Palmblad et al - Supplemental Material

1. **Supplemental material on subjects and methods, on Results**
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Supplemental material on subjects and methods, and on Results

Subjects and methods

The databases. Two databases were used. The first, The Stockholm Neutropenia Study (SNeuPS) contains data on all neutropenic patients (appr 360 persons) referred to the hematological outpatient clinics of Depts. of Stockholm South Hospital (1972-1997) and Karolinska University Hospital Huddinge (1997-2018), Stockholm, Sweden (data to be published). Those who had a family basis in Africa, the Near/Middle East and the Mediterranean area as well as any person with familial neutropenias (NPs) were screened for ADAN. Before 2015, only a full blood group genotyping was available to identify the rs2814778 variation (also known as the Fy*B_GATA SNP) but from 2015, a specific PCR for this SNP was introduced at the Karolinska University Hospital Blood Bank and was used for subsequent genotypings.

Referrals of neutropenic individuals originated from GPs or secondary hospitals in the Stockholm County area (covering approximately 1.5 to 2.3 million inhabitants over the actual time-period) but also from all over Sweden. Supplemental Figure 1 gives the evaluation algorithm.[21]

Migrants from Near/Middle East and East Africa (mostly Eritrea, Ethiopia and Iraq; see Supplemental Table 1) constituted the majority of referred ADAN persons during 2010-2018. One person had a mixed Swedish and Maldivian background.

The second database included the laboratory records of the Platelet and Granulocyte Laboratory, Dept of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden, performing the ADAN genetic testing. Since those samples originated from different regions in Sweden, clinical data were unavailable for analysis (n=24). These persons were, however, also included for evaluations of autoantibodies to neutrophils and hCAP-18/pro-LL-37 plasma levels.

As healthy controls served three cohorts: The first consisted of 94 healthy blood donors used for the Pro-LL-37 analysis, as described and published previously[22]. The second cohort was made up from 102 healthy doors for the GIFT analysis (this study). It consisted of 42 males (mean age 52,5 years, range 19-76) and 62 females (mean age 50,5 years, range 22-74) representing all blood groups. Samples for this cohort were retrieved anonymized from standard control blood typing tubes collected from the donors according to standard procedures. (3) 20 healthy controls included in a study on the role of NK cells in NP. [23]

Inclusions and exclusions. Patients were initially evaluated for the cause of NP by methods available at that time, but also re-evaluated continuously with novel techniques. Basically, we followed the algorithm in Supplemental Figure 1. All patients were tested for a CBC, hepatic and renal functions, cobalamin and folate blood levels, serum electrophoresis, HIV and hepatitis A, B and C serology, as well as screened for antinuclear (ANA) and related autoantibodies for autoimmune disorders. Most were also tested for antineutrophil antibodies, usually several times (vide infra). Patients who were referred for characterization of a NP but had a previous diagnosis of a viral disorder or an autoimmune systemic disorder (such as rheumatoid arthritis (RA), SLE, Sjögren syndrome, systemic sclerosis etc) were not included in the present analysis. Some ADAN persons (n=28) were evaluated by a bone marrow (BM) biopsy, particularly those with bi- or pancytopenias and all with a suspicion of a hematological malignancy, including MDS. Thus, with exception of a few, where anemia was caused by iron deficiency (which was treated during the course of the follow-up) or was diagnosed due to thalassemia (n=5), or where mild thrombocytopenia (blood platelet count <100 x10^9/L) was part of an autoimmune disorder, all had isolated NP. The follow up was performed by us or, if applicable, by nationwide Swedish Registries for MDS and acute leukemias.

ACKR1-variation analysis, anti-neutrophil antibody testing and hCAP-18/pro-LL-37 quantification. For the *ACKR1*-polymorphism analysis, DNA was extracted from EDTA preserved peripheral blood samples with QIAamp DNA Mini kit (Qiagen, Sollentuna, Sweden) and Fy*B (GATA -67T>C) allele genotyping was performed using TaqMan SNP Genotyping Assay C_15769614_10, according to the manufacturers' instruction (Fisher Scientific, Gothenburg, Sweden). The readout was the presence or absence of the Fy*B_GATA allele (i.e. the rs2814778 SNP in *ACKR1*) and/or the wildtype Fy*B allele.

Plasma extracted from EDTA preserved peripheral blood samples was tested for the presence of anti-neutrophil antibodies using the Granulocyte Agglutination Test (GAT) and the Granulocyte Immunofluorescence Test (GIFT), as described.[26.] Some control samples were also analyzed using bead-based screening method on the Luminex platform, LabScreen Multi (LSM, Thermo Fisher Scientific). Neutrophil-reactive samples were in all cases tested for the presence of anti-HLA-class 1-antibodies as HLA antibodies can obscure interpretations of both GAT and GIFT, using using Lifecode QuikScreen ELISA (ImmuCor) (before Jan 2017), LabScreen Mixed (One Lambda) (December 2017-2019) and LabScreen Multi (One Lambda) (from January 2020). If positive GAT or/and GIFT tests were noted, and/or the person had HLA-

antibodies, a monoclonal-antibody-specific immobilization of granulocyte antigens (MAIGA) test was used for detection of specific antibodies to CD16, CD11a/b and CD177.[21,26,36] Thus, if GIFT/GAT and HLA tests all were positive, the neutrophil autoantibody was considered negative, unless also specific antibodies to CD11a/b, CD16a/b and/or CD177 were detected.

Plasma levels of the antibacterial peptide cathelicidin (hCAP-18 or Pro-LL-37), previously shown to discriminate SCNP from other NP forms[27], was analyzed using an in-house ELISA.[22]

Blood monocyte phenotyping

Frozen PBMC samples from seven ADAN subjects and eleven age-matched healthy controls were thawed and washed with ice-cold PBS supplemented with 2% fetal calf serum and 1 mM EDTA. Cells were stained on ice for 20 min using the following antibodies (clones given in brackets): anti-CD3 APC-Cy7 (SK7), anti-CD11c PE-Cy7 (B-ly6), anti-CD14 V450 (MΦP9), anti-CD16 APC (3G8), anti-CD19 APC-H7 (HIB19), anti-CD20 APC-Cy7 (L27), anti-CD45 Alexa Fluo700 (HI30), anti-CD56 PE (MY31), anti-CD123 PerCP-Cy5.5 (7G3), anti-HLA-DR V500 (G46-6) (BD Biosciences, San Diego, CA, USA); anti-SLAN FITC (DD-1) (Miltenyi, Bergisch Gladbach, Germany). The cells were washed as before and 7-AAD (BD Biosciences) was added to the samples to identify dead cells. Cells were acquired immediately after staining on a BD LSR II Fortessa (BD Biosciences) and the data was analyzed using FlowJo v9.9.6 (Treestar, Ashland, OR, USA). The gating of different populations is described in Supplemental figure 2.

Bone marrow examinations (BME).

Twenty-eight ADAN subjects had been evaluated by a BM biopsy, including a BM flow cytometry, particularly those presenting with a clinical suspicion of a hematological malignancy, eg. MDS. Retrieved BM samples (n=17) were re-evaluated in 2019-20 regarding signs of a neutrophil maturation arrest at the (pro)myelocyte stage (typical for severe congenital NP due to *ELANE* or *HAX-1* mutations[6,24,25]), signs of MDS or other pathologies. The maturation index (MI) of the neutropoiesis was calculated according to the formula (myeloblasts + promyelocytes + myelocytes) / (metamyelocytes + band and segmented neutrophils); the normal value is 1:3-1:5 (i.e. 0.20-0.33).[24,25] BM marrow cellularity was assessed according to routine evaluations at the Dept of Clinical Pathology, Karolinska University Laboratory (the normal values for adults being 40-60%).[24,25]

Supplemental Results

Clinical findings

Patients, initially presenting with mild iron or folate deficiency, did not change the ANC upon replenishing iron or folate stores. Four subjects presented with mild microcytic alpha-thalassemia. Seven subjects displayed mild, stable thrombocytopenia (130-150 $x10^9$/L) and one with mild immunologic thrombocytopenia.

Bone marrow examinations (BME)

Upon referral to the tertiary center for evaluation of neutropenia, 28/66 persons with available clinical data underwent a BME as part of the routine work-up, particularly due to the clinical suspicion of MDS. The original reports concluded that insignificant dysplasias, or features suggesting large granular lymphocytic syndromes, could be seen in 9/28 samples. Complementary flow cytometry analyses of BM cells in these persons showed no signs of abnormal cell populations. Also, remaining suspicions of MDS conferred repeated BME in several individuals (prior to the ADAN diagnosis). However, upon a systematic re-evaluation of the BME, as part of this study, no evidence of diagnostic dysplastic features, maturation arrests in the myeloid series or abnormal cellularity could be observed. Thus, the BMs showed no evidence of pathologies related to MDS or any other hematological disorder.

References

References and their numbers in the Supplemental material are identical to those in the main text.

Supplemental tables

Supplemental Table 1. Countries of origin for the ADAN subjects*

Brazil	1	Liberia	1
Cameroun	1	Morocco	1
Congo-Kinshasa	1	Maldives	1
Egypt	3	Nigeria	1
Eritrea	16	Senegal	3
Ethiopia	7	Somalia	1
Gambia	2	Togo	2
Ghana	3	Tanzania	2
Ivory Coast	1	Uganda	3
Iraq	4	USA	1**
Jordan	1		

Three ADAN persons were born in Sweden by parents from one of the listed countries*;* all others were 1st generation migrants. One had a mixed Swedish-Maldivian background. **denote an African-American person with ADAN siblings.

Supplemental Table 2. **Other diseases in the ADAN cohort**.

Gastrointestinal disorder
- Peptic ulcer 2
- Esophagitis 1
- Celiac disease 1
- Colonic polyposis 1

Respiratory tract/Allergic disorder
- Asthma 2
- Nasal polyposis 1
- Lung cancer 1
- Anaphylactoid reaction 1
- Food allergy 1

Cardiovascular disorder
- Cerebral vascular malformation 1
- Aortic valve insufficiency 1
- Hypertension 2

Skin and muscle disorder
- Bullous pemphigoid disease 1
- Fibromyalgia 1

Psychiatric disorder
- Schizophrenia 1

Nervous system/eye and ear disorder
- Headache /migraine 3
- Ocular cavernoma 1
- Sudden deafness 1
- Glaucoma 1

Endocrine disorder
- Diabetes type 2 1
- Prolactinoma 1
- Thyroid disease 2

Blood disorder
- ITP 1
- Chronic myeloid leukemia 1*
- Essential thrombocythemia and polycythemia vera 2*
- Thalassemia minor 4

Bleeding tendency NUD 1

Autoimmune disease
- SLE 1*

*excluded from the clinical evaluation. – The chronic myeloid leukemia, the essential thrombocythemia and the polycythemia vera cases were diagnosed according to WHO criteria, including appropriate genetic markers.

Supplemental Table 3. Plasma cytokines analyzed by Olink.

Proteins	HD median	ADAN median	p values	Comments
NEMO	8,01	7,41	1,7374E-05	controlling and upregulating NFkB function
IL-18R1	7,35	6,785	0,00338792	
HO-1	12,89	12,135	0,0038371	possible positive regulator of myelopoiesis
CEACAM1	7,16	6,81	0,00493259	angiogenesis promotor; integrin-mediated signaling
CCL23	10,49	9,61	0,00595196	
PDL1	4,955	4,41	0,00668246	
VEGFR-2	6,99	6,78	0,00842328	angiogenesis promotor
ERBB2/HER2	6,67	6,4	0,00938016	tumor progession incl angiogenesis
MIA	9,68	10,12	0,01048218	
IL-7	5,24	4,36	0,01178444	
CYR61	5,54	6,48	0,01277318	reprogramming macrophages towards M1 polarization
TRANCE	4,57	3,755	0,01336345	
CD244	7,45	6,995	0,01466654	
FGF-BP1	4,69	5,11	0,01896533	chemotactic glycoprotein, growth factor
HB-EGF	6,965	5,945	0,01938931	blocking neutrophil/endothelial adhesion
ITGAV	3,16	2,92	0,02068506	phagocytosis regulations, integrin-mediated adhesion
PTX3	2,82	2,425	0,02236387	phagocytosis regulation, neutrophil degranulation
OSM	4,065	2,77	0,02277433	
PSGL-1	4,615	4,18	0,02428952	regulating neutrophil/endothelial adhesion
FS	11,03	10,165	0,02843263	
CASP-8	4,535	5,64	0,0300567	
ANG-1/ ANGPT-1	10,295	10,56	0,03070843	angiogenesis driver, reducing duration of neutropenia
IL-16	6,315	5,74	0,03307128	pro-inflammatory pleiotropic cytokine
CEACAM8	4,115	2,665	0,03573217	marker for neutrophils, released on cell activation
IL-1RA	6,72	6,21	0,03906528	anti-inflammatory, protects against neutrophil damage
FCRLB	0,98	2,4	0,04269162	Fc receptor family, regulating phagocytosis, ADCC
MERTK	5,28	4,825	0,04450037	driving monocyte polarization towards a M2 phenotype
KIM-1/HAVRC1	6,62	5,77	0,04790331	tissue injury marker, response to viral infections

	BNP	CD40-L	CXCL10
4E-BP1	CA5A	CD70	CXCL11
5'-NT	CA9	CD84	CXCL13
ABL1ACE2	CCL3	CD160	CXL17
ADAM 8	CCL4	CD207	DECR1
ADAM-TS13	CCL11	CEACAM5	DNER
ADAM-TS 15	CCL17	CPE	DCN
ADM	CCL20	CSF-1	Dkk-1
AGRP	CCL25	CST5	DKN1A
ANXA1	CCL28	CTRC	DLL1
AR	CD4	CTSL1	DNER
ARTN	CD5	CTSV	EGF
AXIN1	CD6	CXCL1	EN-RAGE
Beta-NGF	CD27	CXCL5	EPHA2
BDNF	CD40	CXCL6	ERBB3

ESM-1	IL1RL2	PAR-1	TFPI-2
FABP2	IL-4RA	PARP-1	TGF-alpha
FADD	IL-6	PDGF subunit B	TGF-b1
FASLG	IL-8	PD-L2	TGFR-2
FcRII-IgG	IL-10	PlGF	TGM2
FGF-5	IL-10RB	PigR	THBS2
FGF-19	IL-10RA	PODXL	THPO
FGF-21	IL-12B	PPY	TLR3
FGF-23	IL-17C	PRELP	TM
Flt3L	IL-18	PRSS8	TNFB
FR-alpha	IL-24	PRSS27	TNFRSF4
FR-gamma	IL-27	Protein BOC	TNFRSF9
FURIN	ITGB5	PVRL4	TNFRSF10A
Gal-1	ITGB1BP2	RAGE	TNFRSF11A
Gal-9	KLK13	REN	TNFRSF13B
GIF	LAP	RSPO3	TNFRSF19
GH	LEP	S100A4	TNFSF14
GLO1	LIF-R	SCAMP3	TRAIL-R2
GPNMB	LOX-1 LPL	SCF	TWEAK
GT	LY9	SERPINA12¨	TXLNA
GZMB	LYN		VEGFR-3
GZMH	LYPD3	SEZ6L	VEGF-D
HAOX1	MAD homolog 5	SIRT2	WIF-1
HGF	MCP-2	SLAMF1	VIM
hK8	MCP-3	SLAMF7	WFDC2
hK14	MetAP 2	SORT1	WISP-1
hK11	MIC-A/B	SPARC SRC	XCL1
hOSCAR	MMP-1	ST1A1	XPNPEP2
HSP 27	MMP-7	STAMPB	
IDUA	MSLN	SYND1	
IFN-g-R1	MUC-16	TCL1A	
IGF1R	PAPPA	TF	

Abbreviations and color codes: ADAN=ACR1/DARC associated neutropenia (n=4-6, where those with n=4 are marked in grey letters, not discussed further). HD=Healthy donors (n=19-20).

Proteins of interest for inflammation, myelopoiesis, neutrophil and monocyte function and differentiation (n=12) are marked in red letters, those of interest for vascular biology/apoptosis (n=3) are marked in blue letters. Three genes, implicated in both set of processes, are marked in red/blue. Green letters in the ADAN column denote 5 up-regulated genes in ADAN; all others are down-regulated. Yellow overlay mark proteins assumed to be involved in the "cytokine sink hypothesis".[38] The comments refer to involvement in biological processes related to ADAN. Proteins above the dividing line (====) mark those with a p-value <0.05 (n=28) and proteins under the line were measured but have a p-value >0.05. NB that none of the differences in this table showed a p-value <0.05 when adjusted for multiple comparisons, with exception of NEMO (p=0.004).

Supplemental Table 4. Serum concentrations of G-CSF, M-CSF and GM-CSF in ADAN subjects (n=7) and healthy donors (HD; n=6).

Growth factor	ADAN	HD	P value
G-CSF, pg/mL	61.8±20.1	42.4±23.3	>0.05
M-CSF, pg/mL	39.0±39.4	35.5±15.0	>0.05
GM-CSF, pg/mL	0.93±0.22	1.58±0.90	>0.05

Mean ±SD values. HD=healthy donors

Supplemental Table 5. Gene ontology (GO) terms for the ADAN Olink protein network.

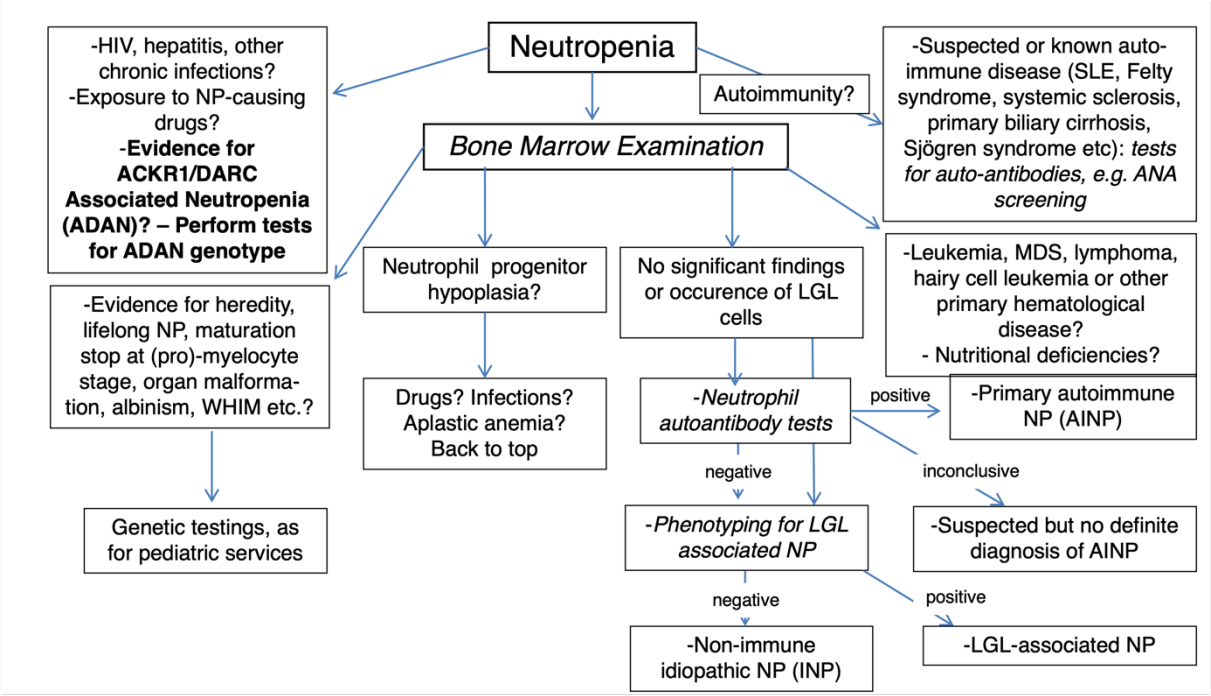
Filtered for FDR <0.01 and fold enrichment above 10.

Gene ontology (GO) term	# of proteins[1]	hits[2]	fold enrichement	p-value
regulation of cytokine production	737	11	12,8	8,49E-07
regulation of leukocyte activation	667	10	12,86	4,62E-06
regulation of cell activation	722	10	11,88	6,53E-06
cytokine-mediated signaling pathway	384	8	17,87	9,38E-06
response to cytokine	819	10	10,47	1,14E-05
positive regulation of cytokine production	477	8	14,39	2,82E-05
cellular response to cytokine stimulus	725	9	10,65	3,58E-05
hemopoiesis	617	8	11,12	1,16E-04
hematopoietic or lymphoid organ development	666	8	10,3	1,93E-04
regulation of cell-cell adhesion	471	7	12,75	2,71E-04
positive regulation of cell adhesion	476	7	12,62	2,85E-04
regulation of ERK1 and ERK2 cascade	300	6	17,16	3,24E-04
inflammatory response	528	7	11,37	4,70E-04
wound healing	335	6	15,36	5,27E-04
negative regulation of leukocyte activation	189	5	22,7	6,51E-04
positive regulation of locomotion	566	7	10,61	6,94E-04
regulation of leukocyte cell-cell adhesion	356	6	14,46	6,99E-04
negative regulation of cell activation	211	5	20,33	1,00E-03
positive regulation of acute inflammatory response	26	3	98,99	1,08E-03
epithelial cell proliferation	94	4	36,51	1,08E-03
negative regulation of extrinsic apoptotic signaling pathway	97	4	35,38	1,15E-03
regulation of epithelial cell migration	228	5	18,81	1,28E-03
regulation of phagocytosis	102	4	33,64	1,30E-03
leukocyte migration	233	5	18,41	1,37E-03
negative regulation of immune system process	420	6	12,26	1,41E-03
regulation of phosphatidylinositol 3-kinase signaling	107	4	32,07	1,47E-03
response to wounding	441	6	11,67	1,79E-03
positive regulation of protein kinase B signaling	116	4	29,58	1,93E-03
female sex differentiation	117	4	29,33	1,95E-03
regulation of interferon-gamma production	117	4	29,33	1,97E-03
positive regulation of leukocyte activation	458	6	11,24	2,12E-03
ERK1 and ERK2 cascade	37	3	69,56	2,20E-03
positive regulation of cell activation	475	6	10,84	2,36E-03
protein kinase B signaling	39	3	65,99	2,42E-03
positive regulation of MAPK cascade	481	6	10,7	2,45E-03
negative regulation of cytokine X activation	282	5	15,21	2,66E-03
regulation of angiogenesis	285	5	15,05	2,77E-03

positive regulation of kinase activity	495	6	10,4	2,80E-03
regulation of vasculature development	289	5	14,84	2,90E-03
chemotaxis	513	6	10,03	3,32E-03
positive regulation of inflammatory response	142	4	24,17	3,34E-03
regulation of leukocyte differentiation	306	5	14,02	3,59E-03
regulation of acute inflammatory response	48	3	53,62	3,83E-03
regulation of extrinsic apoptotic signaling pathway	153	4	22,43	4,16E-03
positive regulation of NF-kappaB transcription factor activity	154	4	22,28	4,20E-03
negative regulation of lymphocyte activation	159	4	21,58	4,58E-03
negative regulation of secretion	165	4	20,8	5,03E-03
regulation of epithelial cell proliferation	337	5	12,73	5,06E-03
regulation of production of molecular mediator of immune response	169	4	20,3	5,34E-03
regulation of endothelial cell migration	169	4	20,3	5,38E-03
positive regulation of fever generation	7	2	> 100	5,40E-03
regulation of protein kinase B signaling	169	4	20,3	5,43E-03
positive regulation of neutrophil activation	7	2	> 100	5,44E-03
regulation of adaptive immune response based on somatic recombination of immune receptors built from Ig superfamily domains	176	4	19,5	6,01E-03
natural killer cell activation	61	3	42,19	6,26E-03
regulation of immune effector process	363	5	11,82	6,35E-03
leukocyte differentiation	363	5	11,82	6,40E-03
regulation of T cell activation	366	5	11,72	6,46E-03
regulation of fever generation	9	2	> 100	7,57E-03
positive regulation of heat generation	9	2	> 100	7,62E-03
regulation of adaptive immune response	191	4	17,97	7,53E-03
positive regulation of I-kappaB kinaseg /NF-kappaB signalin	190	4	18,06	7,54E-03
negative regulation of cell-cell adhesion	192	4	17,87	7,63E-03
positive regulation of epithelial cell proliferation	194	4	17,69	7,89E-03
regulation of hemopoiesis	386	5	11,11	7,84E-03
positive regulation of chemokine production	71	3	36,25	8,78E-03
MAPK cascade	202	4	16,99	8,96E-03
positive regulation of macroautophagy	73	3	35,26	9,26E-03
positive regulation of phagocytosis	73	3	35,26	9,32E-03
cell chemotaxis	208	4	16,5	9,69E-03

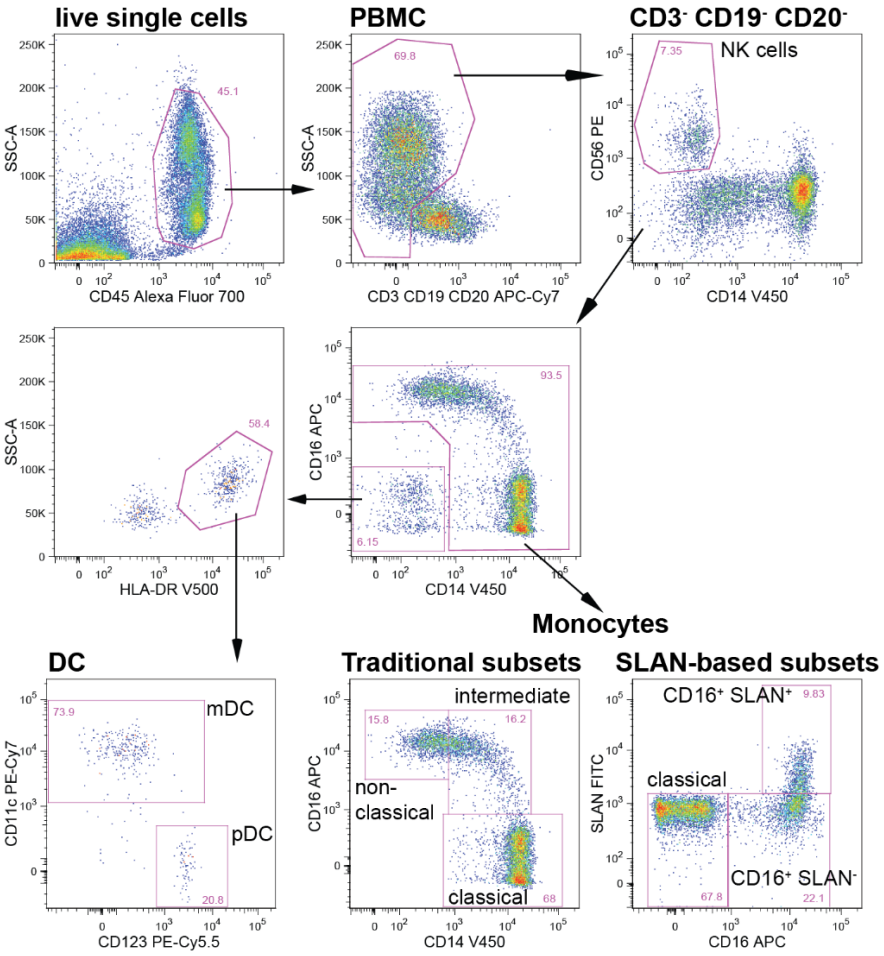
Supplemental figures

Supplemental figure 1. Algorithm for evaluation of an adult patient with neutropenia. Adapted from reference [21].



Supplemental figure 2. Gating strategy for monocyte and dendritic cell phenotyping.

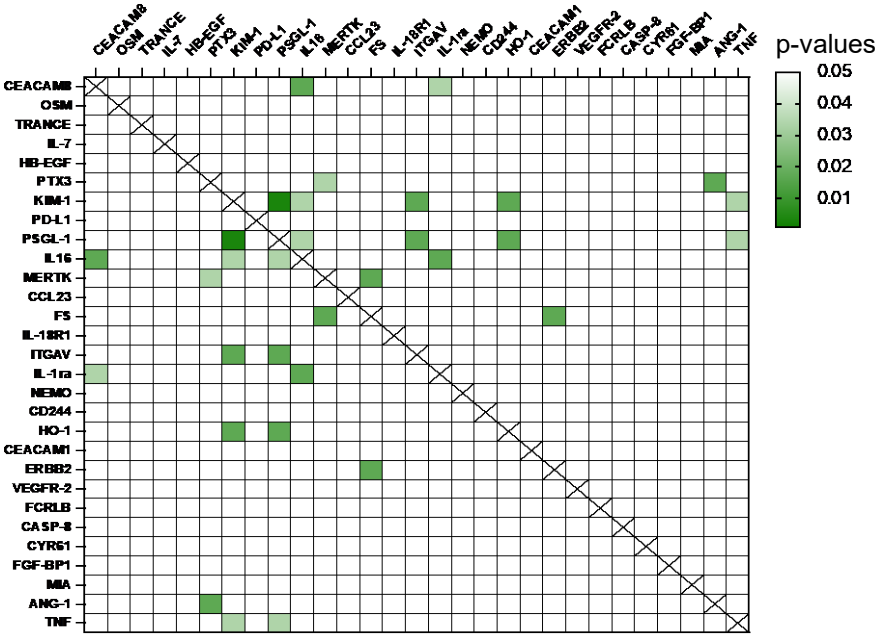
Events were gated on single cells using FSC-Area vs. FSC-Height, and SSC-Area vs. SSC-Height. Live cells were gated as 7-AAD-negative events from single cells.



Supplemental figure 3. Spearman correlation analysis for the 28 proteins significantly differentially regulated in ADAN.

Panel A shows the significant (P<0.05) correlation analyses, where green fields denote a significant correlation. *Panel B* shows the r-values, where a blue field represents a positive correlation and a red a negative, according to the scale on the right side of the figure.

A



B