Assessment of type I interferon signatures in undifferentiated inflammatory diseases: A Japanese multicenter experience.

The Journal of Clinical Immunology

Takayuki Miyamoto*[1], Yoshitaka Honda*[1,2,3], Kazushi Izawa**[1], Nobuo Kanazawa[4], Saori Kadowaki[5], Hidenori Ohnishi[5], Masakazu Fujimoto[6], Naotomo Kambe[7], Naoya Kase[8], Takeshi Shiba[9], Yasuo Nakagishi[10], Shuji Akizuki[11], Kosaku Murakami[11], Masahiro Bamba[12], Yutaka Nishida[13], Ayano Inui[14], Tomoo Fujisawa[14], Daisuke Nishida[15], Naomi Iwata[15], Yoshikazu Otsubo[16], Shingo Ishimori[17], Momoko Nishikori[18], Kiminobu Tanizawa[19], Tomoyuki Nakamura[20], Takeshi Ueda[21], Yoko Ohwada[22], Yu Tsuyusaki[23], Masaki Shimizu[24], Takasuke Ebato[25], Kousho Iwao[26], Akiharu Kubo[27], Toshinao Kawai[28], Tadashi Matsubayashi[29], Tatsuhiko Miyazaki[30], Tomohiro Kanayama[30], Masahiko Isa-Nishitani[1], Hiroshi Nihira[1], Junya Abe[1,31], Takayuki Tanaka[1,32], Eitaro Hiejima[1], Satoshi Okada[33], Osamu Ohara[34], Megumu K Saito[8], Junko Takita[1], Ryuta Nishikomori[35], Takahiro Yasumi[1].

the 16-digit ORCIDs of the authors
Takayuki Miyamoto: 0000-0002-3832-5201
Yoshitaka Honda: 0000-0001-6273-0648
Kazushi Izawa: 0000-0003-1080-0936
Kosaku Murakami: 0000-0001-5981-4648
Satoshi Okada: 0000-0002-4622-5657

[1] Department of Pediatrics, Kyoto University Graduate School of Medicine, Kyoto, Japan.
[2] Institute for the Advanced Study of Human Biology (ASHBi), Kyoto University, Kyoto, Japan
[3] Department of Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan
[4] Department of Dermatology, Hyogo College of Medicine. Hyogo, Japan.
[5] Department of Pediatrics, Gifu University Graduate School of Medicine, Gifu, Japan.
[6] Department of Diagnostic Pathology, Kyoto University Hospital, Kyoto, Japan
[7] Department of Dermatology, Kyoto University Graduate School of Medicine, Kyoto, Japan
[8] Department of Clinical Application, Center for iPS Cell Research and Application, Kyoto University, Kyoto, Japan.
[9] Department of Pediatrics, Tenri Hospital, Tenri, Japan.
[10] Department of Pediatric Rheumatology, Hyogo Prefectural Kobe Children's Hospital, Kobe, Japan.
[11] Division of Clinical Immunology and Cancer Immunotherapy, Center for Cancer Immunotherapy and Immunobiology, Graduate School of Medicine, Kyoto University, Kyoto, Japan

[12] Department of Pediatrics, Kawasaki Municipal Hospital, Kawasaki, Japan
[13] Department of Pediatrics, Gunma University Graduate School of Medicine, Maebashi, Japan.
[14] Department of Pediatric Hepatology and Gastroenterology Saiseikai Yokohamashi Tobu Hospital, Kanagawa, Japan.
[15] Department of Infection and Immunology, Aichi Children's Health and Medical Center, Aichi, Japan.
[16] Department of Pediatrics, Sasebo City General Hospital, Sasebo, Japan.
[17] Department of Pediatrics, Takatsuki General Hospital, Takatsuki, Japan.
[18] Department of Hematology and Oncology, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[19] Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[20] Department of General Medicine, Osaka City Hospital Organization Osaka City General Hospital, Osaka, Japan
[21] Department of Emergency and General Internal Medicine, Rakuwakai Marutamachi Hospital, Kyoto, Japan
[22] Department of Pediatrics, Dokkyo Medical University School of Medicine, Tochigi, Japan.
[23] Department of Neurology, Kanagawa Children's Medical Center, Yokohama, Japan.
[24] Department of Pediatrics and Developmental Biology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan.
[25] Department of Pediatrics, Kitasato University, School of Medicine, Kanagawa, Japan.
[26] Department of Internal medicine, Division of Rheumatology, Infectious diseases and Laboratory Medicine, University of Miyazaki, Miyazaki, Japan.
[27] Department of Dermatology, Keio University School of Medicine, Tokyo, Japan.
[28] Division of Immunology, National Center for Child Health and Development, Tokyo, Japan.
[29] Department of Pediatrics, Seirei Hamamatsu General Hospital, Hamamatsu, Japan.
[30] Department of Pathology, Gifu University Hospital, Gifu, Japan.
[31] Department of Pediatrics, Kitano Hospital, Tazuke Kofukai Medical Research Institute, Osaka, Japan
[32] Department of Pediatrics, Otsu Red Cross Hospital, Otsu, Japan.
[33] Department of Pediatrics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, Japan.
[34] Department of Applied Genomics, Kazusa DNA Research Institute, Kisarazu, Japan.
[35] Department of Pediatrics and Child Health, Kurume University School of Medicine, Kurume, Japan.

*These authors contributed equally to this work
**Corresponding author

Corresponding author:

Kazushi Izawa, MD, PhD
Department of Pediatrics, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507, Japan.
E-mail: kizawa@kuhp.kyoto-u.ac.jp
Phone: +81-75-751-3290

Methods

Nuclear factor-kB (NF-κB) reporter gene activity assay

NF-κB reporter gene activity was measured as previously reported[30]. HEK293T cells, in 96-well plates, were transfected with 10 ng per well of the following vectors: a pcDNA3.1+ mock vector, pcDNA3.1+ containing WT myc-A20, or pcDNA3.1+ containing myc-A20 variants. DNA transfection was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The NF-κB luciferase reporter construct and a *Renilla* luciferase control were co-transfected into the cells. After transfection, cells were incubated for 24 hours, and then stimulated with 20 ng/mL tumor necrosis factor α (TNF-α) (R&D Systems, Inc., Minneapolis, MN) for 6 hours. Luciferase reporter activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The activity values for WT and each variant were normalized to that of the mock vector transfection, stimulated with 20 ng/mL TNF-α. Values are expressed as the mean of three independent experiments with technical triplicates ± standard deviation (SD).

Proteasome activity detection

Proteasome activity was measured, with some modifications, as previously described [31]. For the detection of chymotrypsin activity, peripheral blood mononuclear cells (PBMCs), obtained by a Lymphoprep™ (STEMCELL Technologies, Vancouver, Canada) separation, were seeded at a concentration of 1×10^4 cells per well in a 96 well white plate, and then DMSO or 1 μM ONX-0914 (Adooq Bioscience, Irvine, CA) was added. Three hours later, TNF-α and IFN-γ (100 ng/mL) were added to stimulate the cells. After culturing the cells for 21 hours, 100 μL of Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay (Promega, Madison, WI) was added. A one-hour incubation at 37 ºC was then completed before chemiluminescence was detected using the 2104 EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA).

To detect β5 and β5i subunit activity, 3×10^4 cells per well were seeded into 96-well white plates and DMSO or 1 μM ONX-0914 was then added to the wells. Three hours later, the cells were stimulated with TNF-α and IFN-γ (100 ng/mL). After culturing the cells for 21 hours, Ac-Trp-Leu-Ala-AMC (R&D Systems, Inc., Minneapolis, MN) for β5 activity, or Ac-Ala-Asn-Trp-AMC (R&D Systems, Inc., Minneapolis, MN) for β5i activity, was added to a final concentration of 50 μM. After an incubation at 37 ºC for the indicated time (Fig. 3B), AMC fluorescence was detected using a 2104 EnVision Multilabel Plate Reader (PerkinElmer, Waltham, MA).

Table S1: Genotypes and clinical phenotypes of patients with A 20 haploinsufficiency.

Patient	Gender	Age at IS analysis	Disease-onset age	IS	genotype	Initial diagnosis before genetic analysis	CRP at IS analysis (mg/L)	Symptoms at IS analysis	Treatment at IS analysis
1	F	37y	Infancy	40.3	c.986+1 G>T, p.Lys329Asn*1 hetero	SLE (Recurrent fever, lymphadenitis, arthritis)	N/D	None	PSL, Hydroxychloroquine
2	M	15y	4y	41.0	c.133C>T, p.Arg45* hetero	Crohn's disease	N/D	None	Colchicine, Mesalamin
3	F	6y	7m	51.7	c.1747G>T, p.Gly583* hetero	Recurrent fever with arthralgia and abdominal pain	7	None	No treatment
4	M	10y	1y1m	48.2	c.133C>T, p.Arg45* hetero	PFAPA-like recurrent fever → intestinal Behçet's disease	5	Stomatitis	Colchicine
5[a]	F	5y	6m	46.2	c.2209delC, p.Gln737Serfs*79 hetero	FMF like autoinflammatory disease	<0.2	None	Etanercept
6[a]	F	29y	5y	56.0	c.2209delC, p.Gln737Serfs*79 hetero	Crohn's disease, Hashimoto's disease	7.1	None	No treatment
7[a]	F	68y	Early childhood	44.3	c.2209delC, p.Gln737Serfs*79 hetero	Recurrent stomatitis, Hashimoto's disease	N/D	None	No treatment
8	F	19y	11y	27.1	c.252delC, p.Trp85Glyfs*11 hetero	RF negative pJIA → intestinal BD	0.8	None	ADA, PSL, Celecoxib, Iguratimod
9	M	4y	1y	24.7	c.2088+5G>C, p.His636Glufs*55 hetero	sJIA → Psoriatic arthritis	11.3	Skin erythema, adrenal insufficiency	IFX, MTX, PSL, Naproxen, Colchicine

a: Patients 6 and 7 are the mother and grandmother of patient 5, respectively.

M: male, F: female, PSL: prednisolone, ADA: adalimumab, IFX: infliximab, MTX: methotrexate

Table S2: Clinical phenotypes of patients with idiopathic pulmonary hemorrhage.

Patient	Gender	Age at IS analysis	Disease-onset age	IS	Clinical manifestation at diagnosis				Extrapulmonary manifestations	Symptoms at IS analysis	Auto-antibodies	Hemosiderin-laden macrophages	Treatment	Genotype
					Anemia	Cough	Dyspnea	Hemoptysis						
1	F	15y	4y	93.7/48.6	+	+	+	+	None	None	ANA 1:320, Anti-ssDNA, Anti-dsDNA, Anti-SS-A	+ (gastric aspirate)	PSL, AZA, ICS, CAM	No pathogenic mutations found in the NGS-based gene panel test
2	F	13y	4y	11.2	+	+	−	+	None	None	ANA 1:80	+ (sputum)	PSL, ICS, CAM	Not analyzed
3	F	20y	2w	1.62	+	+	+	+	Arthralgia, morning stiffness, annular erythema	None	ACPA, RF	N/D	Iguratimod (for arthralgia)	No mutations in the *COPA* gene

ACPA: anti-cyclic citrullinated peptide antibody, AZA: azathioprine, ICS: inhaled corticosteroid, CAM: clarithromycin, NGS: next-generation sequencing

Table S3: Clinical phenotypes of patients with chronic active Epstein-Barr virus disease (CAEBV) and hypersensitivity to mosquito bites (HMB).

Patient	Gender	Age at IS analysis	Disease-onset age	IS	diagnosis	Clinical manifestation at sampling				EBV DNA load in whole blood (copy/μgDNA)	Infected cell type	Treatment
						fever	Skin rash	lymphadenopathy	hepatosplenomegaly			
1	F	1y4m	1y1m	12.2	CAEBV	+	+	+	+	2400	T cell	PSL
2-1	F	3y4m	2y4m	3.3	HMB	−	+	−	−	32000	NK cell	None
2-2	F	3y10m	2y4m	13.6	HMB	−	++	−	−	52000	NK cell	None

Table S4：A summary of histological and immunohistochemical findings for the 10 samples studied.

Patient	1	2	3-1	3-2	4	5	6	7	8	9	10
CD163	++	++	N/D	+++	+++	+++	+++	++	++	++	+++
MPO	+++	++	++	+++	+	++	++	+	-	+	++
CD15	+	-	N/D	+++	+	+	-	+	-	+	++
CD123	+	-	N/D	-	-	-	++	-	-	++	++
CD3	+	++	N/D	++	+	++	++	+	++	+++	++
CD20	-	+	N/D	+	+	+	+	-	-	+	+
Dermal infiltrate pattern	Perivascular and interstitial	Perivascular and interstitial	Perivascular	Perivascular	Perivascular and interstitial	Perivascular	Perivascular and interstitial	Perivascular	Superficial perivascular	Perivascular	Perivascular and interstitial
Epidermal change	-	-	-	-	-	-	-	Vacuolar degeneration	Vacuolar degeneration	-	-
Vasculitis	-	-	-	+	-	-	-	-	-	-	-
Panniculitis	-	-	N/D	Septal	-	-	-	Lobular	-	-	-

N/D: no data

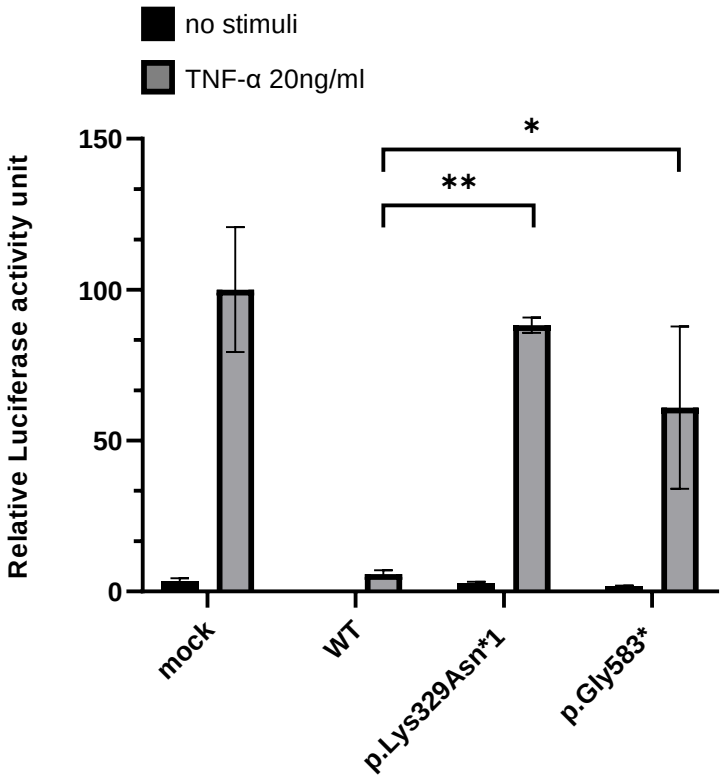
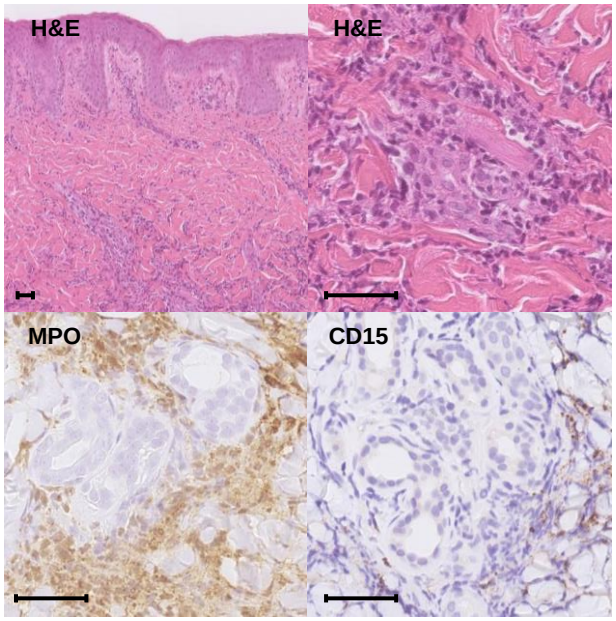
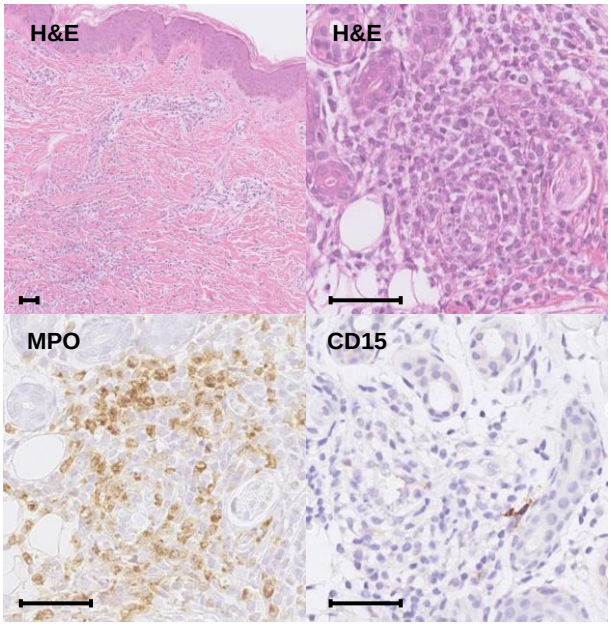


Figure S1- *Results of the NF-κB reporter gene activity assay.* Statistical significance of the difference between the WT and variants stimulated with TNF-α was measured by luciferase activity and analyzed using a one-way ANOVA with Dunnett's multiple comparisons test. Calculated p values are indicated; $p < 0.05$ with * and $p < 0.001$ with **. Suppression of the TNF-α induced NF-κB activity by the *TNFAIP3* variants was significantly lower than that seen in the WT.

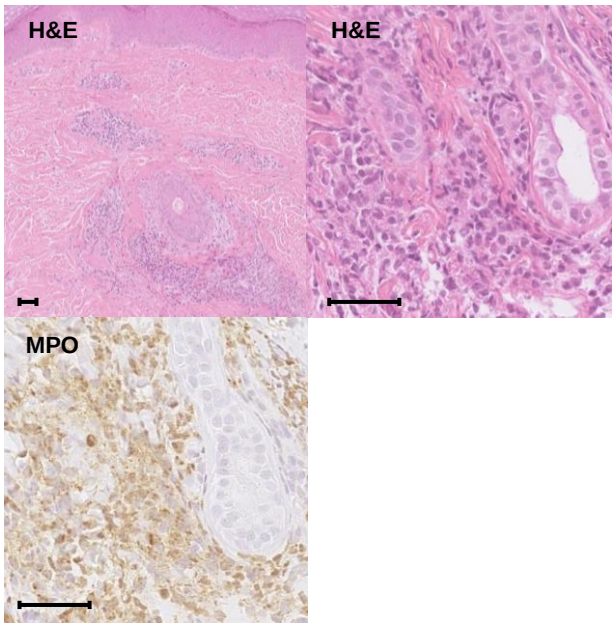
P1



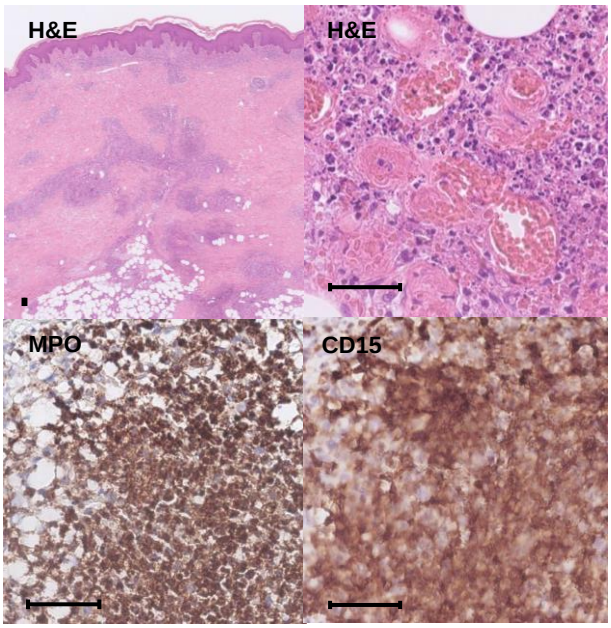
P2



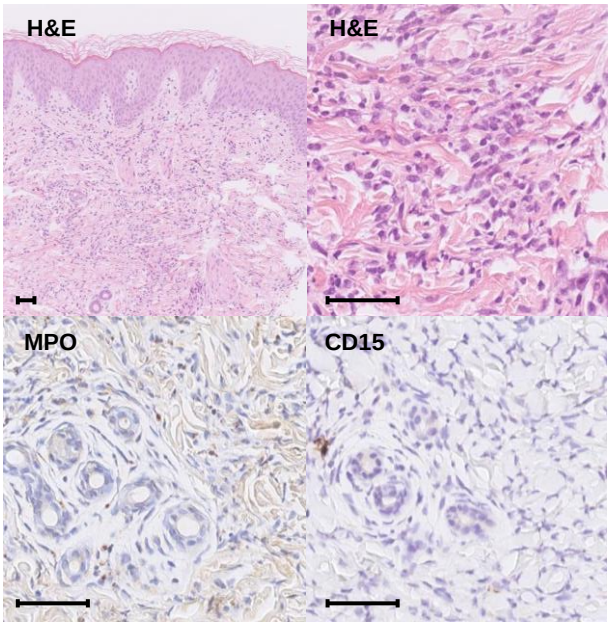
P3-1



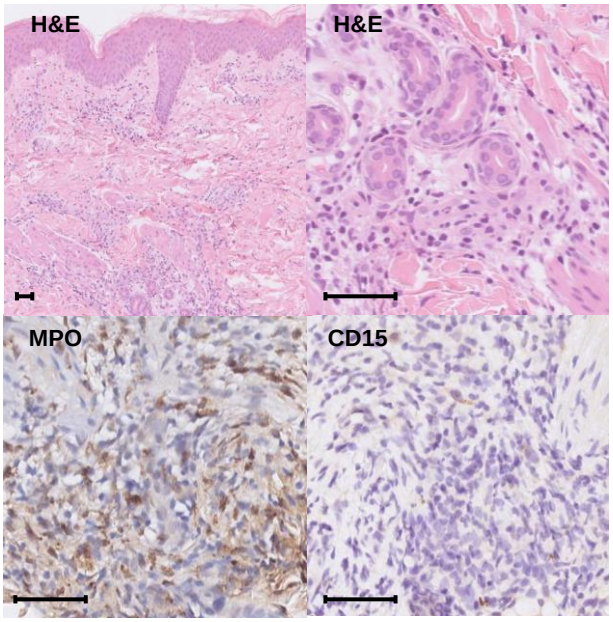
P3-2



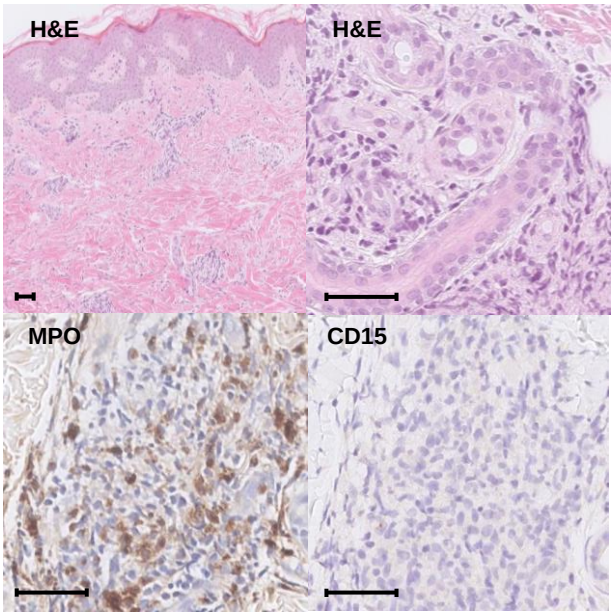
P4



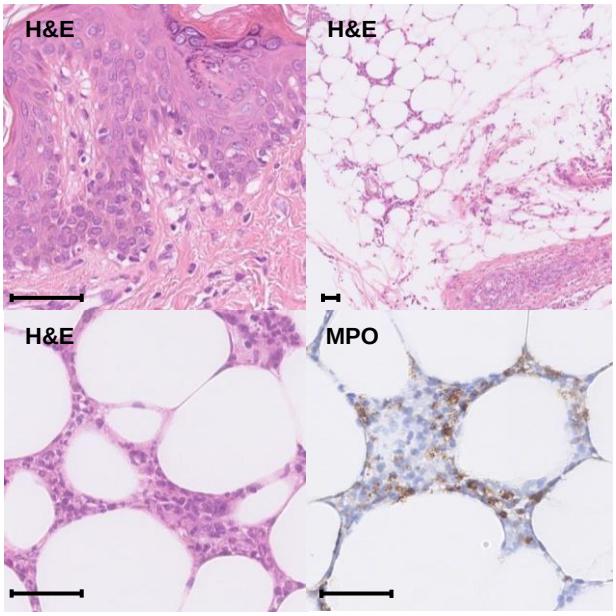
P5



P6



P7



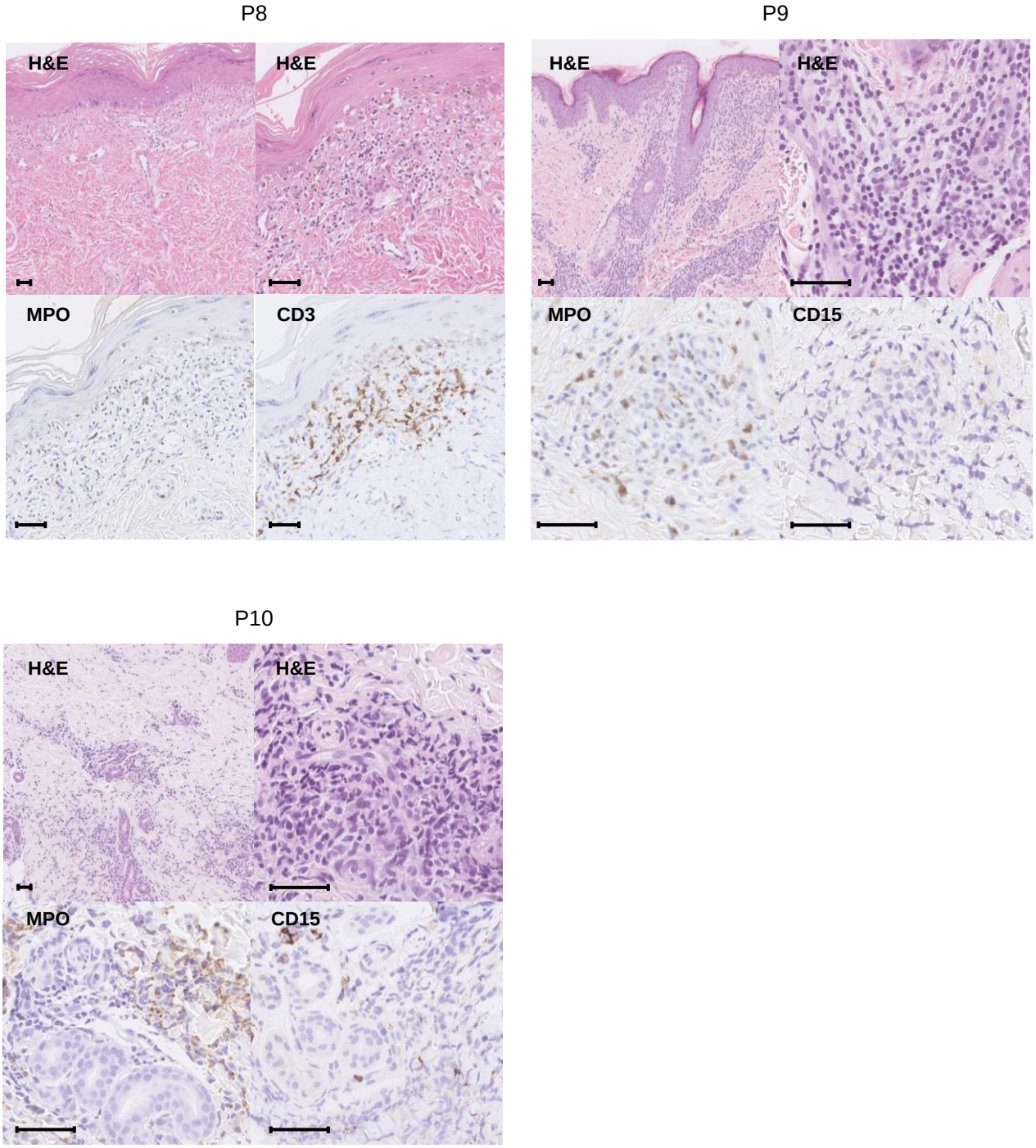


Figure S2- *H&E stained sections of patients.* With the exception of the sections for patients P3-2, P7, and P8, all sections showed superficial and deep perivascular dermal infiltrates without epidermal changes, containing mononuclear cells, many of which were MPO-positive and CD15-negative. The skin specimen for P3-2 showed leukocytoclastic vasculitis and septal panniculitis, in addition to dermal infiltrates consisting of matured neutrophils, histiocytes, and lymphocytes. The H&E stained section for patient 7 showed lobular panniculitis, and superficial and deep perivascular dermatitis with vacuolar changes. Cellular infiltrates mainly consisted of MPO-positive and CD163-positive mononuclear cells. The H&E stained section for patient 8 showed superficial perivascular dermatitis with vacuolar degeneration of the basal layer, and exhibited CD3-positive T cell, but not CD20-positive B cell infiltration. The scale bar for all sections represents 50 μm.