Additional File 1: Design T cell panel, antibodies and detection method per marker.

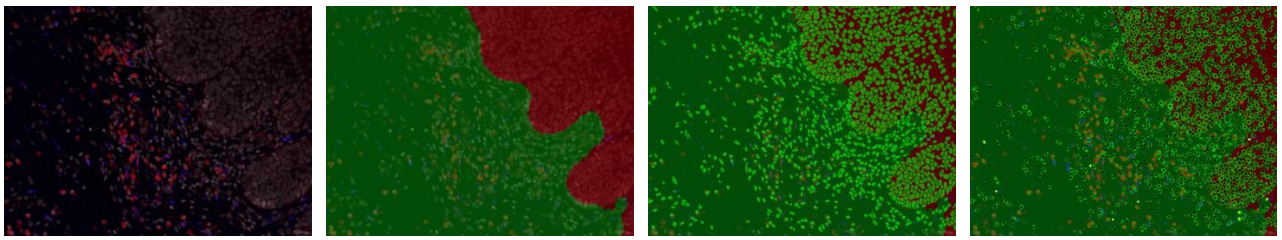
Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-1	D4W2J Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 570	Opal tyramide signal amplification
Tbet	D6N8B XP Cell Signaling Technology	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
FoxP3	236A/E7 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 633	Secondary antibody fluorochrome labelled
CD8	4B11 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
Tim3	D5D5R XP Cell Signaling Technology	Rabbit IgG	Alexa goat anti rabbit IgG 680	Secondary antibody fluorochrome labelled
CD3	D7A6E Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe

Additional File 2: Design myeloid cell panel, antibodies and detection method per marker.

Marker	Primary antibody clone and company	Primary antibody isotype	Secondary antibody	Detection method
PD-L1	SP142 Spring Bioscience	Rabbit IgG	Poly-HRP and Opal 520	Opal tyramide signal amplification
CD14	D7A2T Cell Signaling Technology	Rabbit IgG	CF donkey anti rabbit IgG 633	Secondary antibody fluorochrome labelled
CD33	PWS44 Novocastra	Mouse IgG2b	Alexa goat anti mouse IgG2b 647	Secondary antibody fluorochrome labelled
CD163	10D6 Invitrogen ThermoFisher	Mouse IgG1	CF goat anti mouse IgG1 680	Secondary antibody fluorochrome labelled
CD11c	EP1347Y Abcam, directly labelled with Alexa 546	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
CD68	D4B9C XP Cell Signaling Technology, directly labelled with Alexa 594	Rabbit IgG	-	Directly fluorochrome labelled primary antibody
DAPI	ThermoFisher	-	-	Fluorescent probe

Additional File 3: inForm analyses

A



1 **2** **3** **4**

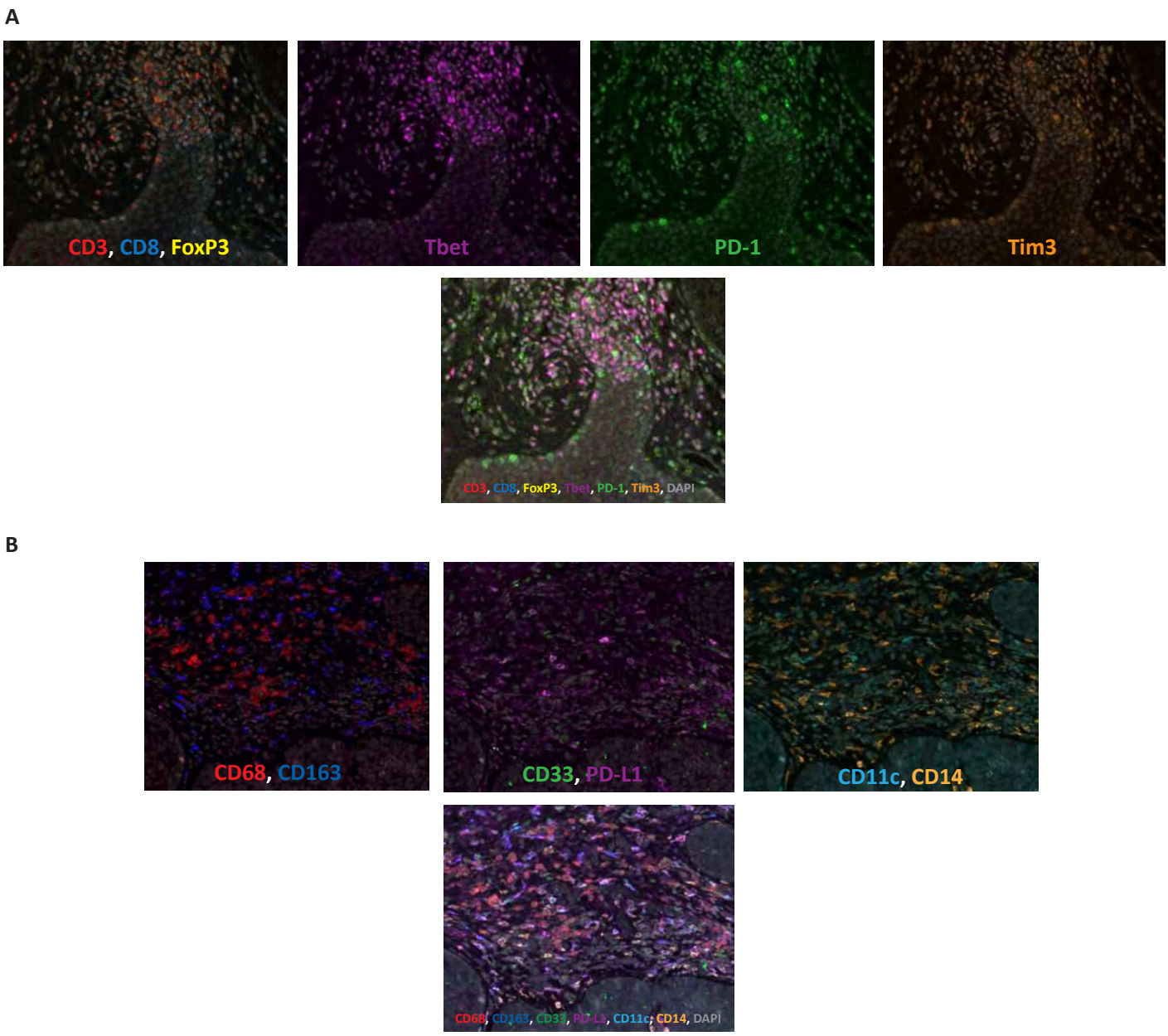
A) Multispectral immunofluorescence image analysis workflow with inForm, representative example of a subanalysis. InForm uses a stepwise training approach, each step adding a layer of differentiation.

Step 1: Signal extraction	(example subanalysis here shows CD68 in red, CD163 in blue)
Step 2: Tissue segmentation	(epithelium in red, stroma in green)
Step 3: Cell segmentation	(cells are marked in light green)
Step 4: Cell phenotyping	($CD68^+CD163^-$ cells are assigned a red dot, $CD68^+CD163^+$ cells a blue dot, $CD68^-CD163^+$ cells a yellow dot, and $CD68^-CD163^-$ cells a black dot)

B

T cell panel		**Myeloid cell panel**	
<u>Subanalysis 1:</u>		<u>Subanalysis 1:</u>	
$CD3^+CD8^-$ $FoxP3^-$	(T helper cell)	$CD68^+CD163^-$	(M1 macrophage)
$CD3^+CD8^+FoxP3^-$	($CD8^+$ T cell)	$CD68^+CD163^+$	(M2 macrophage)
$CD3^+CD8^-$ $FoxP3^+$	(regulatory T cell)	$CD68^-CD163^+$	($CD163^+$ non-macrophage)
$CD3^-CD8^-FoxP3^-$	(no T cell)	$CD68^-CD163^-$	(non-macrophage)
<u>Subanalysis 2:</u>		<u>Subanalysis 2:</u>	
$Tbet^+$	(IFNγ producing T cell)	$CD11c^+CD14^-$	(dendritic cell, DC)
$Tbet^-$	($Tbet^-$ T cell)	$CD11c^+CD14^+$	(inflammatory DC)
		$CD11c^-CD14^+$	(inflammatory myeloid cell)
<u>Subanalysis 3:</u>		$CD11c^-CD14^-$	($CD11c^-$ and $CD14^-$ myeloid cell)
$PD1^+$	(activated PD1 expressing T cell)		
$PD1^-$	($PD1^-$ T cell)	<u>Subanalysis 3:</u>	
		$CD33^+PDL1^-$	(immature myeloid cell)
<u>Subanalysis 4:</u>		$CD33^-PDL1^+$	(mature myeloid cell expressing PDL1)
$Tim3^+$	(activated Tim3 expressing T cell)	$CD33^-PDL1^-$	($CD33^-$ and $PDL1^-$ myeloid cell)
$Tim3^-$	($Tim3^-$ T cells)		

B) Subanalyses of the T cell and myeloid cell panel as used in inForm. The full seven marker panels were divided into multiple subanalyses, to preclude the exclusion of possible relevant phenotypes. Subsequently, the output of the multiple subanalyses were merged per cell based on X,Y-positions to obtain the full seven marker expression profile of each cell.



Additional File 4: Examples of immune cell subtypes analyzed.
A) Analysis of the T cell panel consisted of four sub-analyses; first CD3, CD8, FoxP3 positivity was assessed, secondly Tbet, thirdly PD-1, and fourthly Tim3.
B) Analysis of the myeloid cell panel consisted of three sub-analyses: first CD68 and CD163 positivity was assessed, secondly CD33 and PD-L1, and thirdly CD11c and CD14.

Additional File 5: Descriptive statistics of immune cells in healthy vulvar tissue.

Immune cell phenotype	Number of cells/mm² epithelium (median with 95% CI)	Number of cells/mm² stroma (median with 95% CI)
CD3+CD8-	9,53 (3,08-26,57)	22,9 (9,76-59,4)
CD3+CD8-TBET+	28,74 (8,04-52,88)	40,4 (20,1-76,8)
CD3+CD8-TBET+TIM3+	-	0,510 (0,100-1,06)
CD3+CD8-PD1+TBET+	8,09 (2,02-15,73)	10,7 (3,28-20,2)
CD3+CD8-FOXP3+	0,43 (0,00-2,86)	2,91 (0,980-6,73)
CD3+CD8+	6,00 (2,18-14,08)	10,3 (5,27-14,3)
CD3+CD8+TIM3+	-	0,200 (0,00-1,39)
CD3+CD8+TBET+	-	10,2 (3,66-26,8)
CD14+CD68-CD163-	36,20 (14,35-57,23)	138 (88,7-239)
CD14-CD68-CD163+	-	2,39 (0,360-5,86)
CD14-CD68+CD163-	1,26 (0,00-3,24)	11,4 (4,98-18,8)
CD14+CD68+CD163-	2,26 (0,49-6,13)	24,9 (11,0-49,6)
CD14-CD68+CD163+	-	9,07 (2,32-18,4)
CD14+CD68+CD163+	1,49 (0,67-2,58)	63,0 (25,6-89,1)

N=27 healthy vulvar tissue samples; CI=confidence interval
A threshold of a median cell count of ≥10 cells/mm² per tissue compartment was applied, to only select relevant phenotypes for analyses.

Additional File 6: Descriptive statistics of immune cells in pre-vaccination vHSIL biopsies as one group and based on clinical response upon vaccination.

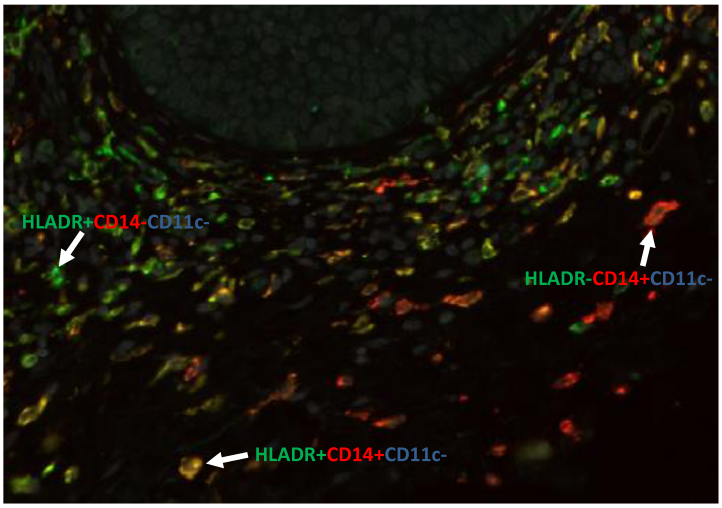
Immune cell phenotype	Number of cells/mm² epithelium (median with 95% CI)					Number of cells/mm² stroma (median with 95% CI)				
	vHSIL all	NR	PR	CR	Statistically significant differences	vHSIL all	NR	PR	CR	Statistically significant differences
CD3+CD8-	5,94 (4,14-20,2)	5,83 (1,71-11,1)	10,6 (0,00-49,9)	14,3 (4,14-49,0)	-	63,6 (13,0-104)	30,7 (5,90-107)	68,8 (13,0-155)	41,9 (0,00-287)	-
CD3+CD8-TBET+	4,14 (0,00-12,2)	0,00 (0,00-5,50)	3,87 (0,00-53,8)	6,51 (0,00-33,4)	NR***H	23,6 (0,730-57,2)	5,20 (0,00-57,2)	32,1 (0,00-68,4)	48,8 (0,00-368)	NR*H
CD3+CD8-TBET+TIM3+	-	-	-	-	-	0,00 (0,00-2,33)	0,00 (0,00-3,12)	0,400 (0,00-3,90)	1,39 (0,00-18,0)	-
CD3+CD8-PD1+TBET+	0,00 (0,00-1,71)	0,00 (0,00-1,38)	0,00 (0,00-5,51)	13,5 (0,00-25,8)	NR***H, PR**H, NR*CR, PR*CR	4,08 (0,00-10,2)	0,260 (0,00-8,38)	1,32 (0,00-10,2)	23,3 (4,85-73,2)	NR**H, PR*H, NR**CR, PR**CR
CD3+CD8-FOXP3+	6,30 (3,51-12,7)	10,7 (2,75-13,1)	6,63 (3,35-16,3)	3,76 (1,43-32,5)	NR***H, PR***H, CR**H	39,3 (20,1-67,5)	47,5 (8,84-82,6)	60,4 (20,1-89,4)	13,0 (1,34-57,8)	NR****H, PR****H, PR*CR
CD3+CD8+	1,71 (0,00-4,14)	0,00 (0,00-2,46)	1,72 (0,00-9,35)	5,43 (1,50-22.8)	NR***H, NR**CR	6,22 (2,49-26,4)	3,84 (2,45-27,3)	10,1 (0,00-30,5)	26,4 (0,670-310)	-
CD3+CD8+TIM3+	-	-	-	-	-	0,800 (0,00-3,48)	0,00 (0,00-6,70)	0,845 (0,00-4,16)	2,53 (0,00-10,4)	CR*H
CD3+CD8+TBET+	-	-	-	-	-	0,520 (0,00-2,23)	0,260 (0,00-1,18)	0,500 (0,00-4,95)	6,91 (0,00-16,0)	NR****H, PR***H
CD14+CD68-CD163-	4,18 (1,18-11,0)	2,64 (0,00-24,0)	4,26 (0,00-16,8)	5,44 (0,00-30,5)	NR***H, PR***H, CR**H	94,1 (55,6-155)	56,6 (16,7-144)	128 (67,6-352)	124 (6,18-290)	NR**H
CD14-CD68-CD163+	-	-	-	-	-	6,64 (1,67-12,0)	16,5 (0,480-64,5)	7,10 (1,78-12,0)	3,08 (0,00-26,7)	PR*H
CD14-CD68+CD163-	10,4 (6,12-22,4)	8,98 (3,24-22,6)	10,5 (6,12-26,1)	10,8 (1,38-73,1)	-	58,1 (32,1-127)	46,4 (22,4-142)	69,8 (43,5-161)	35,5 (16,2-1054)	NR***H, PR****H, CR***H
CD14+CD68+CD163-	1,29 (0,00-3,54)	0,840 (0,00-2,53)	1,78 (0,00-5,46)	3,22 (0,00-14,8)	-	24,5 (5,50-54,4)	5,06 (2,47-13,5)	45,4 (18,1-60,5)	79,1 (2,06-124)	NR**H, NR**PR
CD14-CD68+CD163+	-	-	-	-	-	53,1 (37,6-105)	56,2 (10,0-145)	64,7 (35,0-196)	43,9 (2,39-280)	NR***H, PR****H, CR**H
CD14+CD68+CD163+	0,00 (0,00-2,43)	0,00 (0,00 2,43)	0,465 (0,00-3,20)	3,89 (0,00- 27,4)	NR***H, PR***H, CR**H	123 (74,1-176)	78,7 (31,7-142)	147 (89,2-222)	176 (25,1-322)	PR****H, CR*H, NR*PR

vHSIL all (n=29); non-responders (NR, n=12); partial responders (PR, n=10); complete responders (CR, n=7); CI=confidence interval, H=healthy vulva

A threshold of a median cell count of ≥10 cells/mm^2 per tissue compartment was applied, to only select relevant phenotypes for analyses. Differences between two groups were calculated with a Mann-Whitney test, statistical significance is indicated with asterisks: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

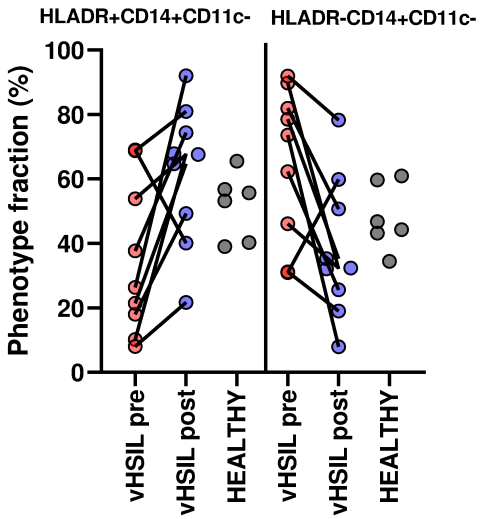
Additional File 7: CD14⁺ inflammatory myeloid cells co-expressing HLA-DR, in pre- and post-vaccination vHSIL and in healthy vulva.

A



Example of HLA-DR expressing CD14⁺ myeloid cells in vHSIL. For this staining, a panel consisting of HLA-DR (clone TAL1B5, directly labelled with Alexa fluor 594), CD14 (clone D7A2T, indirectly detected with CF donkey anti rabbit IgG 633), CD11c (clone EP1347Y, directly labelled with Alexa fluor 546) and DAPI was designed.

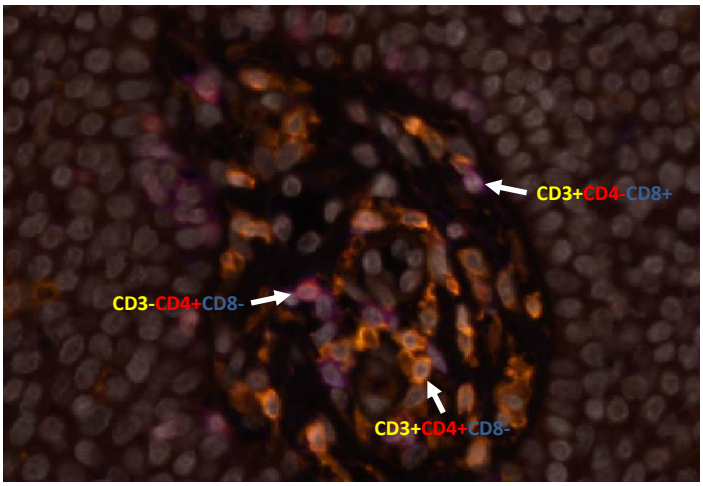
B



6 healthy vulva samples and the pre- and post-vaccination biopsies of 9 vHSIL patients (3 NR, 3 PR, 3 CR) were stained and analyzed with this panel. Samples for staining were selected based on high CD14⁺ cell counts. Upon vaccination, the fraction of CD14⁺ cells co-expressing HLA-DR increases.

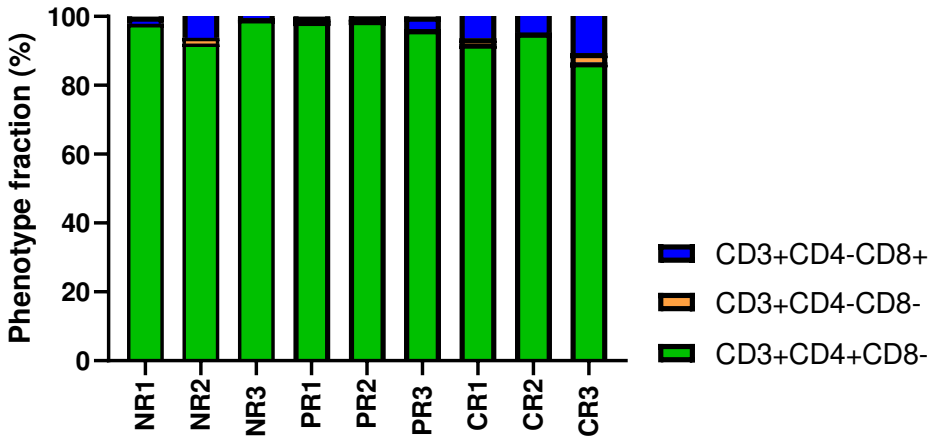
Additional File 8: almost all CD3$^+$CD8$^-$Foxp3$^-$ T cells co-express CD4.

A



Example of CD3, CD4 and CD8 expression in a vHSIL biopsy. For this staining, a panel consisting of CD3 (clone D7A6E, directly labelled with Alexa fluor 594), CD4 (clone EPR6855, indirectly detected with Alexa fluor goat anti rabbit IgG 546), CD8 (clone 4B11, indirectly detected with Alexa fluor goat anti mouse IgG2b 647) and DAPI was designed.

B



9 pre-vaccination vHSIL samples (3 NR, 3 PR, 3 CR) were stained and analyzed with the CD3, CD4, CD8, DAPI panel. Samples for staining were selected based on high CD3$^+$CD8$^-$Foxp3$^-$ cell counts. Of all CD3+CD8- cells, 99.1% was CD3+CD4+ (median, range 97-100%).

Additional File 9: Descriptive statistics of immune cells in post-vaccination vHSIL biopsies as one group and based on clinical response upon vaccination.

Immune cell phenotype	Number of cells/mm² epithelium (median with 95% CI)					Number of cells/mm² stroma (median with 95% CI)				
	vHSIL all	NR	PR	CR	Statistically significant differences	vHSIL all	NR	PR	CR	Statistically significant differences
CD3+CD8-	5,82 (2,75-21,7)	4,92 (0,93-26,5)	11,1 (3,13-63,3)	3,26 (0,00-48,0)	-	29,7 (10,4-65,6)	35,1 (9,03-65,6)	35,7 (5,25-248)	12,2 (1,79-156)	-
CD3+CD8-TBET+	10,6 (4,64-43,2)	4,64 (0,00-10,9)	42,5 (5,65-72,3)	52,4 (10,4-80,0)	NR**H, NR**PR	60,1 (21,7-147)	21,7 (5,51-63,2)	147 (58,7-677)	133 (31,6-336)	PR**H, NR**PR, NR*CR
CD3+CD8-TBET+TIM3+	-	-	-	-	-	0,00 (0,00-2,48)	0,00 (0,00-0,00)	2,46 (1,58-6,89)	14,7 (0,00-36,3)	NR****H, PR***H, NR****PR, NR**CR
CD3+CD8-PD1+TBET+	-	-	-	-	-	0,650 (0,00-10,5)	0,00 (0,00-0,55)	10,7 (1,59-39,4)	8,45 (0,60-10,8)	NR****H, NR***PR, NR**CR
CD3+CD8-FOXP3+	-	-	-	-	-	15,8 (1,59-52,7)	52,7 (25,9-171)	0,860 (0,00-6,42)	3,72 (0,00-13,4)	NR****H, NR**PR, NR**CR
CD3+CD8+	0,260 (0,00-1,79)	0,00 (0,00-2,21)	0,520 (0,00-6,17)	18,8 (0,00-38,4)	NR***H, PR*H	12,0 (3,07-23,4)	11,3 (0,55-20,0)	19,0 (1,58-69,3)	42,0 (1,79-186)	-
CD3+CD8+TBET+	-	-	-	-	-	0,00 (0,00-1,17)	0,00 (0,00-0,00)	0,00 (0,00-5,45)	2,14 (0,00-25,4)	NR****H, PR**H, NR**CR
CD14+CD68-CD163-	4,71 (0,00-20,3)	0,00 (0,00-2,65)	20,3 (7,48-33,6)	37,2 (3,79-130)	NR****H, NR****PR, NR**CR	57,0 (18,2-268)	17,4 (5,16-37,2)	426 (66,1-1671)	208 (136-855)	NR****H, NR***PR, NR***CR
CD14-CD68+CD163-	6,10 (0,00-14,1)	0,00 (0,00-14,1)	11,2 (0,00-25,7)	3,92 (0,00-58,0)	PR*H	40,7 (15,4-101)	21,5 (2,48-233)	58,8 (22,0-453)	34,4 (14,0-91,8)	PR****H, CR*H
CD14+CD68+CD163-	0,00 (0,00-3,28)	0,00 (0,00-0,00)	3,28 (0,92-19,8)	2,94 (0,00-32,1)	NR****H, NR****PR	7,68 (1,58-45,9)	1,40 (0,00-2,74)	86,4 (10,5-185)	19,7 (17,2-45,9)	NR****H, NR****PR, NR**CR
CD14-CD68+CD163+	-	-	-	-	-	1,67 (0,00-5,22)	5,22 (0,00-22,0)	1,61 (0,00-4,20)	0,860 (0,00-2,29)	CR*H
CD14+CD68+CD163+	-	-	-	-	-	6,66 (2,29-16,2)	5,97 (0,00-31,6)	7,65 (2,98-24,2)	1,93 (0,00-62,0)	NR****H, PR***H, CR*H

vHSIL all (n=24); non-responders (NR, n=11); partial responders (PR, n=9); complete responders (CR, n=4); CI=confidence interval, H=healthy vulva

A threshold of a median cell count of ≥10 cells/mm² per tissue compartment was applied, to only select relevant phenotypes for analyses. Differences between two groups were calculated with a Mann-Whitney test, statistical significance is indicated with asterisks: *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.