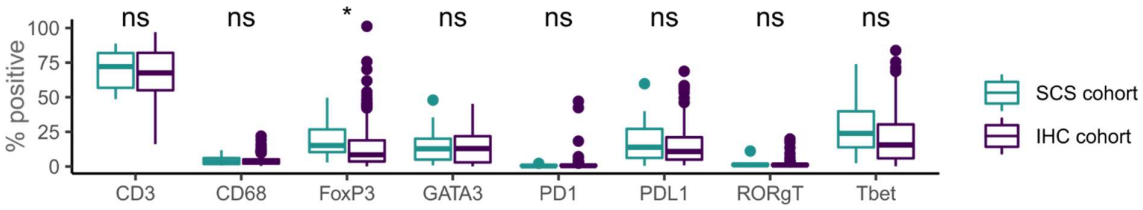
Legend: Comparison of stain quality between IHC and IMC in paired section.

Figure S4: PD1 IHC Antibody Clone Validation



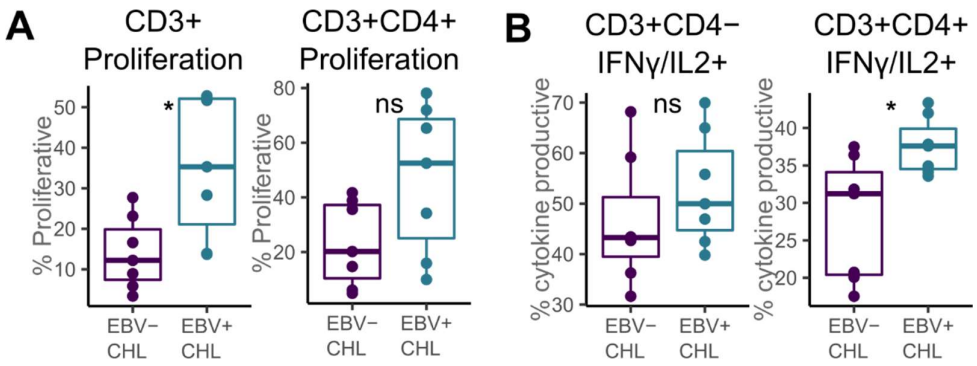
Legend: PD1 clones vary in sensitivity. Superior identification of weak PD1 expression is seen with anti-PD1 clones D4W2J and EH33 as compared to NAT105. IHC in serial tonsil sections. NAT105 (1:25 dilution), D4W2J (1:50 dilution), EH33 (1:50 dilution). Clone D4W2J was used for analysis in this study due to its high sensitivity. Results were validated with clone NAT105 which is approved for diagnostic use.

Figure S5: The CHL Single Cell Suspension Cohort was Similar to the Wider IHC Cohort



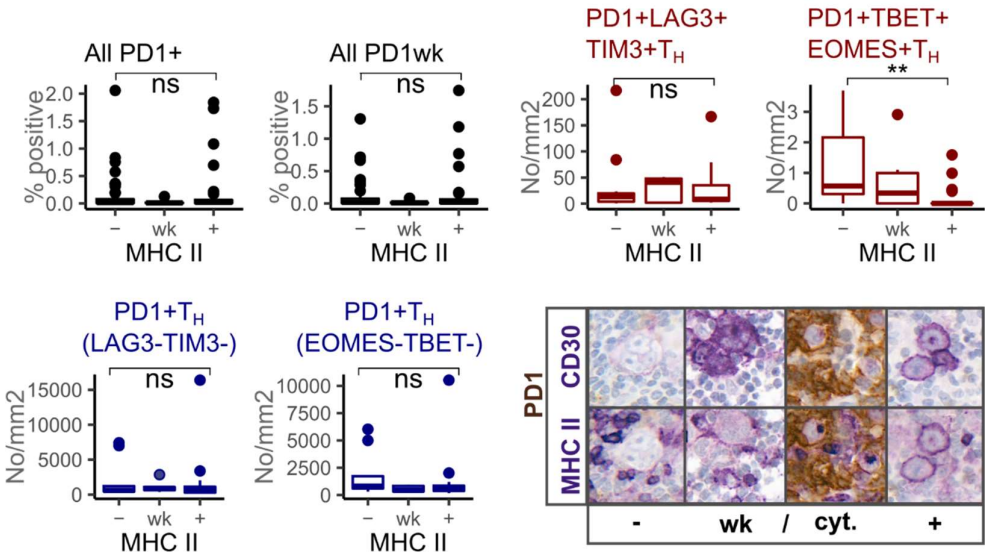
Legend: The Single Cell Suspension (functional) cohort is representative of the IHC cohort. Paired Formalin-Fixed Paraffin-Embedded presenting biopsies of cases with single cell suspension samples used for functional testing were compared by IHC to the wider IHC cohort. The functional cohort was similar in terms of CD3, PD1, PDL1, CD68, TBET, GATA3 and RORγT expression. Significantly more FOXP3 (associated with T_{Reg}) was detected in the functional cohort as compared to the wider IHC cohort (comparison by Mann Whitney, p = 0.044).

Figure S6: CHL Proliferation and Cytokine-competence Varies by EBV Status



Legend: EBV^+CHL is associated with higher T cell proliferation and higher T_H cytokine responsiveness than EBV^-CHL. EBV^+CHL T cells were more proliferative than EBV^-CHL T cells to CD3/CD28 stimulation in vitro by CFSE assay, Mann Whitney, EBV^- n = 8, EBV^+ n = 7, p = 0.017). Similarly, a greater fraction of EBV^+CHL T_H cells produced IFNγ or IL2 to PMA/Ionomycin stimulation in vitro than EBV^-CHL T cell. (Mann Whitney, EBV^- n = 8, EBV^+ n = 7, p = 0.017)

Figure S7: PD1 does not associate with CHL MHC II expression



Legend: PD1 is not associated with CHL MHC-II expression. CHL cases were manually scored as MHC-II positive, MHC-II weak or cytoplasmic or MHC-II negative (image: Top - CD30 purple, PD1 brown. Bottom – MHC-II purple, PD1 brown). No significant association was detected between either PD1 or $PD1^{weak}$ to MHC-II overall (MHC-II- n = 47, MHC-II+ n = 54) and a significant inverse relationship was detected between CHL MHC=II and $PD1^{+}TBET^{+}EOMES^{+}T_H$ frequency (MHC-II- n = 12, MHC-II+ n = 17, p = 0.0024). Statistical comparison by Mann Whitney between positive and negative group because weak category is of indeterminate significance. MHC-II staining was scored manually.