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CHAPTER 7

ANALYSIS OF MYELOID CELLS AND LYMPHOCYTES BY EXPRESSION OF CO-INHIBITORY MOLECULES AND PHOSPHORYLATION OF SIGNAL TRANSDUCTION ACTIVATORS OF TRANSCRIPTION (STAT) AFTER STIMULATION IN PERIPHERAL BLOOD OF PATIENTS WITH HIGH GRADE USUAL VULVAR INTRAEPITHELIAL NEOPLASIA

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Abstract

Different topical or systemic immunotherapies have shown partial clinical success in the treatment of human papillomavirus (HPV)-induced usual vulvar intraepithelial neoplasia (uVIN). The clinical response of patients with HPV16-induced uVIN to HPV type 16 synthetic long peptide (SLP) vaccination was associated with a stronger HPV16-specific T-cell response and a distinct peak in cytokine levels directly after the first vaccination. This suggested that differences in capacity to respond to HPV16-SLP vaccination are related to the immune status of patients before therapeutic intervention. Because cytokines and co-inhibitory molecules are important regulators of the immune response, circulating myeloid and lymphoid cells of uVIN patients were phenotyped and their phosphorylation of signal transduction activators of transcription (STAT) proteins (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) upon stimulation with different cytokines were analysed. A decreased number of circulating dendritic cells (DCs) and higher numbers of immature DCs and type 2 monocytes are related to recurrent disease. Increased frequencies of CD4+CD94+ and CD4+CD94+NKG2a+ T cells and lower numbers of CD8+TIM3+ T cells were related to the absence of recurrent disease. No differences were found in pSTAT response upon stimulation with IFNγ, IL-2, IL-4, IL-5, IL-6, IL-7 and IL-10. However, the CD14+CD33+ monocytes of uVIN patients responded differently when stimulated with IFNα and GM-CSF, specifically with respect to the response rate or levels of pSTAT5 and total pSTAT induction, suggesting that there are differences in the capacity of these immature immune cells to induce pro-inflammatory cytokine responses. Interestingly, the CD14+CD33+ cells of uVIN patients, who responded with a distinct peak in IFNγ upon HPV 16 SLP vaccination, displayed a stronger pSTAT1 response to IFNα stimulation. Altogether, our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients.

Introduction

Human papilloma virus (HPV) induced usual vulvar intraepithelial neoplasia (uVIN) is caused by a failure of the immune system to clear a persistent HPV infection and is related to the majority of uVIN lesions[1]. The incidence of uVIN is higher in immunocompromised patients, indicating the pivotal role of the immune system in viral clearance and lesion regression[2-5]. Indeed, the presence of HPV-specific T-cell reactivity is associated with protection against development and progression of HPV-induced neoplasia, but is lacking in most uVIN patients[6-9]. In addition, treatment of uVIN with immunotherapy aimed at the reinforcement of the immune response against HPV, shows promising clinical successes although the number of complete clinical responses varies up to 50%[10-13]. Furthermore, local immunity is associated with clinical outcome. The epithelium of uVIN lesions is characterised by lower numbers of infiltrating CD8+ T cells, a reduction of Langerhans cells (LCs) and an increase in CD14+ macrophages[14,15] and CD4+ regulatory T cells (Tregs)[12-16]. Importantly, a high number of intraepithelial macrophages are an independent prognostic factor for rapid recurrences[16] and high Treg numbers are related to non-responsiveness to immunotherapy[11-13]. Furthermore, a dense stromal infiltration by dendritic cells (DCs) and Tbet+, CD8+TIM3+ and CD3+NKG2a+ T cells as well as low numbers of Tregs are associated with the prevention of recurrences[14,15,17]. T-cell function has been related to expression of co-inhibitory molecules as CTLA-4, PD1, TIM3 and NKG2a after interaction with their ligands[18-21]. Monocytes and macrophages are important antigen presenting cells and mediate the innate immune responses by phagocytosis and activate and polarise the adaptive immune response depending upon cytokine production[22-24].

We recently showed that a high number of HPV16-positive uVIN patients clinically responded to treatment with a HPV16-SLP vaccine and that these clinical responses were strongly correlated with the strength of HPV16-specific proliferative T-cell responses characterised by high amounts of interferon gamma (IFNγ) and interleukin-5 (IL-5)[10,25]. The HPV-specific T-cell response in the clinical complete responders was characteristically accompanied by a distinct peak in cytokine levels three weeks after the first vaccination[10,25], suggesting that already before treatment a difference in the patients' capacity to respond to therapeutic vaccination may determine final outcome. Cytokines are well-known important immune regulators and the cytokine milieu plays an essential role in the functional differentiation of immune cells, including T cells and antigen presenting cells [22,26,27]. Binding of cytokines to their receptor triggers distinct signal transduction activators of transcription (STAT) pathways [28-30]. Phosphorylation of STAT (pSTAT) within cells modulates their activity and phosphorylation either leads to the activation of the protein, and triggers downstream activation of different regulatory pathways as NF-kB, MAP kinases and PI3K/Act[31] involved in differentiation, proliferation and apoptosis[32]. The JAK/STAT pathway is facing different challenges of the immune system from resisting infections and enforcement of barrier

function to maintenance of immune tolerance and protection against cancer (reviewed in[33]). Therefore, measurement of cytokine-induced pSTAT may form a method to analyse differences in responsiveness of the immune system between subjects. Due to the many different cell types involved in the immune response, the evaluation of changes in responsiveness requires quantification of phosphorylation events in individual cells by flow cytometry[29].

We hypothesized that differences in patients with respect to the response of immune cells to cytokines may offer an explanation for the observed difference in vaccine responses and associated clinical outcome. Because cytokines and co-inhibitory molecules are important regulators of the immune response we phenotyped circulating myeloid and lymphoid cells in uVIN patients and healthy controls as well as analysed the phosphorylation of STAT proteins (pSTAT1, pSTAT3, pSTAT5 and pSTAT6) in these immune cells upon stimulation with different cytokines.

Our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients and that the measurement of circulating type 2 monocytes is related to recurrent disease, which is similar to our previous finding on the association between recurrences and type 2 macrophages in the local microenvironment.

Materials and Methods

Patient material

Patients presenting with histologically proven high-grade uVIN at the Leiden University Medical Center (LUMC) were enrolled in the Circle study, which is approved by the medical ethical committee of the LUMC and investigates cellular immunity against HPV-induced neoplasia. After meaningful signed informed consent was obtained, venous heparinized blood (60 mL) was collected prior to the therapeutic intervention and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation, cryopreserved at 10 million cells per vial and stored in the vapour phase of liquid nitrogen according to standard operating procedures (SOPs) in the laboratory of the Department of Clinical Oncology. Serum was collected in a clotting tube and frozen at -20°C until use.

Patient characteristics are summarized in Table 1. Fourteen patients with high-grade uVIN were included in this study (mean age at inclusion 51.1 year ± 49.5) of whom in 3 cases a micro-invasive carcinoma (<1mm invasion) was diagnosed after therapy. In 7 cases a recurrence of uVIN was diagnosed after Circle inclusion of which 2 patients were excluded from recurrence analysis because of a persistent uVIN lesion despite therapy. In the remaining 7 cases no recurrence occurred after Circle inclusion.

Table 1: Patient Characteristics

Characteristic	Healthy Controls (n=8)	uVIN patients (n=14)	HPV 16 SLP ISA101 vaccination patients (n=10)
Lesion histology			
High grade uVIN	-	11 (78.6%)	10 (100%)
Microinvasive carcinoma	-	3 (21.4%)	-
No dysplasia	8 (100%)	-	-
Age at diagnosis (years)	n.a.		
Mean		44.4	35.4
Median		42.0	36.0
SD		12.5	7.3
Range		30-71	25-47
Age at inclusion (years)			
Mean	46.4	51.1	41.3
Median	50.5	49.5	41.0
SD	13.1	9.8	7.4
Range	26-64	34-71	28-55
Follow up time from 1st diagnosis (in months)	n.a.		
Mean		129.5	84.4
Median		94.5	90.0
SD		98.4	49.1
Range		18-265	16-154
Time from first diagnosis until inclusion (in months)	n.a.		
Mean		77.2	70.0
Median		14.0	77.0
SD		100.4	47.9
Range		0-247	0-137
Follow up time from inclusion (in months)	n.a.		
Mean		51.5	15.0
Median		30.0	16.0
SD		45.2	4.1
Range		9-158	5-19
Recurrences before inclusion	n.a.		
Yes		7 (50%)	2 (20%)
No		7 (50%)	8 (80%)
Treatment type at inclusion	n.a.		
Excision		8 (57.1%)	-
Laser		5 (35.7%)	-
Laser and Excision		1 (7.1%)	-
HPV 16 SLP vaccination		-	10 (100%)
Recurrences after inclusion	n.a.		n.a.
Yes		6 (42.9%)	
No		8 (57.1%)	
Carcinoma	n.a.		
Before inclusion		2 (14.3%)	0
Diagnosed at inclusion		3 (21.4%)	0
Progression after inclusion		1 (7.1%)	2 (20%)*
HPV type	unknown		
16		9 (64.3%)	10 (100%)
33		4 (28.6%)	-
6 (low risk)		1 (7.1%)	-

The patient characteristics of the subsequent groups are given: the healthy controls, uVIN patients included in the Circle study and the uVIN patients who participated in the HPV 16 SLP ISA101 vaccination trial.

*Of the overall inclusions of the vaccination trial only 3 patients progressed, in this cohort however apparently a higher number was included.

Analyses were performed according to the occurrence of a recurrent uVIN lesion after Circle inclusion. The Circle patients were compared to a cohort of age-matched healthy controls (mean age at inclusion 46.4 year ± 13.1) with no known HPV medical history.
In order to analyse effects of STAT phosphorylation in patients who received therapeutic vaccination, an additional uVIN patient cohort was selected which included a cohort of 10 HPV16-positive uVIN patients who received 4 vaccinations with HPV16-SLP (ISA101) with or without application of imiquimod on the vaccination site. Pre-vaccination PBMC samples of 3 patients, who displayed a distinct peak of IFNγ production after the first vaccination and 5 patients without an IFNγ peak upon the first vaccination (determined by a median IFNγ production above 1000pg/ml in the cytokine bead array) were analysed[34,35].

HPV typing

HPV typing was performed on formalin-fixed, paraffin embedded tissue, resected during surgery or on a biopsy taken at enrolment in the vaccination study by HPV16 PCR with a HPV16-specific primer set followed by HPV genotyping using the INNO-LiPA HPV genotyping *Extra* line probe assay (Innogenetics, Ghent, Belgium) in case of HPV16 negativity[36,37]. Notably, at the same time the blood samples were collected.

Cell proliferation assay

The overall capacity of the T cells to proliferate upon mitogenic stimuli (i.e. phytohaemagglutinin (PHA), HA 16 Remel, Murex Biotech, Dartford, UK) was tested in a 3-days proliferation assay where the PBMC samples of uVIN patients and healthy controls were analysed against medium (Iscove's Modified Dulbecco Medium (IMDM, Life Technologies, Bleiswijk, the Netherlands) +10% Human AB serum (HAB, Greiner, Alphen a.d. Rijn, the Netherlands)) only (negative control) or after PHA stimulation (0.5 µg/mL), using a previously described method which was slightly modified[38]. The proliferation of PBMCs were measured in quadruplicate wells (50.000 cells/well) after 3 days of incubation by adding ^{3}H-thymidine for the last 16-18 hours of the incubation period after which its incorporation (expressed as counts per minute (cpm)) is measured. A cut-off was calculated by mean cpm of the 4 wells with unstimulated PBMCs (medium only) plus 3 times standard deviation (SD). The stimulation index (SI) was calculated by the ratio of the mean cpm of 4 wells with PHA stimulated PBMCs divided by the mean cpm of the 4 wells containing unstimulated PBMCs. A SI ≥3 plus mean cpm of the PHA stimulated wells above cut-off is considered a positive response.

Antigen presenting capacity of antigen presenting cells

The antigen-presenting capacity of antigen-presenting cells (APC) was tested in a mixed lymphocyte reaction (MLR). 1.5x10^{6} PBMCs of uVIN patients resuspended in 5 mL IMDM-

10%HAB were irradiated at 3000 rad to prevent proliferation of these cells. Thereafter, the cells were centrifuged, the supernatant removed and the cells resuspended in 1.5 mL IMDM-10%HAB. The irradiated patient's PBMCs were independently co-cultured with PBMCs obtained from two different healthy donors (differently from the healthy subjects) in quadruplicate wells (100.000 cells/well). Cells were incubated for 6 days at 37°C, 5% CO_2 in a humified incubator before supernatants (100 uL/well pooled) were harvested and stored at -20°C until cytokine level determination by the Th1/Th2 cytometric bead array kit (CBA, BD Biosciences, Breda, the Netherlands). Cells were pulsed with ^{3}H-thymidine for 16-18 hours and the proliferation was measured by ^{3}H-thymidine incorporation. The proliferative capacity of the two individual healthy donors co-cultured with the irradiated patients PBMCs is a measure for the APC quality of the patient. The SI was calculated by the ratio of the mean cpm of irradiated patient's PBMCs co-cultured with PBMCs of one healthy donor divided by the mean cpm of that particular healthy donor unstimulated PBMCs and a SI ≥3 is considered positive.

Phenotyping of PBMCs for macrophages, myeloid-derived suppressor cells and co-inhibitory molecule expression by flow cytometry analysis

The composition of the immune cells in the Circle and healthy control PBMC samples was determined by phenotyping for macrophages (macrophages set), myeloid-derived suppressor cells (MDSC set) and expression of co-inhibitory molecules on T cells (inhibitory T-cell set). Three sets of antibodies (10-11 antibodies per set) directed to specific surface markers for myeloid cells and T cells were used to stain these cells, which were thereafter analysed by multiparameter flow cytometry (LSR Fortessa, BD Biosciences). Thawed PBMCs were washed two times with Phosphate Buffered Saline (PBS)/0.5% Bovine Serum Albumin (BSA) and incubated on ice with PBS/0.5%BSA/10% Fetal Bovine Serum (FBS). Thereafter, PMBCs were stained for 30 minutes in the dark on ice with the 3 different antibody sets, washed two times with PBS/0.5%BSA and fixed with 1% paraformaldehyde (PA). Compensation beads were made for the different fluorchromes according to the protocol of BD Biosciences to optimize fluorescence compensation settings of the flow cytometer. Surface stainings were analysed using FACSDiva™ Software (BD Biosciences) after identification of the life gate in single cells.

Flow cytometry for analysis of macrophages

The following antibodies were used: anti-CD1a-FITC (Clone HI149, BD Biosciences), anti-CD3-Pacific Blue (PB;Clone UCHT1, DAKO), anti-CD11b-PE (Clone D12, BD Biosciences), anti-CD11c-Alexa Fluor (AF) 700 (Clone B-ly6, BD Biosciences), anti-CD14-PE-Cy7 (Clone M5E2, BD Biosciences), anti-CD16-PE-CF594 (Clone 3G8, BD Biosciences), anti-CD19-Brillian Violet (BV) 605 (Clone SJ25C1, BD Biosciences), anti-CD45-PerCP-Cy5.5 (Clone 2D1, BD Biosciences),

anti-CD163-APC (Clone 215927, R&D systems), anti-CD206-APC-Cy7 (Clone 15-2, Biolegend) and anti-HLA-DR-Horizon (H) V500 (Clone L234, BD Biosciences). In the analysis singlet cells and subsequently those in the life gate and CD45+ cell gate were plotted for CD3 and CD19. The CD45+CD3-CD19- were selected and further gated as HLA-DR+ cells. This population was divided upon differential expression of CD14 and CD11b, which reveals five subpopulations of myeloid cells in the CD45+HLA-DR+ population; activated monocytes (CD14Int+CD11b+ or CD14high+CD11b+), immature DCs/early differentiating monocytes (CD14-CD11b-), activated DCs or monocytes with loss of CD14 (CD14-CD11b+) and non-activated DCs or monocytes (CD14+CD11b-). Within these subcategories CD163, CD16, CD206 and CD11c were plotted for each of the populations to distinguish phenotypes (Supplementary Fig. S1). Differentiation markers used are CD163[39], CD11b (a monocyte activation marker[40]), CD11c (a marker of dendritic cells[41]), CD16 (macrophage Fcγ-Receptor III)[42] and CD206 (a mannose receptor)[42]. CD16 is expressed on IL-6 and IL-10 polarized CD14+ macrophages in co-expression of CD163+[43,44]. CD206 is an early marker for differentiation of monocytes into macrophages[45] and is expressed on IL-4 polarized macrophages where CD14 and CD163 are downregulated and CD200R upregulated[43,44]. Type 2 macrophages are mainly associated with Th2 responses and divided into four sub-categories; M2a macrophages (CD206+) induced by IL-4 and IL-13 associated with allergy and parasitic infections; M2b immunoregulatory macrophages (CD16+) induced by TLR activation and M2c (CD163+) regulatory macrophages induced by IL-10 and M2d macrophages[46-49]. Activation of macrophages may as well result in loss of CD14, CD163 and CD16 expression[42,50]. To compare these data between different patients the numbers of events were calculated as percentage of CD45+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry for analysis of MDSCs

Next to terminally differentiated myeloid cells such as macrophages and DCs, myeloid-derived suppressor cells (MDSCs) were stained and acquired, these immature myeloid cells counteract the anti-tumor immune response (reviewed in [22,51]). Two main MDSC phenotypes have been described: the monocytic (mMDSC) and polymorphonucleair MDSCs (granulocytic MDSC, gMDSC) [22,51], which were analysed using the following antibodies: anti-CD3-PB (Clone UCHT1, DAKO), anti-CD11b-FITC (Clone CBRM1/5, Biolegend), anti-CD14-AF700 (Clone M5E2, BD Biosciences), anti-CD15-PE-CF594 (Clone W6D3, BD Biosciences), anti-CD19-BV605 (Clone SJ25C1, BD Biosciences), anti-CD33-PE-Cy7 (Clone P67.6, BD Biosciences), anti-CD34-APC (Clone 581, BD Biosciences), anti-CD45-PerCP-Cy5.5 (Clone 2D1, BD Biosciences), anti-CD124-PE (Clone HiL4R-M57, BD Biosciences) and anti-HLA-DR-HV500 (Clone L234, BD Biosciences). In the MDSC antibody set CD45+ CD3- CD19-HLA-DR- singlet and viable cells were isolated and herein the CD14+ and CD15+ populations identified. Subsequently CD11b, CD124, CD33 and CD34 were plotted for both CD14+ and CD15+ cells (Supplementary Fig. S2).

We have evaluated these cell types upon CD45 and HLA-DR negative cells for expression of CD14+ and CD15- (mMDSC) and CD14- and CD15+ (gMDSC)[51]. Subsequently, hematopoietic progenitor markers CD33+ and CD34+ in combination with CD11b+ and CD124+ (IL-4Rα) were analysed (reviewed in[51]). Myeloid differentiation of the hematopoietic progenitor cells is characterised by loss of CD34 and expression of CD33[52]. The numbers of events were calculated as the percentage of the CD45+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry for analysis of co-inhibitory molecules on T cells

Expression of co-inhibitory receptors on T cells were analysed by the following antibodies: anti-CD3-PB (Clone UCHT1, DAKO), anti-CD4-PE-CF594 (Clone RPA-T4, BD Biosciences), anti-CD8-APC-Cy7 (Clone SK1, BD Biosciences), anti-TIM3-PE (Clone F38-2E2, Biolegend), anti-NKG2a-AF700 (Clone 131114, R&D Systems), anti-CTLA-4 (CD152)-PE-Cy5 (Clone BNI3, BD Biosciences), anti-CD94-FITC (Clone 131412, R&D Systems) and anti-PD1-BV605 (Clone EH12.2H7, Biolegend). After identification of the singlets and life gate the CD4+ and CD8+ cells were selected within the CD3+ cells. CD94, TIM3, NKG2a, CD152 and PD1 were plotted in these populations (Supplementary Fig. S3). The numbers of events were calculated as percentage of the CD3+ cells. If percentages were below 0.05% these cell populations and differences were considered as irrelevant.

Flow cytometry analysis of STAT phosphorylation in PBMCs

Phosphorylation of signalling proteins within cells modulates their activity and leads to activation of the protein (MAP kinases and STAT transcription factors), whereas in some cases the phosphorylation leaves the proteins in an inactive state. To determine the activation state of PBMCs, intracellular phosphorylation of STAT1, -3, -5 and -6 were measured in CD3+, CD4+ and CD8+ lymphocytes and in CD14+CD33+ and CD14-CD33dim monocytes, after individual stimulation with eight different cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFNγ) or adjuvant immune modulators (IFNα and GM-CSF). PBMCs of uVIN Circle patients, healthy controls and pre-vaccination samples of HPV16-SLP treated uVIN patients were analysed using this phosphorylation cytometry assay. The protocol was adopted from G.P. Nolan (Stanford University, Stanford California)[29] and A. Cesano (Nodality, South San Francisco)[53]. Thawed PBMCs were resuspended in serum-free (SF) IMDM at a concentration of 1-1.5 x10^6/ml and incubated at 37°C for 1 hour to rest. Then, the PBMCs in SF medium were stimulated for 15 minutes at 37°C with the following cytokines and adjuvant immune modulators: IFNγ (20 ng/mL; Immunotools, Friesoythe, Germany), IL-10 (50 ng/mL; Immunotools), IL-2 (50 ng/mL; Aldusleukin 18x10^6IE, Novartis Pharma, Arnhem, the Netherlands), IL-5 (50 ng/mL; Immunotools), IL-6 (100 ng/mL; Immunotools), IL-4 (50 ng/mL; Invitrogen, Life Technologies), IL-7 (50 ng/mL; Peprotech, Huissen, the Netherlands),

IFNα (1000 IU/mL Roferon A, Roche, Woerden, the Netherlands), GM-CSF (4 ng/mL; Immunotools). These concentrations were first determined by titration assays (data not shown). After the stimulation the cells were immediately fixed in 1.5% paraformaldehyde (PA, pharmacy LUMC) for 10 minutes at room temperature (RT) and after centrifugation resuspended in 100 uL phosphate buffered saline (PBS, B. Braun, Melsungen, Germany)/0.5% bovine serum albumin (BSA; Sigma, St Louis, USA)/1.5% PA, and transferred into wells of a V-bottom 96-wells plate (Costar). After another wash step in 100 uL PBS/0.5% BSA the cell surface staining was performed for 30 minutes in the dark on ice with anti-CD3-PB (Clone UCHT, DAKO, Heverlee, Belgium), anti-CD4-HV500 (Clone RPA-T4, BD Biosciences), anti-CD8-APC-Cy7 (Clone SK1, BD Biosciences), anti-CD14-FITC (Clone M5E2, BD Biosciences) and anti-CD33-AF700 (Clone WM53, BD Biosciences). Then, the cells were washed two times with 100 uL PBS/0.5% BSA and permeabilized by adding 100 uL cold 100% Methanol (Sigma) for 10 minutes on ice. Thereafter, cells were washed again two times with 100 uL PBS/0.5% BSA and subsequently the intracellular staining was performed for 30 minutes in the dark at RT again with anti-CD33-AF700 (Clone WM53, BD Biosciences) and with anti-pSTAT1 (Clone 4a, BD Biosciences), anti-pSTAT3 (Clone 49-p-STAT3, BD Biosciences), anti-pSTAT5 (Clone 47, BD Biosciences), anti-pSTAT6 (Clone 18-p-STAT6, BD Biosciences) either coupled to fluorochromes PE or AF647. After this intracellular staining the cells were washed for two times with 100 uL PBS/0.5% BSA and fixated in 1% PA. Compensation beads were prepared for all the fluorochromes according to the protocol of BD Biosciences to properly set up the fluorescence compensation settings for the multicolour flow cytometric analyses by use of LSR Fortessa (BD Biosciences). Phosphorylation data were analyzed using FlowJo software (TreeStar Inc, Ashland, USA, version 7.6.5). The life gate was identified and subsequently CD3+ and CD3-negative populations were selected to distinguish between CD4+ and CD8+ T cells within the CD3+ population and single positive CD14 or CD33 or double positive (CD14+CD33+) myeloid cells within the CD3-negative population (Supplementary Fig. S4). The levels of pSTATs were analyzed as mean fluorescence intensity (MFI) and a fluorescence index (mean of stimulated sample divided by mean of unstimulated control sample) was calculated. A fluorescence index ≥ 2 was considered as a distinct upregulation of pSTAT after stimulation with the cytokine whereas an index between 1.5 and 2 was considered as a small up-regulation. Downregulation was defined as an index ≤ 0.5 or small downregulation when the index was between 0.5 – 0.7. Values between 0.7 and 1.5 were considered as no difference.

Statistical analysis

The non-parametric Mann-Whitney U test was used to compare continuous variables between patient groups and the $\chi 2$ test was used to compare categorical data with the statistical software package SPSS 20.0 (SPSS Inc., Chicago, USA). The Spearman correlation

coefficient was used to detect correlation in the non-parametric data. Patients were divided into groups based on the median of infiltrating cells and a univariate (Log Rank) analysis was performed for recurrence-free survival (RFS) analysis. Because of the small study population no multivariate analysis was performed. Two sided P values <0.05 were considered statistical significant. GraphPad Prism 5.04 (Graphpad Software Inc, LA Jolla, CA, USA) was used to illustrate the data by graphs and figures and for statistics in the total pSTAT induction.

Results

Antigen presenting capacity of APCs and T-cell proliferation of lymphocytes in uVIN patients and healthy donors

The antigen presenting capacity of blood-derived APCs from uVIN patients and healthy controls was tested by mixing them with lymphocytes of two unrelated healthy donors. In addition, the capacity of T cells to proliferate upon PHA stimulation was tested. No differences were observed between patients with uVIN and healthy controls (Supplementary Fig. S5), indicating that there are no overt general defects in the immune reactivity of patients with uVIN that may explain differences in response to immunotherapy.

Circulating myeloid cells and MDSCs in uVIN patients and healthy controls

An extensive panel of markers was used to distinguish monocytes/macrophages, dendritic cells and MDSC. The major populations of myeloid cells consisted of activated CD14+CD11b+ monocytes and account in total for approximately 15% of myeloid cells in both uVIN patients and healthy controls (Supplementary Table S1). The other three remaining populations represent minor categories of circulating myeloid cells and consist of immature DCs/early differentiating monocytes (CD14-CD11b-), activated DCs or monocytes with loss of CD14 (CD14-CD11b+)[42,50] and non-activated DCs or monocytes (CD14+CD11b-) accounting for <1% of CD45+HLA-DR+ cells.

Comparison of the different subpopulations between uVIN patients and healthy controls revealed no differences in the great proportion of activated CD14+ monocytes. Analysis of the smaller myeloid cell groups revealed that the frequency of circulating immature DCs/early differentiating monocytes (CD14-CD11b-, p=0.03) and type 2a and 2c monocytes (CD14-CD11b+CD206+CD16-, p=0.01 and CD14-CD11b+CD163+CD206-, p=0.02) were lower in uVIN patients than in healthy controls (Supplementary Table S1). The frequency of circulating MDSC populations was estimated below 0.05% and therefore not further analysed (Supplementary Table S2).

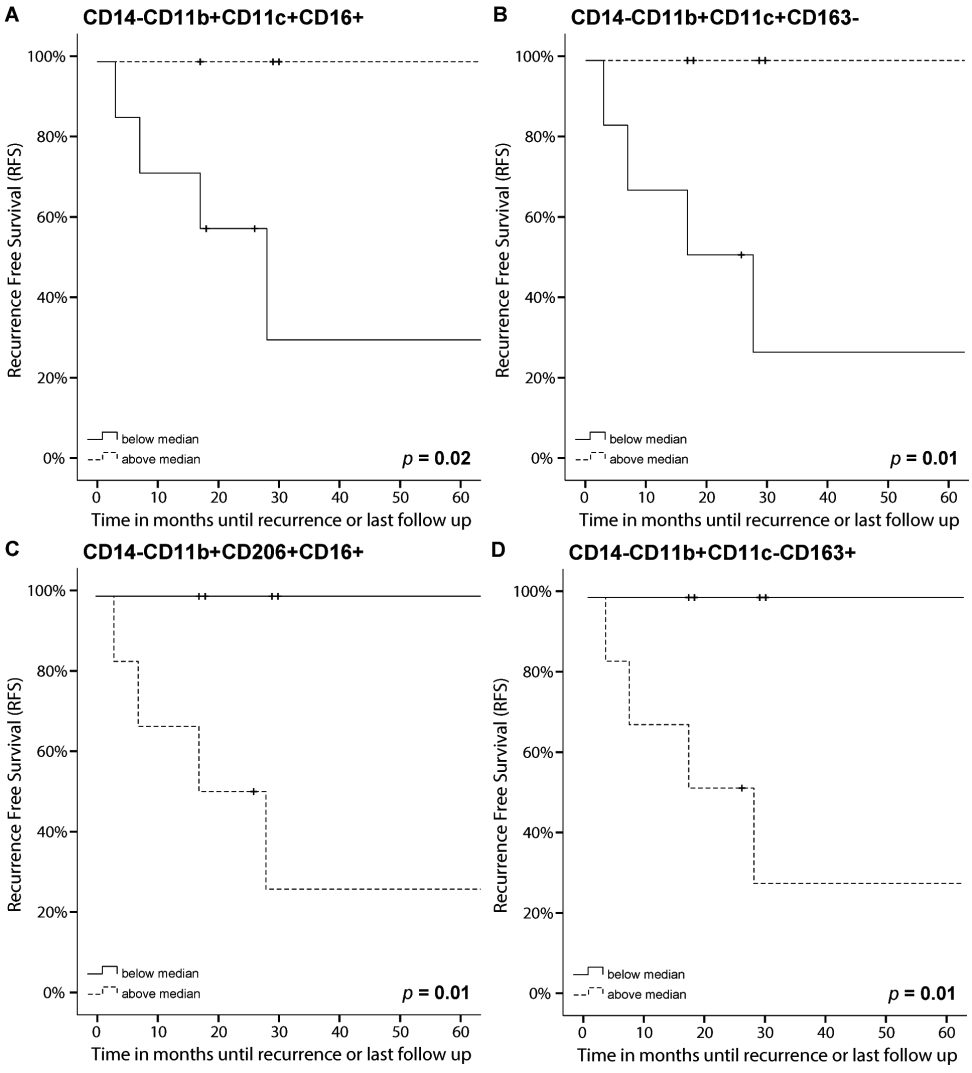


Figure 1: **Influence of systemic DCs and type 2 macrophages on recurrence free survival**

Differences in circulating myeloid cell types between recurrent and non-recurrent uVIN patients were divided based on the median percentage of cells and analysed by univariate Log Rank for their influence on recurrent free survival (RFS). Kaplan-Meir survival curves for activated DCs and type 2 macrophages on RFS are depicted. In A and B a high number of activated DCs (CD14-CD11b+CD11c+CD16+(A) and CD14-CD11b+CD11c+CD163-(B)) is related to an increased RFS and in C and D a high number of type 2a macrophages (CD14-CD11b+CD206+CD16+(C)) and type 2c macrophages (CD14-CD11b+CD11c-CD163+(D)) is related to a decreased RFS.

Previously we observed that the number of CD14+ intraepithelial myeloid cells had a prognostic value with respect to the recurrence and progression of uVIN lesions[16]. Therefore, we evaluated the frequency of circulating myeloid cells in this context. Recurrent uVIN patients have a lower, albeit not significant, percentage of circulating CD14High+CD11b+ activated monocytes compared to non-recurrent uVIN patients (9.5% vs 16.5%). Analysis of DCs by CD11c+ in this CD14High+CD11b+ monocyte population revealed that more DCs are present in non-recurrent uVIN patients, albeit not significant, probably due to the small group of patients analysed. Furthermore, patients with recurrent uVIN lesions displayed increased frequencies of type 2a and 2c monocytes/macrophages (CD14-CD11b+CD206+CD16+, p=0.01, CD14-CD11b+CD163+CD206-, p=0.01, and CD14-CD11b+CD11c-CD163+, p=0.01) and a lower number of activated DCs (CD14-CD11b+CD11c+CD16+, p=0.02 and CD14-CD11b+CD11c+CD163-, p=0.00). Moreover, a high number of activated DCs was associated with a prolonged recurrence free survival (Fig. 1a+b p=0.02/0.01) whereas a high frequency of type 2a and 2c monocytes/macrophages are associated with a decreased recurrence free survival (Fig. 1c+d p=0.01).

Expression of co-inhibitory molecules on PBMC from uVIN patients and healthy controls

In Table 2 the percentage of CD3+ lymphocytes of CD4+, CD8+ and expression of co-inhibitory molecules are represented. The majority of circulating lymphocytes is CD4+ (approximately 70%) and hardly express one of the tested co-inhibitory molecules on their surface, except for PD1 which was expressed in over 4% of CD4+ T cells and in approximately 3% of CD8+ T cells. TIM3 and NKG2a are expressed only by small proportions (<1%) of lymphocytes. Surface CTLA-4 (CD152) expression is scarce (< 0.05% of CD4+ and CD8+ T cells) and was therefore not analysed in detail. Thus, the differences in expression of the co-inhibitory molecules on T cells between healthy controls and uVIN patients are based on small cell populations. In the CD4+ T-cell populations the number of CD4+NKG2a+, either with or without co-expression of TIM3 and/or PD1, T cells is higher in uVIN patients(Supplementary Table S3). There were no differences in co-inhibitory molecule expression on CD8+ T cells observed between uVIN patients and controls (Supplementary Table S3).

Table 2: Expression of co-inhibitory molecules on CD3+ lymphoid cells in healthy controls and uVIN patients

Percentage of CD3+ T cells	PBMCs Healthy controls	PBMCs uVIN
CD4+	68.6%	69.7%
CD8+	23.2%	22.3%
PD1		
CD4+	4.1%	4.7%
CD8+	3.7%	2.7%
TIM3		
CD4+	0.21%	0.25%
CD8+	0.15%	0.13%
NKG2a		
CD4+	0.23%	0.43%
CD8+	0.38%	0.42%
CTLA4 (CD152)		
CD4+	0.04%	0.04%
CD8+	0.03%	0.03%
CD94		
CD4+	0.13%	0.15%
CD8+	1.17%	0.90%

In the microenvironment of the uVIN lesion, stromal expression of CD8+TIM3 and CD3+NKG2a+ T cells was related to an improved clinical outcome in uVIN patients[17]. Hence, we analysed the co-inhibitory molecule expression on lymphocytes in the context of recurrent disease. The main finding was that the frequency of CD4+ T cells expressing CD94+ was higher in patients who did not display a recurrent uVIN lesion. Moreover, a relatively higher number of circulating CD4+CD94+ T cells was associated with a longer recurrence free survival. This was not the case when patients were divided on basis of CD3+CD94+ and CD8+CD94+ T-cell frequencies (Fig. 2a+b+c). Expression of CD94 is related to the NKG2 family of receptors (e.g. NKG2a, -c, -d, -e and -h) by formation of heterodimers with either an activating or inhibitory function and are therefore always co-expressed[54,55]. Our study on the local immune infiltrate revealed that the expression of NKG2a was associated with an increased recurrence free survival (RFS). Therefore, we analysed RFS in the context of CD94+NKG2a+ T cells. The frequency of CD3+CD94+NKG2a+ and CD8+CD94+NKG2a+ T cells was not associated with differences in RFS while relatively higher numbers of CD4+CD94+NKG2a+ T cells were associated to no recurrences (Supplementary Table S3 + Fig. 2d-f). Because the CD3+CD94+NKG2a+/CD3+CD94+NKG2a- cell ratio in the uVIN microenvironment was associated with better clinical performance[17], we also analysed this ratio for cells in the circulation, but this lacked clinical significance (Supplementary Table S3 + Fig. 2g). The expression of TIM3 on circulating T cells was higher in patients with recurrent uVIN lesions albeit not directly related to RFS (Supplementary Table S3 and Fig. 2h-j). However, a relative higher number of circulating CD8+TIM3+ T cells was related to the recurrences and a worse RFS (Supplementary Table S3 + Fig. 2k).

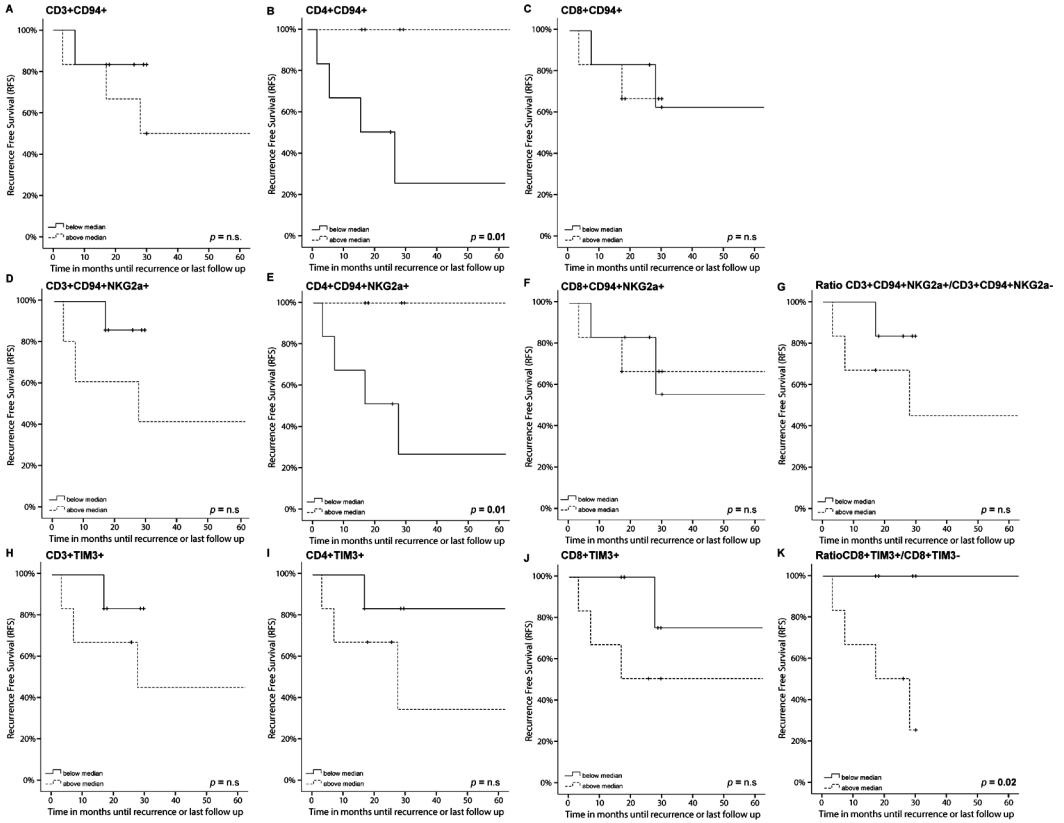
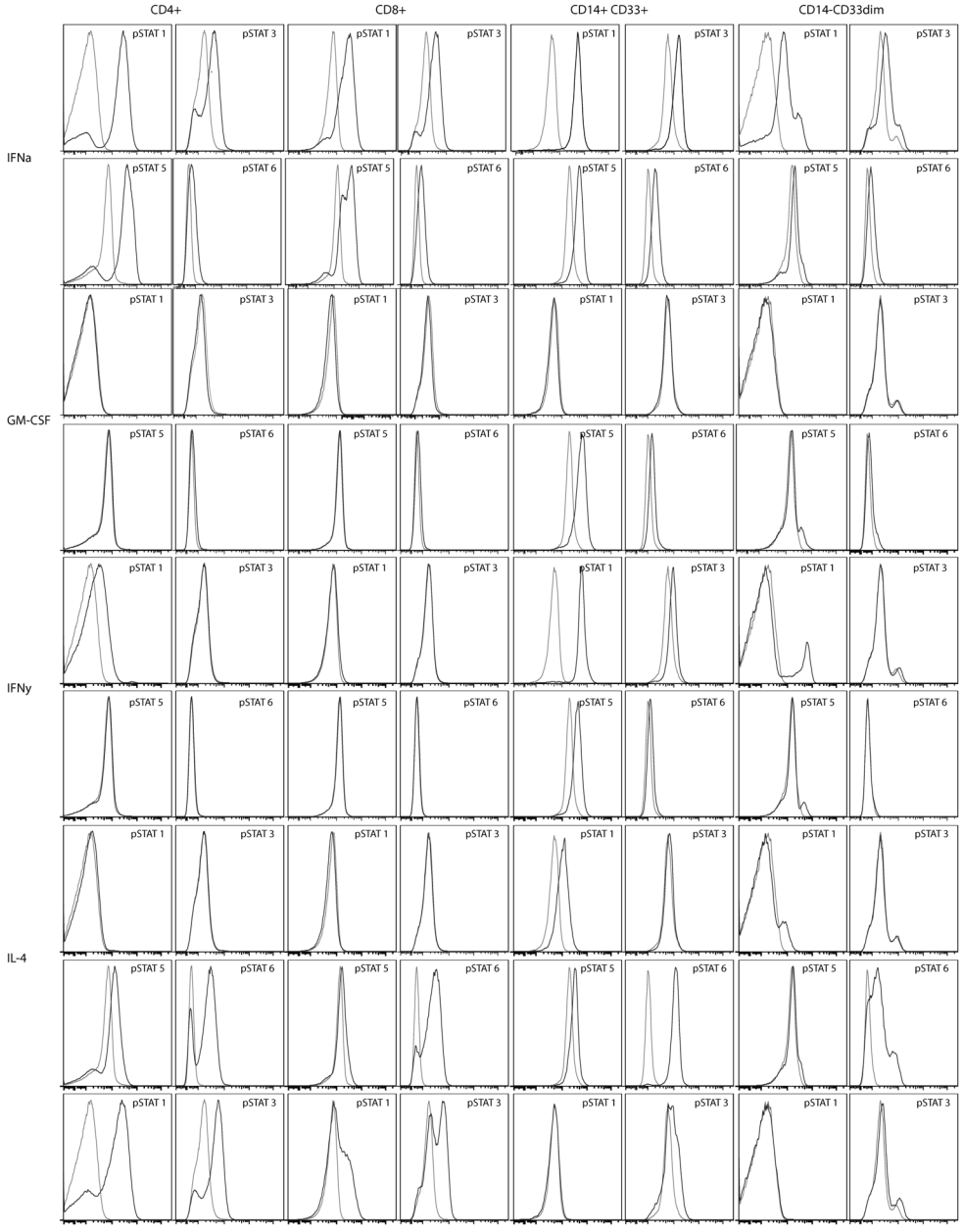


Figure 2: **Influence of CD94+, NKG2a+ and TIM3 expression on systemic lymphocytes on recurrence free survival**

Differences in circulating expression of co-inhibitory molecules on lymphocytes of recurrent and non-recurrent uVIN patients were divided based on the median percentage of cells and analysed by univariate Log Rank for their influence on recurrent free survival (RFS). Kaplan-Meir survival curves for expression of CD94+ on CD3(A), CD4(B) and CD8(C) as well as CD94+NKG2a+ on CD3(D), CD4(E) and CD8(F) and TIM3 on CD3(H), CD4(I), CD8(J) on RFS are depicted. Moreover in G and K, the ratios of CD3+CD94+NKG2a+/CD3+CD94- and CD8+TIM3+/CD8+TIM3- on RFS are depicted. In B and E a high number of CD4+CD94+ and CD4+CD94+NKG2a+ T cells are related to an increased RFS and in K a relative high number CD8+TIM3+ T cells is related to a decreased RFS.

No differences in STAT phosphorylation levels upon stimulation with cytokines

Since pathogens can reprogram intracellular immune-related signalling networks, potentially hindering immune activation, we set out to study the response of APCs and lymphocytes to various cytokines. In Figure 3 an example of STAT phosphorylation (pSTAT) upon cytokine stimulation in different immune cells is depicted.



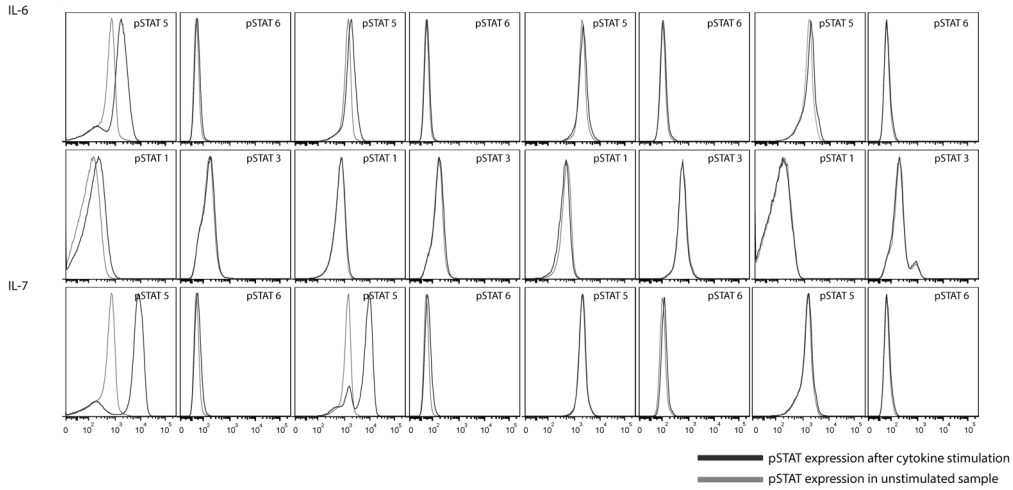


Figure 3: STAT phosphorylation upon stimulation with various cytokines or immune modulators in an example of one patient with uVIN

Intracellular phosphorylation of STAT1, -3, -5 and -6 were measured in CD3+, CD4+ and CD8+ lymphocytes and in CD14+CD33+ and CD14-CD33dim monocytes, after individual stimulation with eight different cytokines (IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFNγ) or adjuvant immune modulators (IFNα and GM-CSF) to determine the activation state of PBMCs of uVIN patients and healthy controls. After identification of the lifegate in the CD3+ populations CD4+ and CD8+ T cells were distinguished whereas in the CD3- population either single or double CD14+ and CD33+ cells were identified. The levels of pSTATs were analyzed as mean fluorescence intensity (MFI) and a fluorescence index (mean of stimulated sample divided by mean of unstimulated control sample) was calculated. A fluorescence index ≥ 2 was considered as a distinct upregulation of pSTAT after stimulation with the cytokine whereas an index between 1.5 and 2 was considered as a small up-regulation. Downregulation was defined as an index ≤ 0.5 or small downregulation when the index was between 0.5 – 0.7. Values between 0.7 and 1.5 were considered as no difference. The differences in pSTAT induction in different immune cell populations upon cytokine stimulation are illustrated by use of FlowJo software (TreeStar Inc, Ashland, USA, version 7.6.5). The results for Il-2, IL-5 and IL-10 are depicted in Supplementary Fig. S7.

IFNγ is expected to induce a high increase in the levels of pSTAT1 in monocytes and somewhat lower levels in T cells[53]. Indeed, a strong pSTAT1 increase was uniformly detected in CD14+CD33+ monocytes and in 37.5% of CD4+ T cells in all uVIN patients and healthy controls. In addition, IFNγ induced increased levels of pSTAT5 in CD14+CD33+ monocytes in approximately half of patients and control subjects (Fig. 4 and Supplementary Fig. S7). IL-2 hardly showed an effect on STAT phosphorylation, except for the upregulation of pSTAT5 in 3 individuals (2 patients and a healthy donor). IL-4 is known to mediate its signals via pSTAT6 [56,57]. Indeed IL-4 stimulation resulted in a strong increase of pSTAT6 in both myeloid cells and T cells of uVIN patients as well as healthy controls (Fig. 4 and Supplementary Fig. S7). IL-5 is known for pSTAT5 induction[57], but this was not observed. IL-6 is both a pro-inflammatory as anti-inflammatory cytokine, which is well known for its activation of STAT3. Whereas IL-6 upregulated pSTAT3 in T cells, predominantly in the CD4+ T cell population in line with IL-6R

expression[58], it did not influence pSTAT3 levels in myeloid cells (Fig. 4 and Supplementary Fig. S7). Furthermore, IL-6 increased the levels of pSTAT1 and pSTAT5 levels in T cells, albeit that pSTAT5 upregulation was more infrequently observed. The upregulation of pSTAT1 was more pronounced in CD4+ than CD8+ T cells (Fig. 4 and Supplementary Fig. S7). IL-7 is known to induce pSTAT5[57]. Indeed CD4+ and CD8+ T cells but not myeloid cells expressed pSTAT5 (Fig. 4 and Supplementary Fig. S7). IL-10 is known to induce pSTAT3[57]. Approximately half of the uVIN patients and controls displayed increased levels of pSTAT3 in CD4+ and CD8+ T cells whereas in 53% of patients and 75% of controls pSTAT3 was increased in the CD14+CD33+ monocyte population upon IL-10 stimulation (Fig. 4 and Supplementary Fig. S7). Overall, we observed no strong differences in the activation or expression of pSTAT to cytokine stimulation in the immune cells between healthy controls and uVIN patients.

STAT phosphorylation upon stimulation with immune modulators that are used to enhance the efficacy of immunotherapy

IFNα and GM-CSF are used in clinical trials as immune modulators to boost and polarize the Th1/CTL response to therapeutic vaccination and in some cases as monotherapy[59-61]. IFNα is a well-known potent inducer of various pSTATs and it is known to have a close synergic function with IFNγ[53,62,63]. Indeed all types of tested immune cells responded with increased levels of pSTAT1. In T cells the levels of pSTAT5 increased, which was most pronounced for CD4+ T cells. Interestingly, pSTAT5 upregulation was more often observed in the CD8+ T-cell population of healthy controls than that of uVIN patients (88% controls vs 54% patients (*p*=n.s.)). Moreover, whereas the majority of healthy controls (75%) displayed increased pSTAT5 levels in their CD14+CD33+ monocytes, this was only the case in 37.5% of the patients (*p*=0.07). The levels of pSTAT5 expressed by healthy control-derived CD14+CD33+ monocytes were increased as well (*p*= 0.05; Supplementary Fig. S7). IFNα also stimulated the increase of pSTAT3 and pSTAT6, especially in the CD4+ T cells and the CD14+CD33+ monocytes. The immune modulator GM-CSF only induced an increase in pSTAT5 levels in CD14+CD33+ monocytes and in contrast to IFNα this was more often observed in CD14+CD33+ monocytes of uVIN patients (83%) than healthy controls (38%). Also the absolute levels of pSTAT5 were higher in these cells after stimulation with GM-CSF (p=0.018; Fig. 4, Fig. 5 and Supplementary Fig. S7). Comparison of the pSTAT responses to these two immune modulators between uVIN patients and healthy controls revealed differences only in the response of CD14+CD33+ monocytes. Healthy control-derived CD14+CD33+ monocytes displayed a stronger response on the basis of the overall number of pSTATs that were increased upon stimulation with IFNα (Fig. 6). In conclusion, comparison of the fold-increase of pSTATs showed that IFNα induced a stronger increase of pSTAT5 in CD14+CD33+ monocytes of healthy subjects while GM-CSF triggered a stronger upregulation of pSTAT5 in these particular monocytes of uVIN patients (Fig.5).

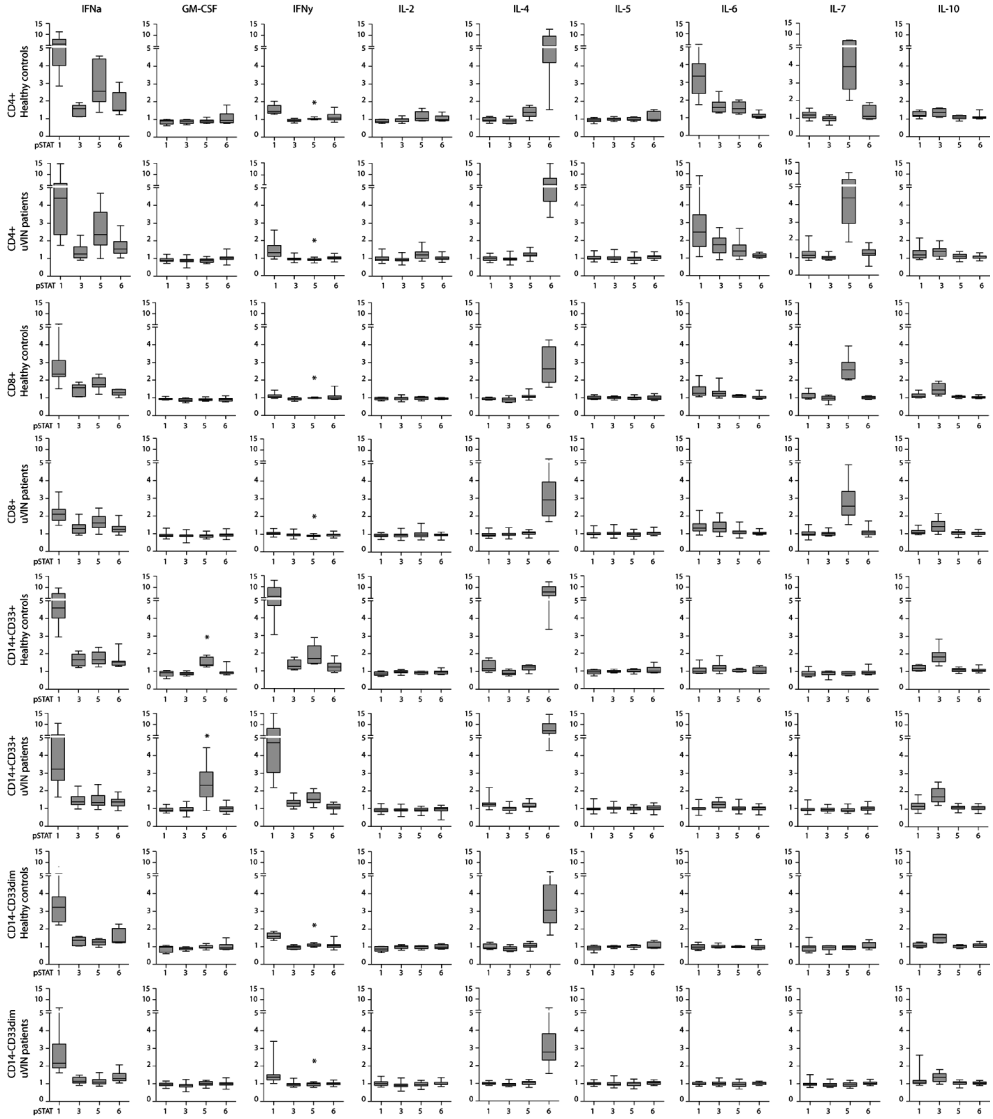


Figure 4 - van Esch et al.

Figure 4: **Boxplots of pSTAT induction upon stimulation with various cytokines or immune modulators in patients with uVIN and healthy donors**

The fold increase of different pSTATs upon cytokine stimulation in all immune cell populations are depicted for uVIN patients and healthy controls. Significant differences by comparison of absolute fold changes of pSTAT in uVIN patients and healthy donors by use of the non-parametric Mann-Whitney U test are indicated by *.

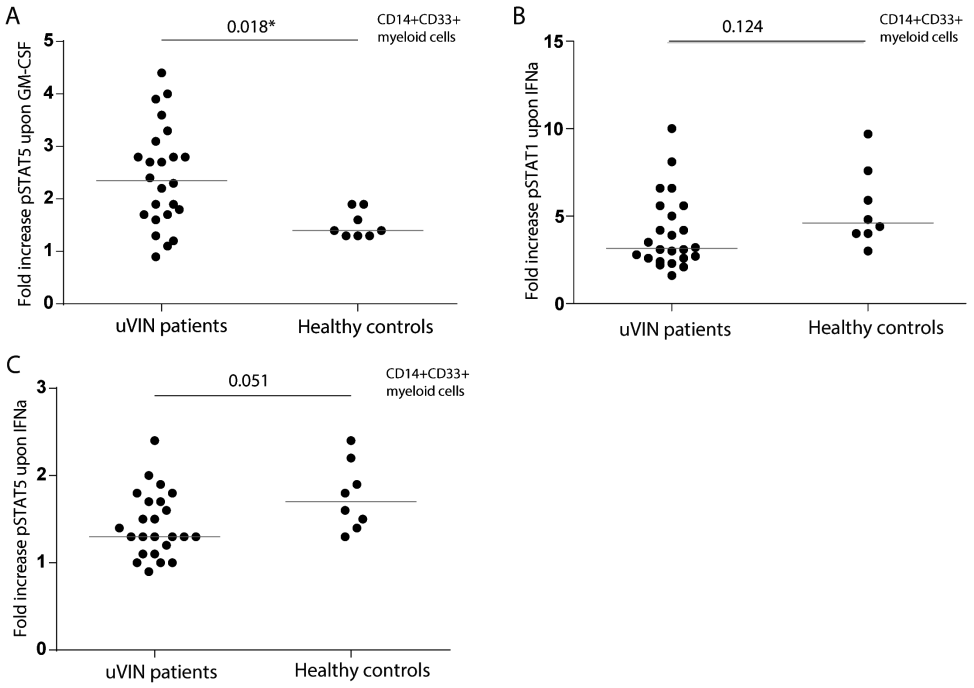


Figure 5: Phosphorylation of pSTAT5 after stimulation with GM-CSF and pSTAT1 and pSTAT5 upon IFNα in CD14+CD33+ monocytes in uVIN patients and healthy donors

The induction of pSTAT1 and pSTAT5 upon stimulation with immunomodulatory agents GM-CSF and IFNα. In figure A, a significant increase in pSTAT5 in CD14+CD33+ myeloid cells upon GM-CSF in comparison to the fold increase in healthy controls is depicted. In figure B the pSTAT1 and in figure C the pSTAT5 induction in CD14+CD33+ myeloid cells upon IFNα is depicted and albeit not significant in uVIN patients the fold induction is lower compared to healthy controls.

STAT phosphorylation of pre-treatment samples of patients treated with HPV-16 SLP ISA 101 vaccination

We then compared the STAT phosphorylation after stimulation with these immune modulators in pre-treatment samples of 3 uVIN patients which responded by a peak in T-cell associated IFNγ production upon the first vaccination with HPV16-SLP ISA101 to those (n=5) that did not[34]. The CD14+CD33+ cells of uVIN patients with a peak IFNγ response displayed a more pronounced increase in pSTAT1 induction upon IFNα stimulation than the other patients. Stimulation with GM-CSF did result in an overt pSTAT5 induction and there were no differences observed between the two patient groups (Fig. 7).

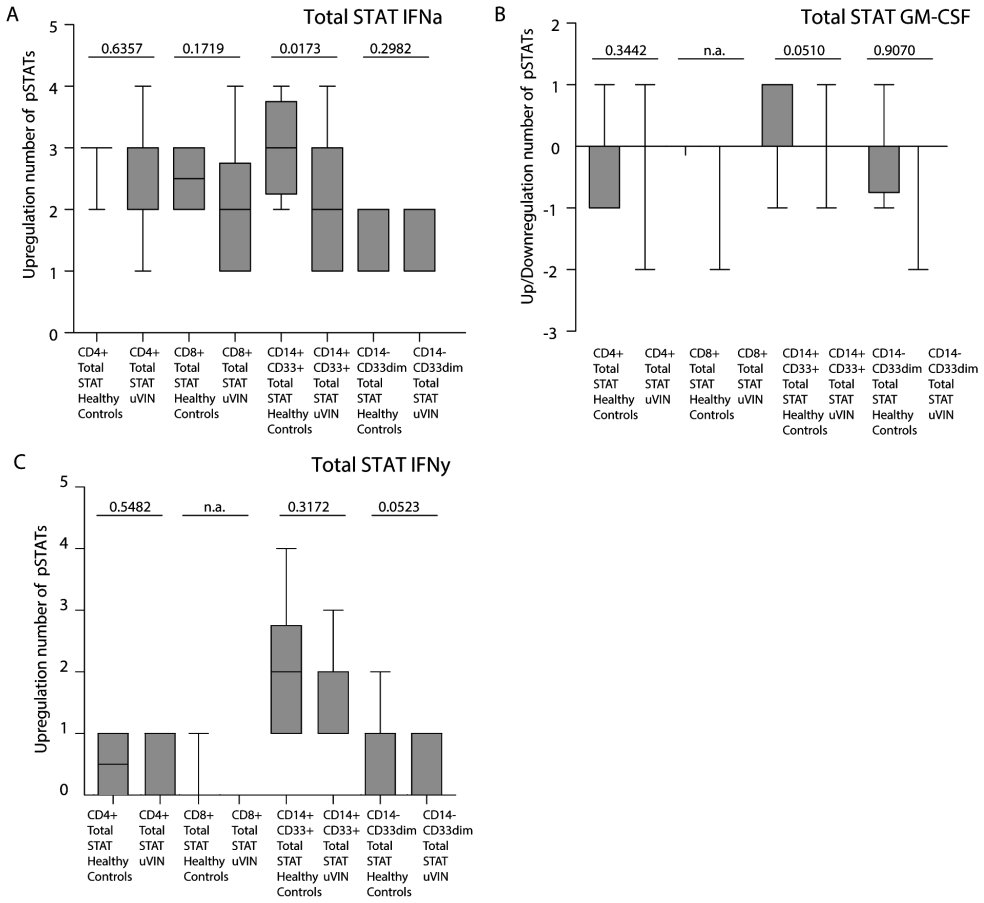


Figure 6: Total pSTAT upregulation in different immune cell types upon stimulation with IFNα, GM-CSF and IFNγ

Total pSTAT upregulation was calculated upon an upregulation or downregulation of one or more STATs upon stimulation with a specific cytokine. In case of upregulation +1 and in case of downregulation -1 was scored revealing a maximum score of 4 in case of pSTAT 1, 3, 5 and 6 upregulation and a minimum score of -4 for each patient. Subsequent analysis of the total pSTATs was performed by an unpaired T test in Graphpad Prism 5. In Figure A the total pSTAT induction upon IFNα is significantly higher in the CD14+CD33+ myeloid cell population in healthy controls. Differences in total pSTAT induction upon GM-CSF (Fig. B) and IFNγ (Fig. C) can be established albeit that IFNγ reveals a distinct upregulation of total pSTAT in CD14+CD33+ myeloid cells of both healthy controls as uVIN patients.

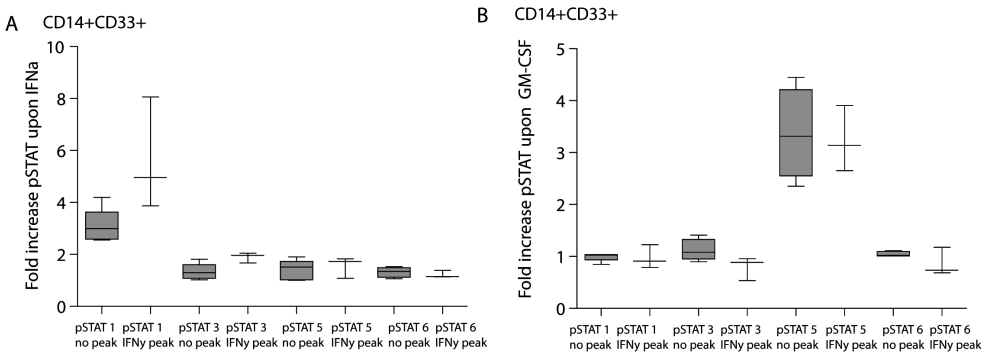


Figure 7: STAT phosphorylation upon stimulation with IFNα and GM-CSF in CD14+CD33+ myeloid cells of patients with and without an IFNγ peak response upon HPV 16 SLP ISA 101 vaccination

In patients where a distinct peak of IFNγ production upon the first vaccination with HPV 16 SLP ISA 101 is found, IFNα reveals a higher fold increase in pSTAT1 of CD14+CD33+ monocytes albeit not significant (A). Stimulation with GM-CS F does result in an overt increase in pSTAT5 in both peak responders and non-responders (B).

Discussion

In this study we analysed the type and function of circulating immune cells by an extensive phenotypic analysis and a novel technology that allows simultaneous measurements of changes in phosphorylation of protein levels of STATs after stimulation[29,53]. Our study revealed no differences in the potential of T cells to proliferate or the capacity of antigen presenting cells to stimulate T-cell responses between uVIN patients and healthy subjects. However, the frequencies of immature DCs or differentiating macrophages and activated monocytes/macrophages are lower in uVIN patients compared to healthy controls. Furthermore, patients with recurrent uVIN lesions display lower frequencies of DCs and higher frequencies of type 2 monocytes/macrophages than patients without recurrent disease. This alteration was also observed at the functional level since CD14+CD33+ monocytes/macrophages of uVIN patients displayed decreased levels of pSTAT5 upon IFNα stimulation but increased pSTAT5 levels when stimulated with GM-CSF when compared to healthy controls. Our data on the response of CD14+CD33+ cells from vaccinated patients to IFNα suggest that this may also be reflected in their capacity to respond to vaccination, but clearly more patients need to be analysed. Importantly, the relation between increased numbers of circulating type 2 monocytes/macrophages in the blood and a worse RFS is in concordance with our previous observation that M2 macrophages are abundantly infiltrating uVIN lesions and associated with recurrent disease as well[16].

A higher frequency of circulating CD4+CD94+(NKG2a+) T cells was found to be associated with the absence of recurrences. If cells express NKG2a than they can also be considered

positive for CD94 since CD94 forms a complex with the NKG2 family[54,55]. In our previous study on the expression of co-inhibitory molecules in the uVIN microenvironment we studied NKG2a expression in the context of CD3+ T cells and showed that its expression was associated with a better RFS[17]. Unfortunately, we did not distinguish between CD4+ and CD8+ T cells expressing NKG2a+ in that study. Our current data suggest that specifically the expression of NKG2a on CD4+ T cells is associated with better clinical outcome. New studies are required to analyse whether the NKG2a+ cells in the uVIN microenvironment are CD4+ or CD8+ and which of the two populations is associated with better RFS. It will be extremely important to know if the analysis of both the type 2 monocytes/macrophages and the CD4+CD94+NKG2A+ T cells in a blood sample could substitute for measurements of these cell types in the tissue as it may allow a relatively simple blood analysis to become a biomarker that is associated with local events and prognosis. Therefore, a new study is warranted in which both blood and tissue of the same patient population is studied to validate our current observations.

In contrast to the similarities between local and systemic NKG2a expression we did not observed such a relationship with respect to the presence of CD8+TIM3+ cells. While in the uVIN microenvironment the presence of CD8+TIM3+ cells correlated with pro-inflammatory T-cell responses, characterised by Tbet and Galectin-9 expression, and was found to be a favourable prognostic factor in recurrence free survival, the frequencies of circulating TIM3+ T-cell subpopulations were not directly associated. However, a relative higher number of circulating CD8+TIM3+ T cells over CD8+TIM3- T cells was found to be associated with worse clinical outcome. These opposite data suggest that either there is no reflection of the changes in the local environment by those measured in the circulation, or that the context in which CD8+TIM3+ cells are analysed is more important as it is expressed both during T-cell activation and exhaustion[64]. Future research should establish the exact role and function of these cells in uVIN patients.

Of interest for future immunotherapeutic studies are our data on the stimulation of immune cells with the immune modulators GM-CSF and IFNα. They are both involved in DC activation and used as adjuvants for different therapeutic vaccines aiming to enhance a Th1 polarized T-cell response[59-61]. The use of mainly IFNα has been proven beneficial to vaccine-induced T-cell responses, whereas GM-CSF resulted in different outcomes[60,61]. IFNα can be seen as a potent immune modulator revealing mainly pro-inflammatory associated STAT1 phosphorylation induction in both healthy controls, premalignant and cancer patients and it is known to have a close synergic effect with IFNγ[62,63]. A type I IFN response is essential for DC maturation and induction of CD4+ Th1 immunity[65]. The higher total induction of pSTAT in healthy subjects compared to uVIN patients may reflect the reduced potency of the precursor cells of patients' DCs and macrophages (CD14+CD33+) to respond to immune modulators. Of particular interest was our observation of opposite pSTAT5 responses in

CD14+CD33+ myeloid cells from uVIN patients and healthy controls when stimulated with IFNα or GM-CSF. Of note, IL-2 is known to induce pSTAT5 in DCs required for their functional maturation[66]. However, an IL-2 induced increase of pSTAT5 was not observed, potentially because the stimulation was for 15 minutes whereas in another study pSTAT5 was shown to be detectable in DCs only after 30 minutes of stimulation[66]. GM-CSF is known to generate granulocyte and macrophage populations from myeloid precursor cells and is of importance in survival, proliferation and differentiation of monocytes and macrophages by activation of JAK2 and STAT5 [24]. The CD14+CD33+ myeloid cells of uVIN patients responded to GM-CSF with higher levels of pSTAT5 but it is unclear how this may impact immunity. In patients with a cytomegalovirus (CMV) infection monocyte-derived DCs lack the ability to secrete IL-12 and to induce Th1 cell activation and this is associated with an impaired response of these monocytes to induce pSTAT5 upon GM-CSF stimulation[67], suggesting that GM-CSF induced pSTAT5 is required for a good immune response. Interestingly, leukaemia patients display an increased pSTAT5 response to GM-CSF stimulation when compared to healthy donors. Similar to what we observed in uVIN patients. This response in leukaemia patients was classified as hypersensitivity and correlated with high risk disease, higher peripheral leukocyte and monocyte count and altered signalling in the Ras pathway[68]. Signalling via STAT5 may result in pro-inflammatory responses but it also essential for the induction of peripheral tolerance[27]. pSTAT5 plays an essential role in Th2 differentiation since pSTAT5 expression results in IL-4 expression by cells lacking pSTAT6 and pSTAT5 induces IL-4 in T cells cultured under Th1 polarizing conditions. Moreover pSTAT5 is involved in regulatory FoxP3+ T-cell (Treg) development, induced by IL-2 and pSTAT5, by binding to the FoxP3 promotor[56]. The role of an altered pSTAT5 expression in myeloid cells of uVIN patients can thus be interpreted along two lines; a tolerance inducing role as well as a pro-inflammatory role when pSTAT5 is expressed in combination with pSTAT1. Interestingly, the increased expression of pSTAT1 upon stimulation with IFNα in uVIN patients with a peak IFNγ response upon vaccination fits this latter role of pSTAT5 well. Unfortunately, it remains difficult to unravel the activated interactive pathways *in vivo* that may explain the difference in the patients capacity to respond to immunotherapy by *in vitro* experiments of phosphorylation of STATs upon single cytokine stimulations due to heterodimerization of simultaneous STATs and crosstalk among the JAK-STAT pathways occur[32,57,62]. For example there is cross talk between type I and type II IFNs as pre-treatment with IFNγ sensitizes cells to IFNα and pre-treatment with IFNγ increases the level of pSTAT1 which can enhance the subsequent IFNγ response during macrophage activation[62,63]. Furthermore, IFNs inhibit IL-4 induced STAT6 expression in human monocytes and IL-10 can inhibit the activity of pro-inflammatory cytokines such as IFNα [62,69,70]. Potentially, combinations of cytokines to stimulate immune cells may better mimic the *in vivo* reaction upon secretion of multiple cytokines in response to infections or therapy.

In conclusion, our data suggest that the circulating myeloid cell population is phenotypically and functionally altered in uVIN patients and that the measurement of circulating type 2 monocytes is related to recurrent disease. This relationship needs to be confirmed as it may serve as a blood biomarker that reflects the local microenvironment and is important for prognosis.

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The authors disclose no potential conflicts of interest.

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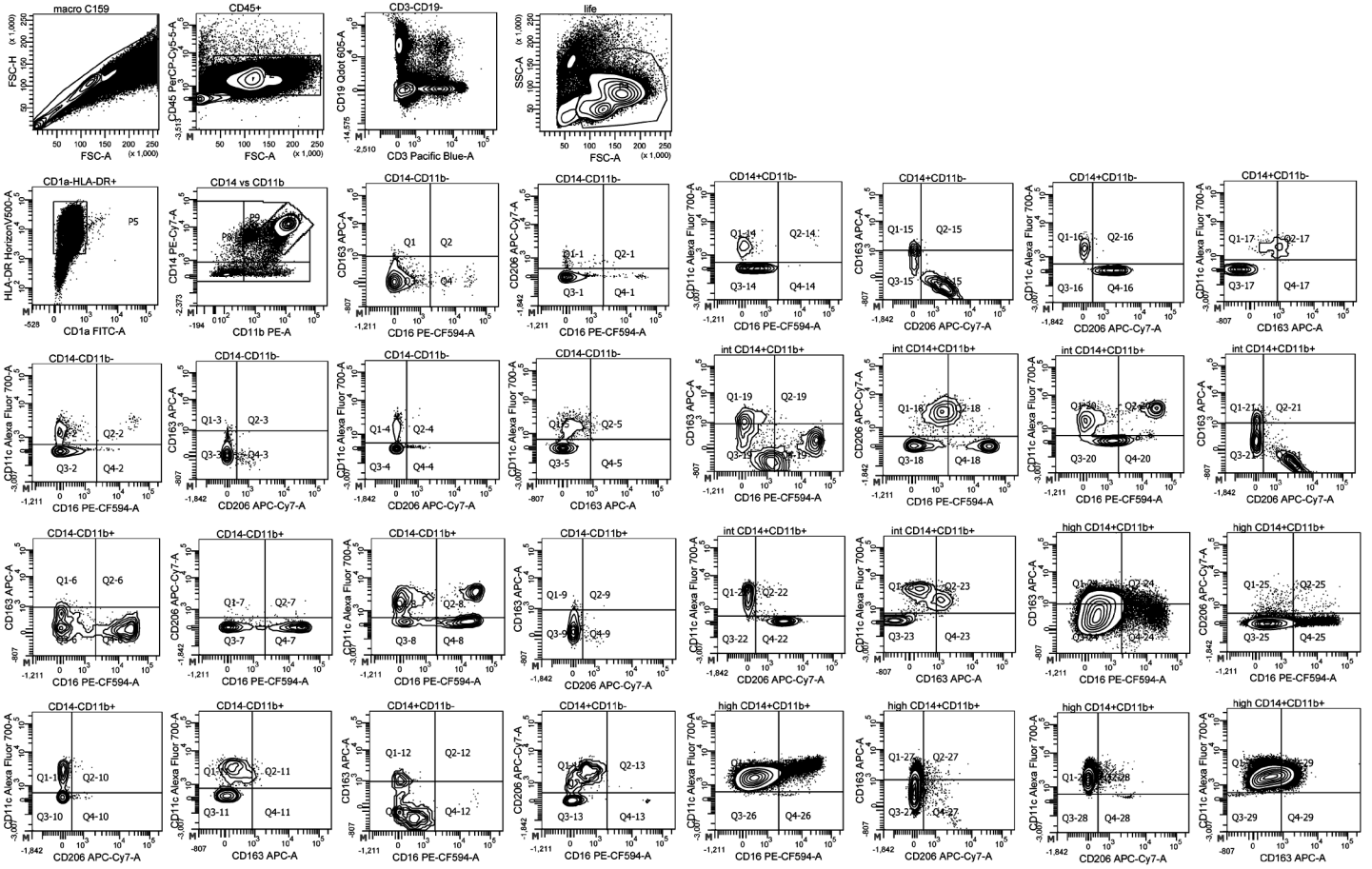
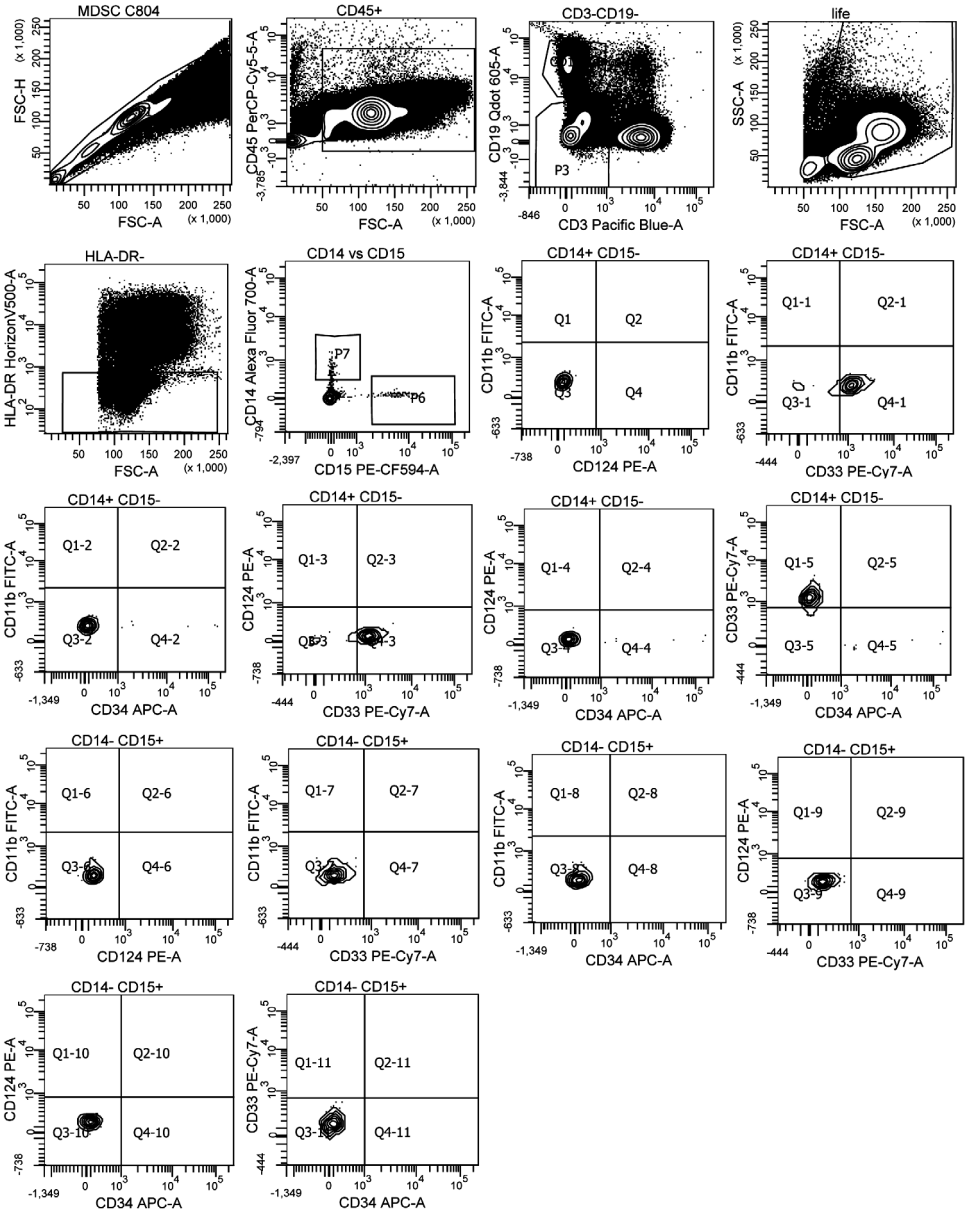


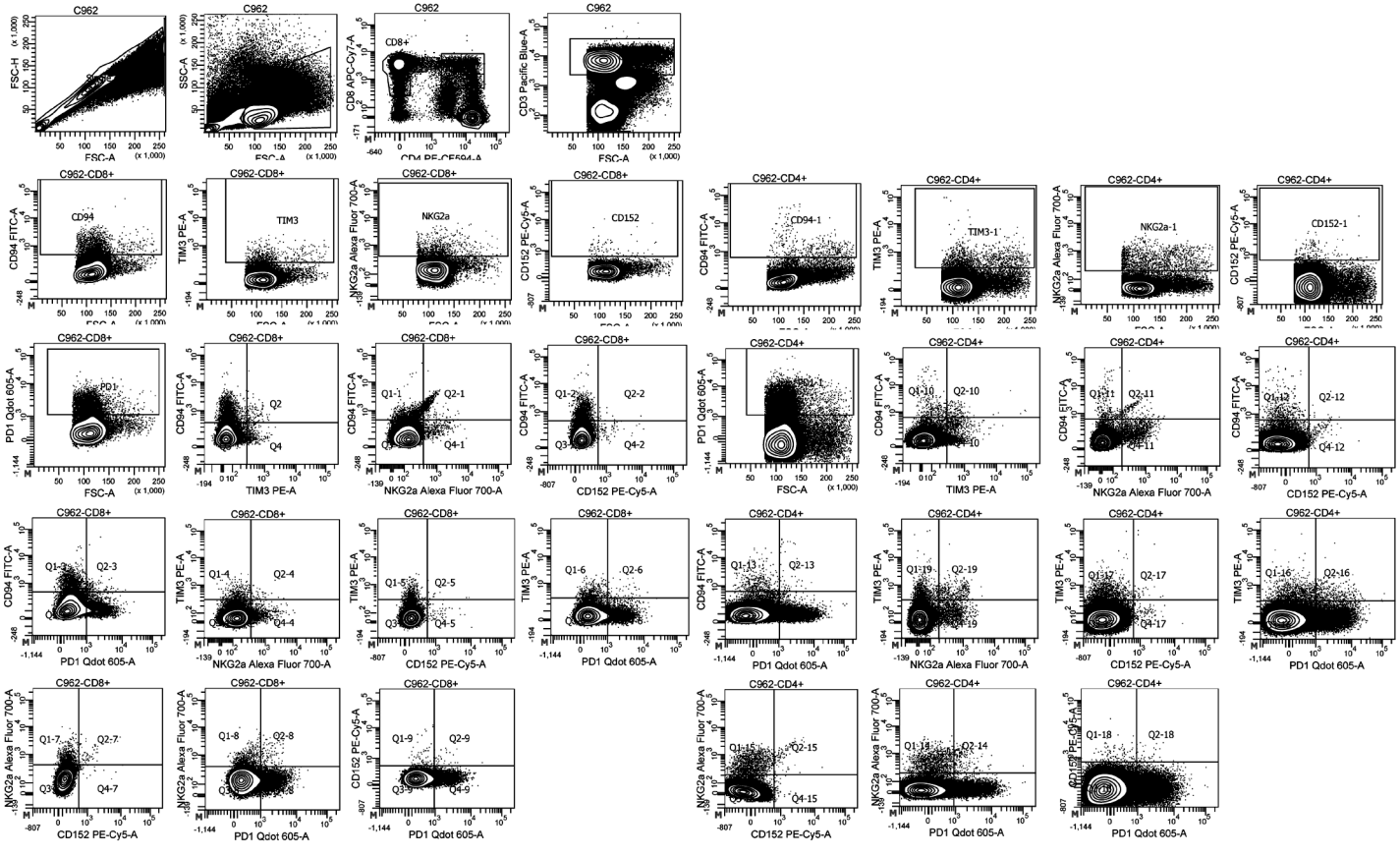
Supplementary Figure S1

Gating strategy example of monocytes/macrophages



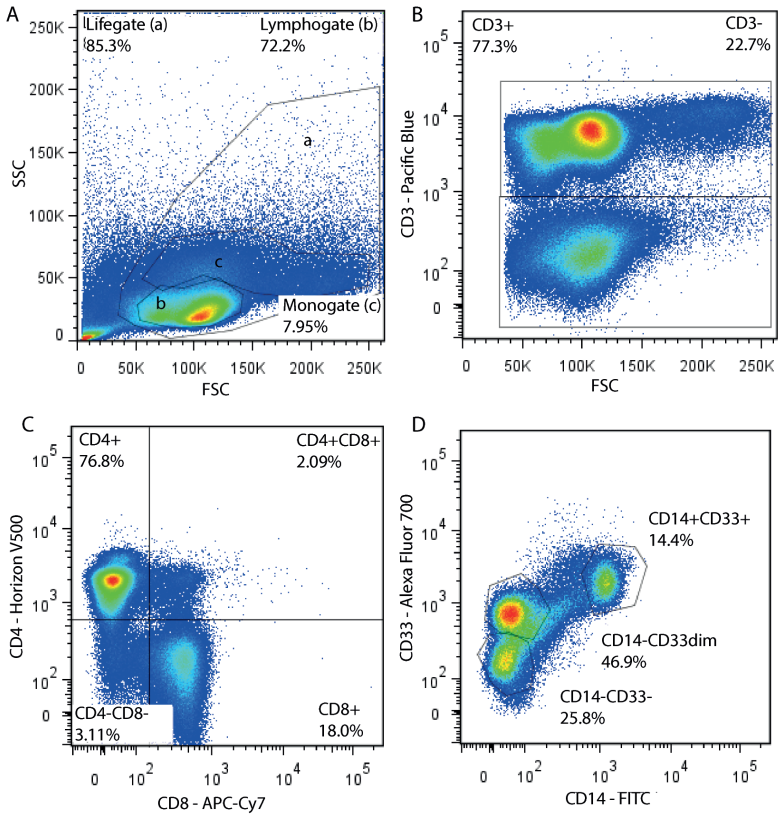
Supplementary Figure S2

Gating strategy example of myeloid derived suppressor cells



Supplementary Figure S3

Gating strategy example of co-inhibitionary molecules



Supplementary Figure S4

Gating strategy example of phosphorylation of STAT

PHA

Patient	SI	SI	SI	SI	SI
Buffy 9	689,0	867,5	495,3	443,6	655,4
Buffy 11	368,5	482,4	304,5	361,2	418,8
C883	539,3				
C889	455,4				
C977	201,6				
C988	423,3				
C612		237,1			
C887		400,9			
C296		491,3			
C333		170,1			
C80			1105,4		
C962			608,1		
C804			196,0		
C159				344,5	
C412				802,6	
C789				603,4	
C313				409,2	
HD ML					980,7
HD PV					245,4
HD SS					240,3
HD SM					768,3

HD = Healthy control
SI = Stimulation Index: calculated by the ratio of the mean of PHA stimulated PBMCs devided by the mean of the unstimulated PBMCs
SI >3 is considered positive (of mean plus 3 times the standarad deviation of unstimulated PBMCs)

MLR

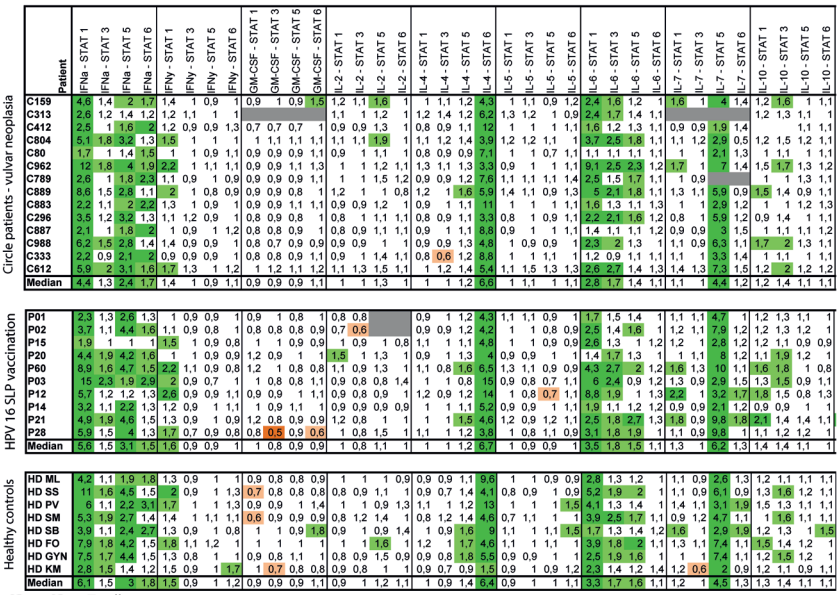
Patient	Buffy	SI	Circle	Buffy	SI	Median SI
C883	B9	44,2	C883	B11	48,4	46,3
C889	B9	33,9	C889	B11	6,6	20,2
C977	B9	49,3	C977	B11	15,6	32,5
C988	B9	8,4	C988	B11	14,3	11,3
C612	B9	23,1	C612	B11	23,9	23,5
C887	B9	23,5	C887	B11	10,2	16,9
C296	B9	65,2	C296	B11	24,8	45,0
C333	B9	3,5	C333	B11	45,2	24,4
C80	B9	2,4*	C80	B11	10,7	10,7
C962	B9	13,1	C962	B11	17,1	15,1
C804	B9	15,3	C804	B11	23,3	19,3
C159	B9	3,8	C159	B11	19,8	11,8
C412	B9	6,4	C412	B11	5,7	6,0
C789	B9	15,2	C789	B11	5,6	10,4
C313	B9	12,6	C313	B11	3,3	7,9
HD ML	B9	17,7	HD ML	B11	13,2	15,5
HD PV	B9	28,7	HD PV	B11	21,9	25,3
HD SS	B9	23,2	HD SS	B11	18,6	20,9
HD SM	B9	5,8	HD SM	B11	8,9	7,3

HD = Healthy control
* HLA typing B9 = HLA-DRB1 401 and C80 = HLA-DRB1 401
SI = Stimulation Index: calculated by the ratio of the mean of irradiated PBMCs co-cultured with healthy donor PBMCs divided by the mean of the healthy donor PBMCs alone
SI >3 is considered positve (of the mean plus 3 times the standard deviationof healthy donors alone)

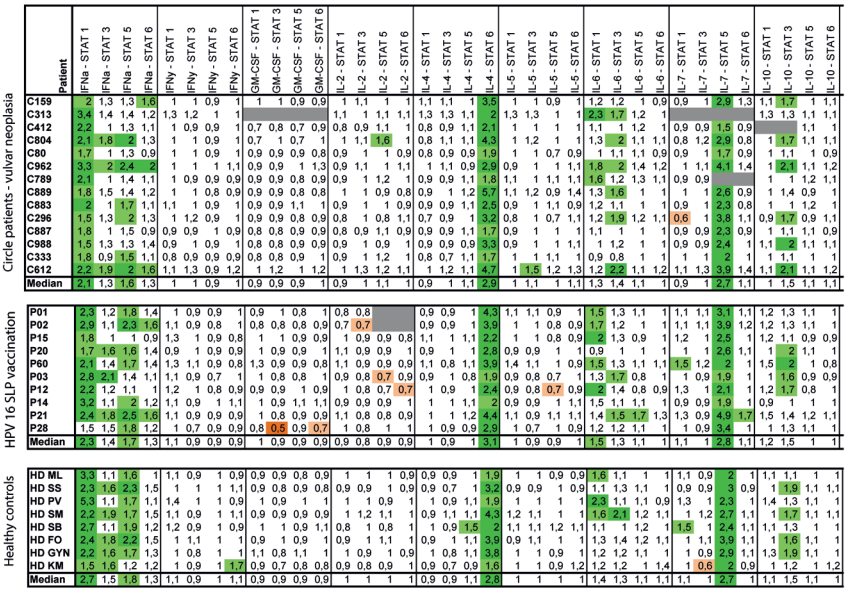
Supplementary Figure S5

Results of proliferation (PHA) and antigen presenting capacity (MLR) of PBMCs of uVIN patients. SI: stimulation index

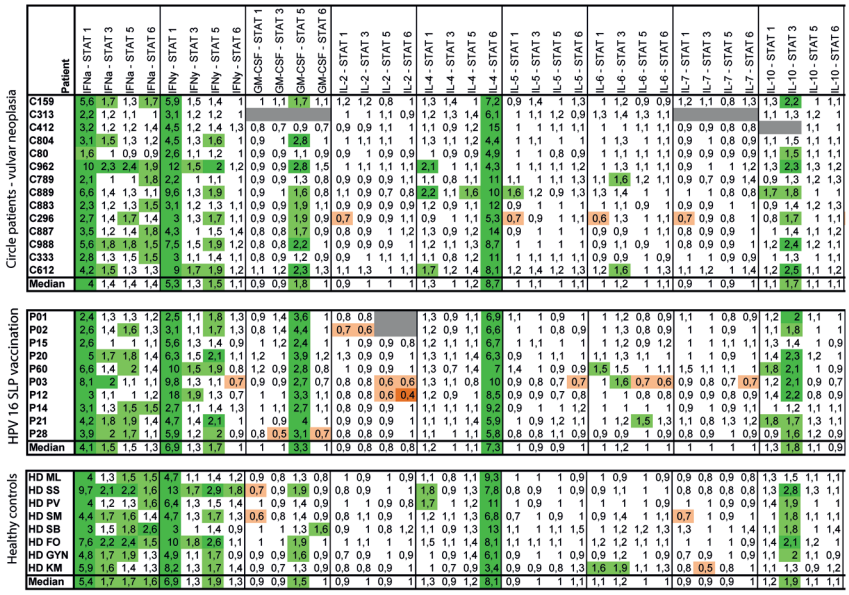
A: CD3+ CD4+ T cells



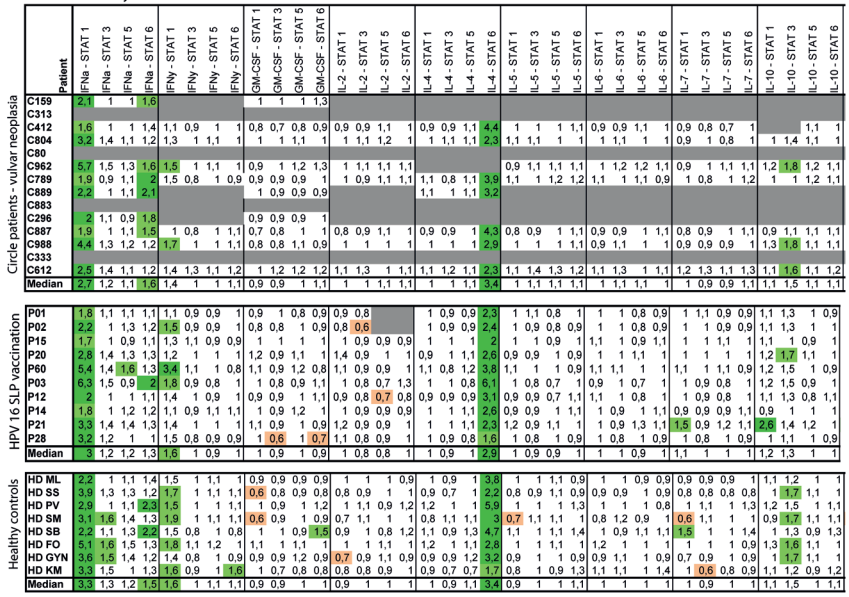
B: CD3+ CD8+ T cells



Supplementary Figure S6

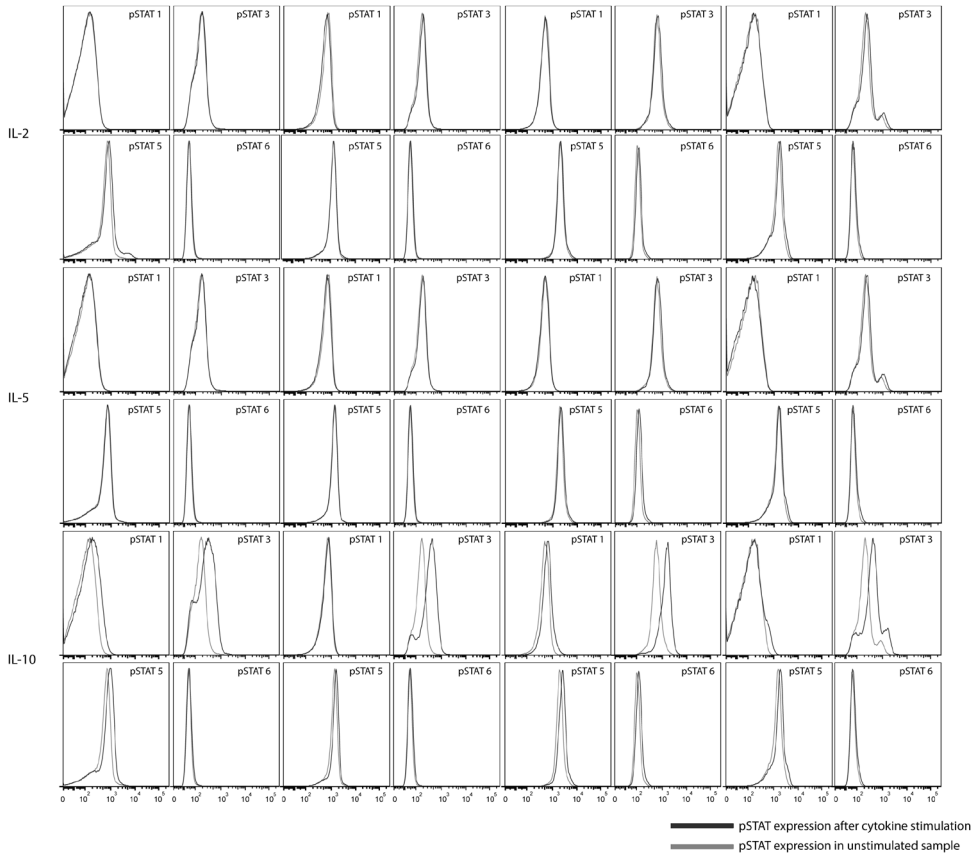


D: CD14- CD33dim myeloid cells



Supplementary Figure S6 - Continued

Overview of pSTAT upregulation in CD4+, CD8+, CD14+CD33+ and CD14-CD33dim immune cells upon stimulation with various cytokines or immune modulators in patients with uVIN and healthy controls



Supplementary Figure S7

Gating strategy example of phosphorylation of STAT for IL-2, IL-5 and IL-10

Supplementary Table S1 - Systemic expression of myeloid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.

*Non-parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant and in **bold** the p-values are marked where the cells numbers are >0.05%.

Myeloid cell type	Controls (median % of CD45+) N=8	uVIN (median % of CD45+) N=14	p =	Recurrent uVIN (median % of CD45+) N=5	Non Recurrent uVIN (median % of CD45+) N=7	p =	RFS
CD14- CD11b-	1.16 (0.58-2.41)	0.85 (0.30-1.72)	**0.029**	0.59 (0.30-1.17)	0.89 (0.42-1.72)	n.s.	0.295
CD163+ CD16-	0.03 (0.01-0.11)	0.004 (0.00-0.03)	0.005	0.005 (0.00-0.01)	0.003 (0.00-0.03)	n.s.	n.t.
CD163+ CD16+	0.0002 (0.00-0.00)	0.0001 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.000 (0.00-0.00)	n.s.	n.t.
CD163- CD16+	0.27 (0.04-1.51)	0.19 (0.02-1.02)	n.s.	0.05 (0.02-0.72)	0.19 (0.06-1.02)	n.s.	n.t.
CD206+ CD16-	0.007 (0.00-0.02)	0.01 (0.01-0.04)	0.042	0.01 (0.01-0.02)	0.02 (0.01-0.03)	n.s.	n.t.
CD206+ CD16+	0.001 (0.00-0.00)	0.003 (0.00-0.02)	n.s.	0.001 (0.00-0.002)	0.004 (0.001-0.01)	0.030	0.021
CD206- CD16+	0.27 (0.04-1.51)	0.19 (0.02-1.02)	n.s.	0.05 (0.02-0.71)	0.19 (0.06-1.02)	n.s.	n.t.
CD11c+ CD16-	0.33 (0.07-0.64)	0.21 (0.07-0.39)	n.s.	0.12 (0.10-0.20)	0.23 (0.07-0.31)	n.s.	n.t.
CD11c+ CD16+	0.27 (0.04-1.51)	0.78 (0.01-1.02)	n.s.	0.04 (0.01-0.71)	0.18 (0.05-1.02)	n.s.	n.t.
CD11c- CD16+	0.003 (0.00-0.03)	0.007 (0.00-0.02)	n.s.	0.003 (0.00-0.02)	0.007 (0.00-0.02)	n.s.	n.t.
CD163+ CD206-	0.03 (0.01-0.11)	0.005 (0.00-0.03)	0.006	0.006 (0.00-0.01)	0.004 (0.00-0.03)	n.s.	n.t.
CD163+ CD206+	0.0003 (0.00-0.00)	0.00 (0.00-0.00)	0.010	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.008 (0.00-0.02)	0.016 (0.01-0.06)	0.020	0.01 (0.0-0.03)	0.03 (0.01-0.03)	n.s.	n.t.
CD11c+ CD206-	0.64 (0.38-1.89)	0.37 (0.13-1.31)	n.s.	0.24 (0.13-0.91)	0.40 (0.17-1.31)	n.s.	n.t.
CD11c+ CD206+	0.004 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	0.001 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	n.t.
CD11c- CD206+	0.006 (0.00-0.01)	0.01 (0.00-0.05)	0.002	0.01 (0.00-0.02)	0.02 (0.01-0.03)	n.s.	n.t.
CD11c+ CD163-	0.60 (0.34-1.87)	0.34 (0.12-1.29)	n.s.	0.20 (0.12-0.89)	0.39 (0.16-1.29)	n.s.	n.t.
CD11c+ CD163+	0.03 (0.01-0.08)	0.005 (0.00-0.03)	0.004	0.006 (0.00-0.01)	0.005 (0.00-0.03)	n.s.	n.t.
CD11c- CD163+	0.0002 (0.00-0.03)	0.0002 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.	n.t.
CD14- CD11b+	0.61 (0.44-1.05)	0.42 (0.15-1.12)	n.s.	0.83 (0.15-1.12)	0.35 (0.24-0.90)	n.s.	0.255
CD163+ CD16-	0.006 (0.00-0.12)	0.005 (0.00-0.08)	n.s.	0.004 (0.00-0.01)	0.006 (0.00-0.02)	n.s.	n.t.
CD163+ CD16+	0.002 (0.00-0.01)	0.002 (0.00-0.01)	n.s.	0.004 (0.00-0.01)	0.001 (0.00-0.01)	n.s.	n.t.
CD163- CD16+	0.26 (0.01-0.73)	0.20 (0.07-1.02)	n.s.	0.64 (0.07-1.02)	0.18 (0.13-0.78)	n.s.	n.t.
CD206+ CD16-	0.18 (0.00-0.42)	0.04 (0.00-0.19)	**0.005**	0.09 (0.06-0.18)	0.003 (0.00-0.19)	**0.048**	**0.011**
CD206+ CD16+	0.22 (0.00-0.66)	0.04 (0.00-1.00)	n.s.	0.34 (0.06-1.00)	0.006 (0.00-0.14)	**0.010**	**0.011**
CD206- CD16+	0.03 (0.00-0.24)	0.17 (0.01-0.77)	n.s.	0.09 (0.01-0.30)	0.17 (0.01-0.77)	n.s.	n.t.

CD11c+ CD16-	0.07 (0.02-0.33)	0.04 (0.00-0.24)	n.s.	0.005 (0.00-0.03)	0.07 (0.04-0.17)	0.003	0.011
CD11c+ CD16+	0.002 (0.00-0.23)	0.043 (0.00-0.77)	n.s.	0.002 (0.00-0.00)	0.15 (0.00-0.77)	**0.018**	**0.021**
CD11c- CD16+	0.008 (0.00-0.01)	0.01 (0.00-0.09)	n.s.	0.009 (0.00-0.01)	0.02 (0.00-0.05)	0.048	0.030
CD163+ CD206-	0.43 (0.12-0.90)	0.10 (0.00-1.08)	**0.016**	0.43 (0.12-1.08)	0.008 (0.00-0.33)	**0.010**	0.084
CD163+ CD206+	0.005 (0.00-0.01)	0.0007 (0.00-0.01)	0.001	0.003 (0.00-0.01)	0.000 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.003 (0.00-0.01)	0.009 (0.00-0.04)	0.003	0.006 (0.00-0.01)	0.01 (0.00-0.01)	n.s.	n.t.
CD11c+ CD206-	0.43 (0.15-0.87)	0.29 (0.10-1.07)	n.s.	0.40 (0.10-1.07)	0.25 (0.18-0.86)	n.s.	n.t.
CD11c+ CD206+	0.05 (0.01-0.14)	0.006 (0.00-0.04)	0.003	0.005 (0.00-0.03)	0.006 (0.00-0.04)	n.s.	n.t.
CD11c- CD206+	0.001 (0.00-0.06)	0.004 (0.00-0.03)	n.s.	0.00 (0.00-0.00)	0.005 (0.00-0.01)	0.018	0.218
CD11c+ CD163-	0.08 (0.02-0.43)	0.11 (0.00-0.84)	n.s.	0.005 (0.00-0.02)	0.22 (0.04-0.84)	**0.003**	**0.011**
CD11c+ CD163+	0.001 (0.00-0.13)	0.004 (0.00-0.08)	n.s.	0.00 (0.00-0.02)	0.008 (0.00-0.02)	n.s.	n.t.
CD11c- CD163+	0.25 (0.00-0.73)	0.03 (0.00-1.02)	n.s.	0.64 (0.07-1.02)	0.00 (0.00-0.16)	**0.010**	**0.011**
CD14+ CD11b-	0.07 (0.02-0.17)	0.08 (0.02-0.20)	n.s.	0.09 (0.02-0.17)	0.07 (0.03-0.20)	n.s.	0.663
CD163+ CD16-	0.003 (0.00-0.01)	0.0082 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD163+ CD16+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.048	n.t.
CD163- CD16+	0.02 (0.00-0.08)	0.018 (0.00-0.06)	n.s.	0.02 (0.00-0.02)	0.02 (0.01-0.06)	n.s.	n.t.
CD206+ CD16-	0.01 (0.00-0.15)	0.02 (0.01-0.12)	n.s.	0.03 (0.01-0.12)	0.02 (0.01-0.03)	n.s.	n.t.
CD206+ CD16+	0.003 (0.00-0.01)	0.003 (0.00-0.01)	n.s.	0.002 (0.00-0.00)	0.005 (0.00-0.01)	n.s.	n.t.
CD206- CD16+	0.009 (0.00-0.08)	0.012 (0.0-0.05)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.05)	n.s.	n.t.
CD11c+ CD16-	0.007 (0.00-0.03)	0.03 (0.00-0.11)	0.029	0.04 (0.00-0.05)	0.03 (0.01-0.11)	n.s.	n.t.
CD11c+ CD16+	0.02 (0.00-0.08)	0.02 (0.00-0.05)	n.s.	0.01 (0.00-0.02)	0.02 (0.00-0.05)	n.s.	n.t.
CD11c- CD16+	0.0004 (0.00-0.00)	0.001 (0.00-0.01)	0.042	0.000 (0.00-0.00)	0.003 (0.00-0.07)	0.030	0.142
CD163+ CD206-	0.002 (0.00-0.01)	0.006 (0.00-0.03)	n.s.	0.009 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD163+ CD206+	0.0002 (0.00-0.00)	0.0004 (0.00-0.00)	n.s.	0.001 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	n.t.
CD163- CD206+	0.02 (0.01-0.16)	0.03 (0.01-0.12	n.s.	0.03 (0.01-0.12)	0.03 (0.01-0.04)	n.s.	n.t.
CD11c+ CD206-	0.02 (0.01-0.08)	0.04 (0.00-0.16)	n.s.	0.04 (0.00-0.05)	0.04 (0.01-0.16)	n.s.	n.t.
CD11c+ CD206+	0.006 (0.00-0.01)	0.005 (0.00-0.01)	n.s.	0.002 (0.00-0.01)	0.006 (0.00-0.01)	n.s.	n.t.
CD11c- CD206+	0.01 (0.00-0.14)	0.02 (0.00-0.11)	n.s.	0.02 (0.01-0.11)	0.02 (0.00-0.03)	n.s.	n.t.
CD11c+ CD163-	0.02 (0.00-0.08)	0.036 (0.00-0.14)	n.s.	0.02 (0.00-0.04)	0.03 (0.02-0.14)	n.s.	n.t.
CD11c+ CD163+	0.002 (0.00-0.01)	0.007 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.02 (0.00-0.03)	n.s.	n.t.
CD11c- CD163+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	n.t.

Intermediate CD14+ CD11b+	0.54 (0.18-0.84)	0.61 (0.27-0.78)	n.s.	0.55 (0.27-0.78)	0.69 (0.40-0.78)	n.s.	0.829
CD163+ CD16-	0.04 (0.02-0.14)	0.035 (0.00-0.19)	n.s.	0.04 (0.00-0.19)	0.03 (0.02-0.04)	n.s.	n.t.
CD163+ CD16+	0.02 (0.00-0.08)	0.03 (0.00-0.08)	n.s.	0.04 (0.01-0.08)	0.03 (0.00-0.07)	n.s.	n.t.
CD163- CD16+	0.20 (0.00-0.50)	0.26 (0.09-0.58)	n.s.	0.30 (0.09-0.58)	0.21 (0.11-0.37)	n.s.	n.t.
CD206+ CD16-	0.09 (0.04-0.12)	0.07 (0.02-0.20)	n.s.	0.05 (0.02-0.08)	0.11 (0.04-0.20)	n.s.	n.t.
CD206+ CD16+	0.002 (0.00-0.01)	0.002 (0.00-0.04)	n.s.	0.002 (0.00-0.04)	0.002 (0.00-0.01)	n.s.	n.t.
CD206- CD16+	0.22 (0.00-0.58)	0.29 (0.11-0.62)	n.s.	0.32 (0.11-0.62)	0.21 (0.12-0.44)	n.s.	n.t.
CD11c+ CD16-	0.18 (0.05-0.26)	0.21 (0.10-0.36)	n.s.	0.15 (0.10-0.19)	0.29 (0.13-0.36)	n.s.	n.t.
CD11c+ CD16+	0.22 (0.00-0.55)	0.27 (0.10-0.58)	n.s.	0.29 (0.10-0.58)	0.21 (0.12-0.43)	n.s.	n.t.
CD11c- CD16+	0.004 (0.00-0.02)	0.008 (0.00-0.07)	n.s.	0.03 (0.00-0.07)	0.005 (0.00-0.02)	n.s.	n.t.
CD163+ CD206-	0.08 (0.04-0.12)	0.06 (0.02-0.21)	n.s.	0.06 (0.02-0.07)	0.10 (0.03-0.21)	n.s.	n.t.
CD163+ CD206+	0.008 (0.00-0.02)	0.004 (0.00-0.02)	n.s.	0.007 (0.00-0.02)	0.005 (0.00-0.02)	n.s.	n.t.
CD163- CD206+	0.09 (0.02 (0.164)	0.07 (0.03-0.26)	n.s.	0.08 (0.04-0.26)	0.06 (0.03-0.09)	n.s.	n.t.
CD11c+ CD206-	0.38 (0.13-0.68)	0.48 (0.17-0.68)	n.s.	0.42 (0.17-0.68)	0.55 (0.31-0.67)	n.s.	n.t.
CD11c+ CD206+	0.05 (0.01-0.11)	0.04 (0.01-0.10)	n.s.	0.03 (0.02-0.08)	0.04 (0.01-0.10)	n.s.	n.t.
CD11c- CD206+	0.04 (0.01-0.12)	0.03 (0.01-0.25)	n.s.	0.03 (0.01-0.25)	0.02 (0.01-0.04)	n.s.	n.t.
CD11c+ CD163-	0.31 (0.03-0.68)	0.40 (0.15-0.67)	n.s.	0.33 (0.15-0.67)	0.38 (0.26-0.56)	n.s.	n.t.
CD11c+ CD163+	0.10 (0.05-0.12)	0.07 (0.02-0.22)	n.s.	0.06 (0.03-0.08)	0.12 (0.04-0.22)	n.s.	n.t.
CD11c- CD163+	0.0001 (0.00-0.01)	0.001 (0.00-0.01)	n.s.	0.001 (0.00-0.00)	0.002 (0.00-0.01)	n.s.	n.t.
High CD14+ CD11b+	11.80 (2.33-18.46)	14.95 (3.96-24.65)	n.s.	9.45 (3.96-24.65)	16.52 (14.76-20.54)	n.s.	0.100
CD163+ CD16-	2.98 (1.15-5.17)	1.49 (0.14-6.76)	n.s.	0.89 (0.14-2.74)	2.05 (1.21-6.76)	n.s.	n.t.
CD163+ CD16+	0.08 (0.01-0.23)	0.09 (0.03-0.81)	n.s.	0.09 (0.03-0.59)	0.10 (0.03-0.29)	n.s.	n.t.
CD163- CD16+	0.35 (0.00-0.95)	0.62 (0.15-4.41)	n.s.	1.04 (0.15-4.41)	0.49 (0.44-0.94)	n.s.	n.t.
CD206+ CD16-	0.16 (0.13-0.33)	0.17 (0.00-0.66)	n.s.	0.09 (0.00-0.23)	0.20 (0.06-0.66)	n.s.	n.t.
CD206+ CD16+	0.04 (0.00-0.11)	0.04 (0.01-0.14)	n.s.	0.03 (0.02-0.06)	0.04 (0.01-0.14)	n.s.	n.t.
CD206- CD16+	0.35 (0.01-0.98)	0.75 (0.14-4.90	n.s.	1.35 (0.14-4.90)	0.66 (0.47-1.03)	n.s.	n.t.
CD11c+ CD16-	11.40 (2.29-17.34)	14.22 (2.55-22.46)	n.s.	8.41 (2.55-22.46)	15.5 (13.9-19.6)	n.s.	n.t.
CD11c+ CD16+	0.39 (0.01-1.10)	0.84 (0.18-4.98)	n.s.	1.39 (0.18-4.98)	0.65 (0.49-1.08)	n.s.	n.t.
CD11c- CD16+	0.0008 (0.00-0.00)	0.001 (0.00-0.01)	n.s.	0.003 (0.00-0.01)	0.000 (0.00-0.00)	n.s.	n.t.
CD163+ CD206-	2.70 (1.02-4.87)	1.30 (0.48-6.90)	n.s.	0.80 (0.48-2.78)	1.99 (1.16-6.90)	n.s.	n.t.
CD163+ CD206+	0.13 (0.04-0.16)	0.05 (0.00-0.36)	n.s.	0.04 (0.00-0.11)	0.04 (0.02-0.36)	n.s.	n.t.

CD163- CD206+	0.19 (0.04-0.44)	0.19 (0.02-0.51)	n.s.	0.18 (0.02-0.25)	0.21 (0.05-0.51)	n.s.	n.t.
CD11c+ CD206-	11.55 (2.15-17.89)	14.73 (3.60-24.45)	n.s.	9.11 (3.60-24.45)	16.23 (14.48-20.0)	n.s.	n.t.
CD11c+ CD206+	0.24 (0.16-0.55)	0.27 (0.02-0.89)	n.s.	0.19 (0.02-0.35)	0.28 (0.08-0.89)	n.s.	n.t.
CD11c- CD206+	0.007 (0.00-0.02)	0.001 (0.00-0.01)	0.000	0.003 (0.00-0.01)	0.001 (0.00-0.00)	n.s.	n.t.
CD11c+ CD163-	9.20 (1.13-15.50)	12.07 (3.27-23.60)	n.s.	7.83 (3.27-23.60)	14.46 (7.54-15.16)	n.s.	n.t.
CD11c+ CD163+	3.14 (1.16-5.49)	1.57 (0.66-7.20)	n.s.	1.02 (0.68-3.16)	2.16 (1.23-7.20)	n.s.	n.t.
CD11c- CD163+	0.001 (0.00-0.01)	0.002 (0.00-0.04)	n.s.	0.00 (0.00-0.00)	0.006 (0.00-0.04)	n.s.	n.t.

Supplementary Table S2: Systemic expression of myeloid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.
*Non parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant

Myeloid cell type	Controls (median % of CD45+ or CD45- cells) N=8	uVIN (median % of CD45+ or CD45- cells) N=14	p =*	Recurrent uVIN (median % of CD45+ or CD45- cells) N=5	Non Recurrent uVIN (median % of CD45+ or CD45- cells) N=7	p = *
CD14+	0.01 (0.00-0.08)	0.02 (0.00-0.30)	n.s.	0.002 (0.00-0.03)	0.02 (0.00-0.30)	n.s.
CD11b+ CD124-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD33+	0.01 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.001 (0.00-0.03)	0.007 (0.00-0.03)	n.s.
CD11b+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD34+	0.0004 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD33+	0.01 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.001 (0.00-0.03)	0.007 (0.00-0.30)	n.s.
CD124+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD34+	0.001 (0.00-0.00)	0.005 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33+ CD34-	0.010 (0.00-0.07)	0.009 (0.00-0.30)	n.s.	0.002 (0.00-0.03)	0.007 (0.00-0.30)	n.s.
CD33+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33- CD34+	0.0004 (0.00-0.00)	0.001 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD15+	0.05 (0.00-0.27)	0.13 (0.01-1.21)	n.s.	0.16 (0.01-1.21)	0.10 (0.02-0.37)	n.s.
CD11b+ CD124-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD124+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	**0.030**
CD11b- CD33+	0.001 (0.00-0.01)	0.003 (0.00-0.05)	n.s.	0.002 (0.00-0.05)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD11b- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD33+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD33+	0.00 (0.00-0.00)	0.003 (0.00-0.06)	n.s.	0.003 (0.00-0.06)	0.000 (0.00-0.00)	**0.030**
CD124+ CD34-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD124- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33+ CD34-	0.001 (0.00-0.01)	0.0002 (0.00-0.05)	n.s.	0.002 (0.00-0.05)	0.000 (0.00-0.00)	**0.030**
CD33+ CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.
CD33- CD34+	0.00 (0.00-0.00)	0.00 (0.00-0.00)	n.s.	0.000 (0.00-0.00)	0.000 (0.00-0.00)	n.s.

Supplementary Table S3: Systemic expression of co-inhibitory molecule expression on lymphoid cells in PBMCs of uVIN patients and healthy controls recurrent vs non-recurrent uVIN patients.
*Non-parametric Mann-Whitney U test was used to determine the differences in expression of systemic myeloid cells between healthy controls and uVIN patients and recurrent and non-recurrent uVIN patients. p<0.05 is considered significant and in **bold** the p-values are marked where the cells numbers are >0.05%.

Results Mann-Whitney U test	Controls (median % of CD3+ T cells) N=8	uVIN (median % of CD3+ T cells) N=14	p =	Recurrent uVIN (median % of CD3+ T cells) N=5	Non Recurrent uVIN (median % of CD3+ T cells) N=7	p =	RFS (Log Rank)
CD3+			x			x	
CD3+ CD94+	4.05 (1.78-5.57)	2.67 (1.05-5.02)	n.s.	3.15 (2.07-4.05)	2.33 (1.88-5.02)	n.s.	0.372
CD3+ TIM3+	0.77 (0.45-2.38)	0.86 (0.58-3.12)	n.s.	1.60 (0.58-1.73)	0.77 (0.58-3.12)	n.s.	0.249
CD3+ NKG2A+	1.93 (0.98-2.79)	1.98 (1.32-5.60)	n.s.	2.13 1.87-3.18)	1.97 (1.32-5.60)	n.s.	0.488
CD3+ CD152+	0.12 (0.11-0.16)	0.16 (0.08-0.27)	n.s.	0.20 (0.17-0.25)	0.11 (0.08-0.27)	**0.048**	0.082
CD3+ PD1+	10.01 (5.78-16.66)	8.70 (4.69-26.03)	n.s.	14.29 (4.69-26.03)	7.35 (4.83-21.215)	n.s.	0.750
CD3+ CD94+ TIM3-	3.39 (1.66-5.32)	2.14 (0.81-3.80)	n.s.	2.15 (1.11-3.80)	2.13 (1.32-3.13)	n.s.	n.t.
CD3+ CD94+ TIM3+	0.35 (0.06-1.50)	0.31 (0.15-2.17)	n.s.	0.98 (0.21-1.12)	0.29 (0.15-2.17)	n.s.	n.t.
CD3+ CD94- TIM3+	0.46 (0.22-0.97)	0.55 (0.31-1.07)	n.s.	0.67 (031-0.91)	0.51 (0.37-1.07)	n.s.	n.t.
CD3+ CD94+ NKG2A-	2.73 (1.22-3.57)	1.50 (0.56-3.28)	n.s.	1.51 (0.88-3.28)	1.49 (1.17-2.43)	n.s.	n.t.
CD3+ CD94+ NKG2A+	1.38 (0.47-2.13)	0.97 (0.53-3.41)	n.s.	1.23 (0.80-1.71)	0.80 (0.55-3.41)	n.s.	0.142
CD3+ CD94- NKG2A+	0.65 (0.44-0.85)	1.05 (0.55-2.21)	**0.002**	1.10 (0.55-1.96)	0.99 (0.76-2.21)	n.s.	n.t.
CD3+ CD94+ CD152-	4.00 (1.76-5.51)	2.61 (0.99-4.94)	n.s.	3.04 (1.99-3.99)	2.32 (1.82-4.94)	n.s.	n.t.
CD3+ CD94+ CD152+	0.07 (0.03-0.10)	0.07 (0.03-0.14)	n.s.	0.10 (0.09-0.14)	0.05 (0.03-0.11)	**0.018**	n.t.
CD3+ CD94- CD152+	0.05 (0.04-0.07)	0.07 (0.05-0.20)	n.s.	0.08 (0.07-0.12)	0.05 (0.05-0.20)	0.048	n.t.
CD3+ CD94+ PD1-	2.90 (1.38-4.84)	1.70 (0.81-4.18)	n.s.	1.84 (1.25-2.55)	1.67 (0.32-4.18)	n.s.	n.t.
CD3+ CD94+ PD1+	0.84 (0.10-1.50)	0.77 (0.20-1.34)	n.s.	1.30 (0.76-1.34)	0.64 (0.20-1.17)	n.s.	n.t.
CD3+ CD94- PD1+	9.48 (5.50-15.78)	8.55 (4.06-25.06)	n.s.	13.28 (4.06-25.06)	7.42 (4.39-20.59)	n.s.	n.t.
CD3+ TIM3+ NKG2A-	0.43 (0.20-1.00)	0.35 (0.27-0.80)	n.s.	0.53 (0.27-0.80)	0.33 (0.29-0.36)	n.s.	n.t.
CD3+ TIM3+ NKG2A+	0.30 (0.20-1.28)	0.44 (0.15-2.83)	n.s.	0.77 (0.15-1.24)	0.44 (0.29-2.83)	n.s.	n.t.
CD3+ TIM3- NKG2A+	1.33 (0.76-2.46)	1.58 (0.92-2.81)	n.s.	1.69 (1.10-2.36)	1.60 (0.92-2.81)	n.s.	n.t.
CD3+ TIM3+ CD152-	0.75 (0.42-2.34)	0.83 (0.48-3.04)	n.s.	1.53 (0.52-1.65)	0.73 (0.48-3.04)	n.s.	n.t.
CD3+ TIM3+ CD152+	0.03 (0.02-0.06)	0.05 (0.03-0.14)	0.010	0.06 (0.03-0.09)	0.03 (0.03-0.14)	n.s.	n.t.
CD3+ TIM3- CD152+	0.08 (0.07-0.10)	0.09 (0.05-0.17)	n.s.	0.13 (0.09-0.17)	0.06 (0.05-0.11)	**0.005**	n.t.
CD3+ TIM3+ PD1-	0.50 (0.31-1.45)	0.67 (0.21-2.64)	n.s.	0.84 (0.31-0.92)	0.38 (0.21-2.64)	n.s.	n.t.

CD3+ TIM3+ PD1+	0.30 (0.05-0.95)	0.34 (0.17-0.88)	n.s.	0.70 (0.20-0.88)	0.38 (0.17-0.52)	n.s.	n.t.
CD3+ TIM3- PD1+	9.86 (5.79-16.60)	8.76 (4.54-25.54)	n.s.	13.72 (4.64-25.54)	7.47 (4.54-21.42)	n.s.	n.t.
CD3+ NKG2A+ CD152-	1.82 (0.86-2.67)	1.84 (1.09-5.42)	n.s.	1.99 (1.72-2.98)	1.86 (1.09-5.42)	n.s.	n.t.
CD3+ NKG2A+ CD152+	0.10 (0.06-0.13)	0.13 (0.05-0.19)	n.s.	0.17 (0.14-0.19)	0.08 (0.05-0.18)	**0.030**	n.t.
CD3+ NKG2A- CD152+	0.02 (0.01-0.04)	0.03 (0.02-0.09)	n.s.	0.03 (0.03-0.07)	0.02 (0.02-0.09)	0.048	n.t.
CD3+ NKG2A+ PD1-	1.30 (0.65-2.22)	1.17 (0.93-4.82)	n.s.	1.25 (1.13-1.53)	0.99 (0.93-4.82)	n.s.	n.t.
CD3+ NKG2A+ PD1+	0.58 (0.16-1.11)	0.78 (0.31-1.60)	n.s.	0.87 (0.70-1.60)	0.77 (0.31-1.11)	n.s.	n.t.
CD3+ NKG2A- PD1+	9.74 (5.72-16.54)	8.45 (4.06-25.03)	n.s.	13.31 (4.22-25.03)	7.05 (4.06-20.13)	n.s.	n.t.
CD3+ CD152+ PD1-	0.05 (0.04-0.06)	0.07 (0.02-0.13)	n.s.	0.07 (0.07-0.09)	0.05 (0.02-0.13)	n.s.	n.t.
CD3+ CD152+ PD1+	0.07 (0.04-0.09)	0.08 (0.05-0.16)	n.s.	0.10 (0.09-0.16)	0.07 (0.05-0.13)	**0.018**	n.t.
CD3+ CD152- PD1+	10.37 (6.00-17.20)	9.06 (4.84-26.51)	n.s.	14.63 (4.84-26.51)	7.76 (5.11-22.10)	n.s.	n.t.
Ratio CD3+NKG2a+/ CD3+NKG2a-	0.02 (0.01-0.03)	0.02 (0.01-0.06)	n.s.	0.02 (0.02-0.03)	0.02 (0.01-0.06)	n.s.	n.t.
Ratio CD3+CD94+NK-G2a+/CD3+CD94+NK-G2a-	0.49 (0.31-0.77)	0.64 (0.24-1.93)	n.s.	0.88 (0.24-1.37)	0.45 (0.37-1.93)	n.s.	n.t.
Ratio CD3+PD1-/ CD3+PD1+	8.99 (5.00-16.30)	10.78 (2.84-20.34)	n.s.	6.00 (2.84-20.34)	12.59 (3.73-19.71)	n.s.	n.t.
Ratio CD3+TIM3+/ CD3+TIM3-	0.008 (0.005-0.024)	0.009 (0.006-0.032)	n.s.	0.016 (0.006-0.018)	0.008 (0.006-0.32)	n.s.	n.t.
CD4+	68.59 (59.79-79.45)	69.74 (55.46-81.11)	n.s.	75.76 (55.46-81.11)	67.74 (59.26-79.89)	n.s.	0.181
CD4+ CD94+	0.13 (0.08-0.27)	0.15 (0.05-0.43)	n.s.	0.07 (0.05-0.13)	0.30 (0.07-0.43)	**0.010**	**0.011**
CD4+ TIM3+	0.20 (0.08-0.43)	0.27 (0.13-0.61)	n.s.	0.34 (0.13-0.61)	0.24 (0.13-0.35)	n.s.	0.100
CD4+ NKG2A+	0.22 (0.09-0.33)	0.44 (0.18-0.82)	**0.005**	0.65 (0.24-0.82)	0.31 (0.18-0.57	n.s.	0.884
CD4+ CD152+	0.04 (0.02-0.07)	0.05 (0.03-0.08)	n.s.	0.05 (0.04-0.08)	0.04 (0.03-0.08)	0.048	0.017
CD4+ PD1+	4.14 (3.10-6.55)	4.42 (2.02-10.50)	n.s.	5.24 (2.02-8.16)	3.64 (2.43-10.50)	n.s.	0.750
CD4+ CD94+ TIM3-	0.11 (0.06-0.22)	0.10 (0.04-0.36)	n.s.	0.05 (0.04-0.10)	0.18 (0.05-0.36)	**0.030**	n.t.
CD4+ CD94+ TIM3+	0.03 (0.01-0.07)	0.05 (0.01-0.22)	n.s.	0.01 (0.01-0.04)	0.09 (0.02-0.13)	0.010	n.t.
CD4+ CD94- TIM3+	0.16 (0.06-0.41)	0.17 (0.08-0.57)	n.s.	0.29 (0.12-0.57)	0.11 (0.08-0.33)	n.s.	n.t.
CD4+ CD94+ NKG2A-	0.06 (0.04-0.21)	0.09 (0.03-0.35)	n.s.	0.05 (0.03-0.09)	0.14 (0.05-0.35)	**0.018**	n.t.
CD4+ CD94+ NKG2A+	0.06 (0.03-0.13)	0.06 (0.02-0.28)	n.s.	0.04 (0.02-0.05)	0.13 (0.03-0.19)	**0.018**	**0.011**
CD4+ CD94- NKG2A+	0.16 (0.06-0.28)	0.32 (0.13-0.77)	**0.006**	0.61 (0.22-0.77)	0.20 (0.13-0.55)	**0.048**	n.t.
CD4+ CD94+ CD152-	0.12 (0.08-0.26)	0.14 (0.05-0.42)	n.s.	0.07 (0.05-0.14)	0.30 (0.07-0.42)	**0.018**	n.t.
CD4+ CD94+ CD152+	0.003 (0.00-0.01)	0.004 (0.00-0.02)	n.s.	0.003 (0.00-0.00)	0.008 (0.00-0.02)	0.003	n.t.
CD4+ CD94- CD152+	0.02 (0.01-0.03)	0.03 (0.01-0.06)	n.s.	0.04 (0.03-0.06)	0.01 (0.01-0.04)	0.018	n.t.
CD4+ CD94+ PD1-	0.11 (0.07-0.22)	0.12 (0.04-0.41)	n.s.	0.06 (0.04-0.10)	0.15 (0.05-0.41)	**0.018**	n.t.

CD4+ CD94+ PD1+	0.02 (0.01-0.06)	0.03 (0.01-0.19)	n.s.	0.02 (0.01-0.04)	0.03 (0.02-0.19)	0.048	n.t.
CD4+ CD94- PD1+	4.60 (3.49-7.27)	4.94 (2.32-11.38)	n.s.	5.59 (2.32-9.10)	4.20 (2.80-11.38)	n.s.	n.t.
CD4+ TIM3+ NKG2A-	0.18 (0.07-0.28)	0.35 (0.14-0.62)	**0.020**	0.55 (0.22-0.62)	0.18 (0.14-0.50)	**0.048**	n.t.
CD4+ TIM3+ NKG2A+	0.03 (0.01-0.06)	0.08 (0.02-0.20)	0.010	0.09 (0.02-0.20)	0.07 (0.04-0.17)	n.s.	n.t.
CD4+ TIM3- NKG2A+	4.63 (3.54-7.25)	4.96 (2.31-11.47)	n.s.	5.52 (2.31-8.99)	4.26 (2.83-11.47)	n.s.	n.t.
CD4+ TIM3+ CD152-	0.20 (0.08-0.31)	0.41 (0.15-0.78)	n.s.	0.61 (0.21-0.78)	0.30 (0.15-0.54)	n.s.	n.t.
CD4+ TIM3+ CD152+	0.02 (0.01-0.02)	0.02 (0.01-0.03)	0.035	0.02 (0.02-0.03)	0.02 (0.01-0.03)	n.s.	n.t.
CD4+ TIM3- CD152+	0.008 (0.01-0.03)	0.01 (0.01-0.04)	0.035	0.01 (0.01-0.03)	0.008 (0.01-0.04)	n.s.	n.t.
CD4+ TIM3+ PD1-	0.16 (0.06-0.31)	0.20 (0.08-0.42)	n.s.	0.24 (0.08-0.42)	0.11 (0.10-0.30)	n.s.	n.t.
CD4+ TIM3+ PD1+	0.04 (0.01-0.11)	0.07 (0.03-0.18)	n.s.	0.08 (0.05-0.18)	0.06 (0.03-0.13)	n.s.	n.t.
CD4+ TIM3- PD1+	4.53 (3.46-7.09)	4.86 (2.24-11.35)	n.s.	5.48 (2.24-8.95)	4.19 (2.78-11.35)	n.s.	n.t.
CD4+ NKG2A+ CD152-	0.20 (0.08-0.42)	0.25 (0.12-0.58)	n.s.	0.32 (0.12-0.58)	0.20 (0.13-0.33)	n.s.	n.t.
CD4+ NKG2A+ CD152+	0.004 (0.00-0.01)	0.008 (0.00-0.05)	0.004	0.01 (0.00-0.02)	0.007 (0.00-0.05)	n.s.	n.t.
CD4+ NKG2A- CD152+	0.02 (0.01-0.03)	0.02 (0.01-0.05)	n.s.	0.03 (0.02-0.05)	0.02 (0.01-0.02)	0.010	n.t.
CD4+ NKG2A+ PD1-	0.02 (0.01-0.03)	0.03 (0.01-0.04)	n.s.	0.03 (0.02-0.04)	0.02 (0.01-0.03)	0.048	n.t.
CD4+ NKG2A+ PD1+	0.005 (0.00-0.01)	0.009 (0.00-0.03)	0.005	0.01 (0.01-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD4+ NKG2A- PD1+	4.75 (3.62-7.44)	5.13 (2.39-11.81)	n.s.	5.67 (2.39-9.31)	4.43 (2.98-11.81)	n.s.	n.t.
CD4+ CD152+ PD1-	0.16 (0.07-0.32)	0.12 (0.07-0.31)	n.s.	0.20 (0.07-0.31)	0.10 (0.07-0.18)	n.s.	n.t.
CD4+ CD152+ PD1+	0.04 (0.01-0.10)	0.09 (0.01-0.43)	0.029	0.07 (0.01-0.43)	0.11 (0.06-0.21)	n.s.	n.t.
CD4+ CD152- PD1+	0.16 (0.08-0.27)	0.32 (0.13-0.67)	**0.008**	0.41 (0.23-0.67)	0.23 (0.13-0.37)	n.s.	n.t.
Ratio CD4+TIM3+/ CD4+TIM3-	0.003 (0.00-0.01)	0.004 (0.00-0.014	n.s.	0.005 (0.00-0.01)	0.004 (0.00-0.00)	n.s.	n.t.
CD8+	23.18 (17.20-31.36)	22.33 (12.28-35.92)	n.s.	20.10 (14.36-35.92)	26.33 (12.28-27.93)	n.s.	0.460
CD8+ CD94+	1.17 (0.76-2.73)	0.91 (0.46-1.78)	n.s.	0.83 (0.47-1.78)	0.92 (0.76-1.69)	n.s.	0.829
CD8+ TIM3+	0.15 (0.06-0.26)	0.13 (0.02-0.67)	n.s.	0.16 (0.13-0.67)	0.11 (0.02-0.15)	**0.010**	0.100
CD8+ NKG2A+	0.38 (0.13-0.96)	0.38 (0.13-0.90)	n.s.	0.67 (0.22-0.90)	0.33 (0.13-0.71)	n.s.	0.625
CD8+ CD152+	0.03 (0.02-0.05)	0.03 (0.01-0.16)	n.s.	0.06 (0.02-0.09)	0.03 (0.01-0.16)	n.s.	0.807
CD8+ PD1+	3.70 (1.09-7.15)	2.71 (0.84-17.88)	n.s.	2.46 (1.07-17.88)	2.72 (0.84-8.19)	n.s.	0.663
CD8+ CD94+ TIM3-	1.33 (0.77-2.75)	0.94 (0.45-2.83)	n.s.	0.95 (0.66-2.83)	0.94 (0.75-1.67)	n.s.	n.t.
CD8+ CD94+ TIM3+	0.01 (0.01-0.03)	0.01 (0.00-0.08)	n.s.	0.03 (0.01-0.07)	0.007 (0.00-0.02)	0.005	n.t.
CD8+ CD94- TIM3+	0.11 (0.03-0.16)	0.08 (0.01-0.40)	n.s.	0.09 (0.07-0.40)	0.08 (0.01-0.10)	n.s.	n.t.
CD8+ CD94+ NKG2A-	0.77 (0.59-1.90)	0.70 (0.31-1.53)	n.s.	0.51 (0.31-1.53)	0.78 (0.55-1.37)	n.s.	n.t.

CD8+ CD94+ NKG2A+	0.24 (0.06-0.92)	0.18 (0.07-0.72)	n.s.	0.17 (0.11-0.72)	0.18 (0.07-0.55)	n.s.	0.684
CD8+ CD94- NKG2A+	0.15 (0.08-0.22)	0.16 (0.08-0.67)	n.s.	0.22 (0.10-0.67)	0.16 (0.08-0.25)	n.s.	n.t.
CD8+ CD94+ CD152-	1.17 (0.79-2.77)	0.91 (0.44-1.73)	n.s.	0.82 (0.46-1.73)	0.91 (0.75-1.68)	n.s.	n.t.
CD8+ CD94+ CD152+	0.005 (0.00-0.01)	0.008 (0.00-0.02)	n.s.	0.008 (0.00-0.02)	0.008 (0.00-0.01)	n.s.	n.t.
CD8+ CD94- CD152+	0.02 (0.01-0.06)	0.03 (0.01-0.16)	n.s.	0.04 (0.02-0.08)	0.02 (0.01-0.16)	n.s.	n.t.
CD8+ CD94+ PD1-	1.05 (0.67-2.59)	0.76 (0.38-1.28)	n.s.	0.78 (0.38-1.28)	0.75 (0.67-1.04)	n.s.	n.t.
CD8+ CD94+ PD1+	0.20 (0.04-0.36)	0.21 (0.02-0.65)	n.s.	0.21 (0.04-0.48)	0.21 (0.02-0.65)	n.s.	n.t.
CD8+ CD94- PD1+	3.84 (1.04-7.21)	2.83 (0.98-18.34)	n.s.	2.63 (1.12-18.34)	2.87 (0.98-8.61)	n.s.	n.t.
CD8+ TIM3+ NKG2A-	0.10 (0.04-0.18)	0.09 (0.01-0.45)	n.s.	0.11 (0.08-0.45)	0.007 (0.01-0.11)	**0.048**	n.t.
CD8+ TIM3+ NKG2A+	0.006 (0.01-0.02)	0.009 (0.00-0.05)	n.s.	0.02 (0.01-0.05)	0.006 (0.00-0.01)	0.018	n.t.
CD8+ TIM3- NKG2A+	0.40 (0.14-1.09)	0.39 (0.14-0.89)	n.s.	0.69 (0.22-0.89)	0.34 (0.14-0.75)	n.s.	n.t.
CD8+ TIM3+ CD152-	0.10 (0.04-0.20)	0.09 (0.02-0.50)	n.s.	0.11 (0.09-0.50)	0.07 (0.02-0.11)	**0.010**	n.t.
CD8+ TIM3+ CD152+	0.002 (0.00-0.00)	0.004 (0.00-0.02)	n.s.	0.004 (0.00-0.01)	0.002 (0.00-0.02)	n.s.	n.t.
CD8+ TIM3- CD152+	0.30 (0.01-0.05)	0.03 (0.01-0.15)	n.s.	0.05 (0.02-0.08)	0.02 (0.01-0.15)	n.s.	n.t.
CD8+ TIM3+ PD1-	0.10 (0.03-0.17)	0.07 (0.02-0.42)	n.s.	0.10 (0.07-0.42)	0.06 (0.02-0.10)	**0.030**	n.t.
CD8+ TIM3+ PD1+	0.01 (0.01-0.04)	0.02 (0.00-0.06)	n.s.	0.03 (0.01-0.06)	0.01 (0.00-0.04)	n.s.	n.t.
CD8+ TIM3- PD1+	4.03 (1.22-7.56)	2.96 (0.97-18.55)	n.s.	2.67 (1.15-18.55)	3.01 (0.97-9.01)	n.s.	n.t.
CD8+ NKG2A+ CD152-	0.38 (0.12-1.04)	0.31 (0.13-0.87)	n.s.	0.61 (0.20-0.87)	0.30 (0.13-0.70)	n.s.	n.t.
CD8+ NKG2A+ CD152+	0.02 (0.01-0.03)	0.02 (0.01-0.14)	n.s.	0.05 (0.02-0.08)	0.02 (0.01-0.14)	n.s.	n.t.
CD8+ NKG2A- CD152+	0.007 (0.00-0.03)	0.009 (0.00-0.03)	n.s.	0.01 (0.00-0.02)	0.01 (0.00-0.03)	n.s.	n.t.
CD8+ NKG2A+ PD1-	0.26 (0.11-0.92)	0.27 (0.10-0.49)	n.s.	0.30 (0.18-0.49)	0.25 (0.10-0.42)	n.s.	n.t.
CD8+ NKG2A+ PD1+	0.11 (0.02-0.18)	0.11 (0.02-0.52)	n.s.	0.21 (0.03-0.52)	0.07 (0.02-0.32)	n.s.	n.t.
CD8+ NKG2A- PD1+	3.96 (1.22-7.40)	2.91 (0.95-18.03)	n.s.	2.63 (1.13-18.03)	2.95 (0.95-8.81)	n.s.	n.t.
CD8+ CD152+ PD1-	0.02 (0.01-0.04)	0.02 (0.00-0.14)	n.s.	0.02 (0.01-0.06)	0.01 (0.00-0.14)	n.s.	n.t.
CD8+ CD152+ PD1+	0.01 (0.00-0.02)	0.01 (0.00-0.04)	n.s.	0.03 (0.01-0.04)	0.01 (0.00-0.03)	n.s.	n.t.
CD8+ CD152- PD1+	4.07 (1.24-7.65)	2.98 (0.98-18.67)	n.s.	2.72 (1.17-18.67)	3.02 (0.98-9.11)	n.s.	n.t.
Ratio CD8+TIM3+/ CD8+TIM3-	0.007 (0.002-0.11)	0.005 (0.001-0.35)	n.s.	0.009 (0.00-0.03)	0.04 (0.00-0.01)	**0.030**	**0.018**
Ratio CD8+CD94+NK-G2a+/CD8+CD94+NK-G2a-	0.33 (0.04-0.56)	0.26 (0.10-1.39)	n.s.	0.42 (0.16-1.39)	0.23 (0.10-0.51)	n.s.	n.t.