# SUPPLEMENTAL

**Figure S1. Conceptual overview of the interplay between immune cells response and melanoma growth with immunosenescence**

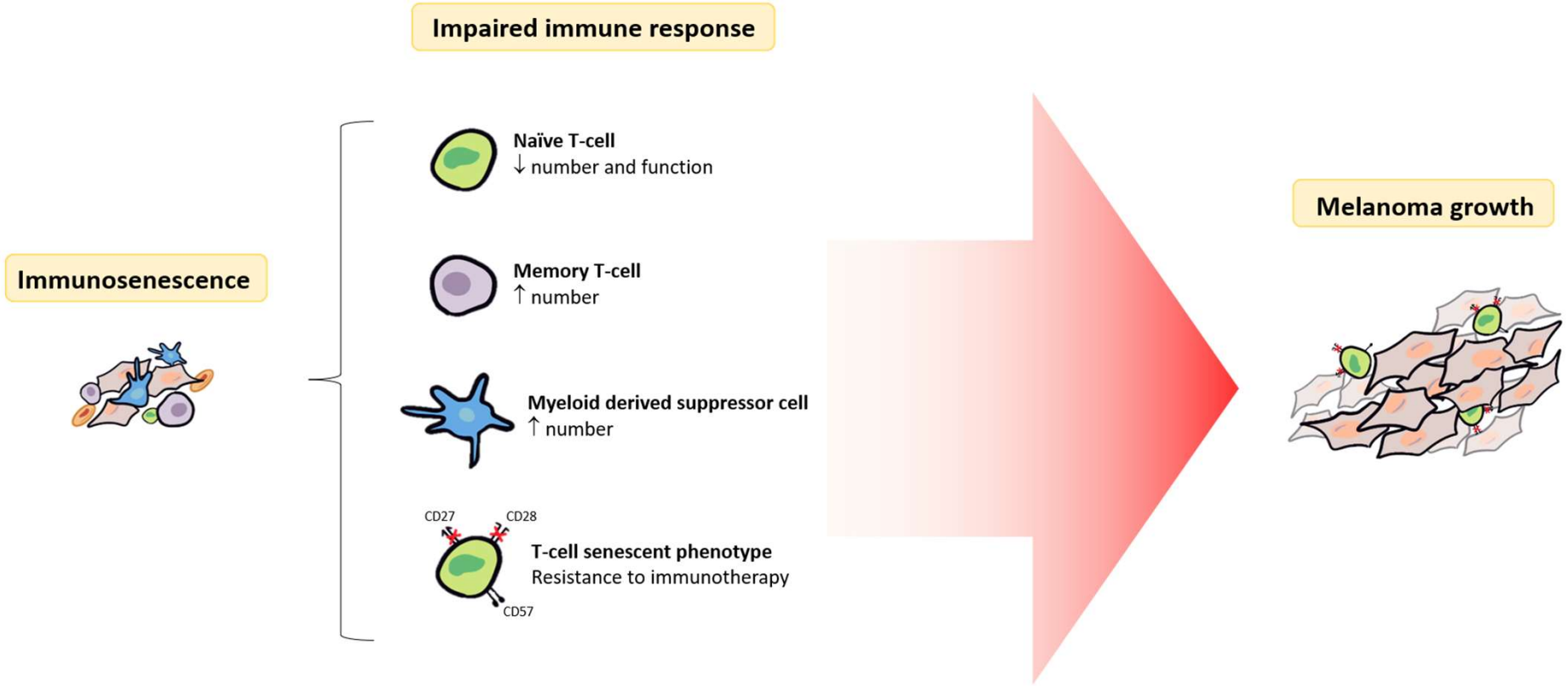
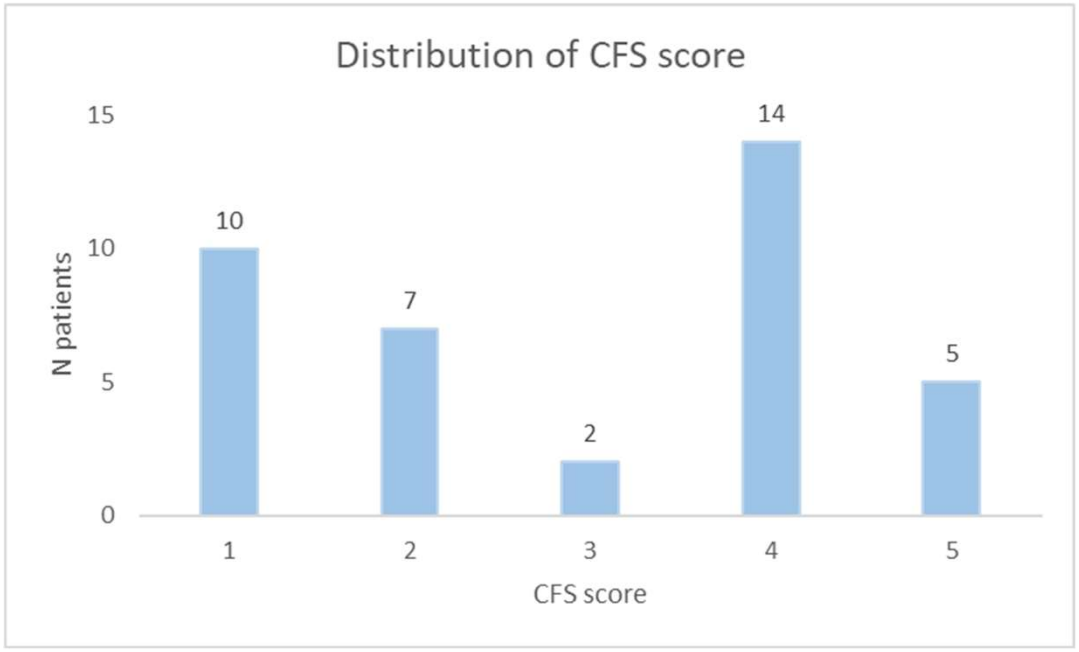



Figure S1. In melanoma, immunosenescence leads to reduced cell number and function of naïve T cells and memory T cells, inducing melanoma growth and metastatization process. Moreover, T cells express an immunosenescent phenotype characterized by reduced expression of CD27 and CD28 and higher expression of CD57, a major marker of immunosenescence. This immunosenescent phenotype has been associated with resistance to immunotherapy treatment.

**Figure S2. Distribution of Clinical Frailty Scale score in patients with frailty**



## Table S1. Panel markers for flow cytometry (1/2)

| Single stain | Laser | Detector | Fluor | Antigen | Characteristics of markers in immunosenescence field [1-3] | | Clone | Lot 1 | Unmixing |
|---|---|---|---|---|---|---|---|---|---|
| s1 | UltraViolet laser | UV2 | BUV395 | CD45RA | Naïve and terminally differentiated effector T cells marker | Lost as T cells become central or effector memory cells; ratio of naïve to these memory T cells is a hallmark of immunosenescence | HI100 | 2077243 | with beads |
| s2 | | UV4 | Zombie UV | L/D | Distinguishes live from dead cells | (to be able to only analyse viable cells as dead cells can non-specifically bind antibodies) | NA | | with cells |
| s3 | | UV7 | BUV496 | CD16 | NK cells marker and is expressed on monocytes and some macrophages | Changes in expression levels can indicate altered immune functions in ageing | 3G8 | 1099494 | with cells |
| s4 | | UV9 | BUV563 | CD39 | Exhausted T cells marker, also expressed in activated antigen-specific CD4 T cells in the tumour [4] | Can lead to increased immunosuppression with age | TU66 | 2069477 | with beads |
| s5 | | UV10 | BUV615 | ICOS | A costimulatory molecule for T cell activation and function | May be involved in the functional decline and activation of T cells in ageing | DX29 | 2077268 | with beads |
| s6 | | UV11 | BUV661 | CD1c | Marker of dendritic cells | Involved in antigen presentation; initiation of immune responses can decline with age | F10/21A3 | 2189988 | with cells |
| s7 | | UV14 | BUV737 | CD86 | Costimulatory molecule on antigen-presenting cells | Essential for effective T cell activation, which can decline with age | 2331 (FUN-1) | 1228590 | with beads |
| s8 | | UV16 | BUV805 | CD8 | Cytotoxic T cells marker | Accumulation of senescent CD8+ T cells is a hallmark of immunosenescence | SK1/HIT8a | 1200765 | with cells |
| s9 | violet laser | V1 | BV421 | CD161 | Expressed on a subset of T cells and NK cells | CD161 is involved in regulating immune responses[5], potentially implicated in age-related immune alterations | HP-3G10 | B334269 | with beads |
| s10 | | V2 | SB436 | CD123 | Plasmacytoid dendritic cells marker | Numerical and functional decline in pDC is associated with ageing | 6H6 | 2305276 | with beads |
| s11 | | V3 | PacBlue | CD15 | Neutrophils marker | Neutrophil functions may decline with ageing, although the neutrophils are hardly present after gradient isolation of blood sample | W6D3 | B273508 | with beads |
| s12 | | V4 | BV480 | CD33 | Early myeloid cells marker | Increased proportions are associated with ageing | P67.6 | 276608 | with beads |
| s13 | | V6 | BV510 | CD11c | Dendritic cells marker | Involved in antigen presentation, initiation of immune responses can decline with age | B-Ly6 | 1344885 | with cells |
| s14 | | V7 | PacOrange | CD3 | T cells marker | Ratio of different T cell subsets changes with age, can indicate immunosenescence | UCHT1 | 539078 | with cells |
| s15 | | V8 | BV570 | CD45RO | Memory T cells marker | Increased proportions suggest an aged immune repertoire | UCHL1 | B354623 | with beads |
| s16 | | V10 | BV605 | CD163 | M2-like macrophages marker | M2 macrophages may increase with age, can promote tumour [6, 7] | GHI/61 | B339795 | with beads |
| s17 | | V11 | BV650 | PD1 | Immune checkpoint inhibitor marker | Increases on T cells as they become activated and exhausted, a hallmark of immunosenescence | EH12.2H7 | B341605 | with beads |
| s18 | | V13 | BV711 | CD103 | Integrin expressed on tissue resident memory T cells | Tissue resident marker, can be upregulated upon activation[5] | Ber-ACT8 | B328658 | with beads |
| s19 | | V14 | BV750 | CD56 | NK cells marker (can also be expressed in T cells) | Changes in NK cell numbers and function are associated with ageing | 5.1H11 | B348832 | with beads |
| s20 | | V15 | BV785 | CD28 | Costimulatory molecule for T cells activation | Loss of CD28 in T cells is a hallmark of immunosenescence | CD28.2 | B344136 | with cells |

For each antibody, the conjugated fluorochrome, clone name and supplier are given.

## Table S1. Panel markers for flow cytometry (2/2)

| Single stain | Laser | Detector | Fluor | Antigen | Characteristics of markers in immunosenescence field [1-3] | | Clone | Lot 1 | Unmixing |
|---|---|---|---|---|---|---|---|---|---|
| s21 | blue laser | B1 | BB515 | CD141 | Expressed in subset of dendritic cells | CD141+ DC may decrease in number with age | 1A4 | 1166028 | with beads |
| s22 | | B2 | AF488 | Foxp3 | Transcription factor for Treg cells | Treg Foxp3+ cells may increase in number with age | 259D | B315166 | with beads |
| s23 | | B3 | Spark Blue 550 | CD14 | Monocytes/macrophages marker | Phenotype and function of monocytes and macrophages change with age | 63D6 | B321401 | with cells |
| s24 | | B8 | PerCP | CD45 | Leukocytes marker | Decreased proportions with age | Hi30 | B331249 | with beads |
| s25 | | B9 | PerCP/Cy5.5 | CD11b | Myeloid cells marker | Changes in expression might reflect shifts in innate immunity with age | ICRF44 | B328101 | with cells |
| s26 | | B10 | PerCP/eF710 | CD274/PD-L1 | PD-1 Ligand marker | Its expression on tumor cells and tumor associated macrophages can contribute to the immunosuppressive environment seen in ageing | MIH5 | 2246625 | with beads |
| s27 | yellow green laser | YG1 | PE | CLec9a | Expressed in subset of dendritic cells | CLEC9A+ DC (cDC1) may decrease in number with age [6] | 8F9 | B309940 | with cells |
| s28 | | YG2 | CF568 | CD4 | Helper T cells marker | Changes in the CD4+ T cell compartment are associated with immunosenescence | C4-206 | 21C0304 | with cells |
| s29 | | YG3 | PE/Dazzle 594 | CD206 | Activated macrophages marker | Activated macrophages increase with age | 15-Feb | B329923 | with beads |
| s30 | | YG4 | PE/Fire640 | CD25 | Expressed on activated T cells and Treg cells | Involved in cell proliferation and regulatory functions that may change with age | M-A251 | B332511 | with cells |
| s31 | | YG5 | PE/Cy5 | Tim3 | Immune checkpoint molecule | Increased expression is associated with T cell exhaustion in ageing | F38-2E2 | B353724 | with beads |
| s32 | | YG6 | PE/Fire700 | CD127 | Memory T cells and effector T cells marker, also expressed in a subset of Treg cells | Memory and effector T cells increase in number with age | AO19D5 | B348589 | with beads |
| s33 | | YG9 | PE/Cy7 | KLRG1 | Expressed on senescent T cells | -Marker of terminal differentiation and senescence in T cells | SA231A2 | B330891 | with cells |
| s34 | | YG10 | PE/Fire810 | HLA-DR | MHC class II molecule marker | Its expression on T cells can mean activated T cells. Its increased expression can be associated with inflammation and ageing | L243 | B341939 | with cells |
| s35 | red laser | R1 | APC | NKG2a | NK cells marker and immune checkpoint molecule on T cells | Increased proportions with age | Z199 | 200056 | with beads |
| s36 | | R2 | Alexa647 | CD68 | Macrophages marker | Decreased proportions with age | Y1/82A | B311503 | with beads |
| s37 | | R3 | Spark NIR 685 | CD19 | B cells marker | Changes in B cell subsets are hallmarks of immunosenescence | HIB19 | B324543 | with beads |
| s38 | | R4 | APC/R700 | Lag3 | Immune checkpoint molecule | Contributes to immune exhaustion and increases with age | T47-530 | 1114707 | with cells |
| s39 | | R7 | APC/Fire750 | CCR7 | Involved in cell migration to lymph nodes, expressed in various cell types | Loss of CCR7 expression on T cells is associated with immunosenescence | G043H7 | B338294 | with cells |
| s40 | | R8 | APC/Fire810 | CD27 | Costimulatory molecule | Loss of CD27 expression on T and B cells is a hallmark of immunosenescence | QA17A18 | B332527 | with beads |

For each antibody, the conjugated fluorochrome, clone name and supplier are given.

**References table S1.**


1. Zou Y, Han M, Wang J, Zhao J, Gan H, Yang Y. Predictive value of frailty in the mortality of hospitalized patients with COVID-19: a systematic review and meta-analysis. *Ann Transl Med* 2022; 10(4):166.
2. Zhang J, He T, Xue L, Guo H. Senescent T cells: a potential biomarker and target for cancer therapy. *EBioMedicine* 2021; 68:103409.
3. Tran Van Hoi E, De Glas NA, Portielje JEA, Van Heemst D, Van Den Bos F, Jochems SP, et al. Biomarkers of the ageing immune system and their association with frailty - A systematic review. *Exp Gerontol* 2023; 176:112163.
4. Kortekaas KE, Santegoets SJ, Sturm G, Ehsan I, van Egmond SL, Finotello F, et al. CD39 Identifies the CD4(+) Tumor-Specific T-cell Population in Human Cancer. *Cancer Immunol Res* 2020; 8(10):1311-1321.
5. Duurland CL, Santegoets SJ, Abdulrahman Z, Loof NM, Sturm G, Wesselink TH, et al. CD161 expression and regulation defines rapidly responding effector CD4+ T cells associated with improved survival in HPV16-associated tumors. *J Immunother Cancer* 2022; 10(1).
6. Villani AC, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* 2017; 356(6335).
7. Santegoets SJ, Duurland CL, Jordanova EJ, van Ham VJ, Ehsan I, Loof NM, et al. CD163(+) cytokine-producing cDC2 stimulate intratumoral type 1 T cell responses in HPV16-induced oropharyngeal cancer. *J Immunother Cancer* 2020; 8(2).

**Figure S3. Frequencies of CD3+ T cell populations between patients having response/no response to treatment**

**(A)**

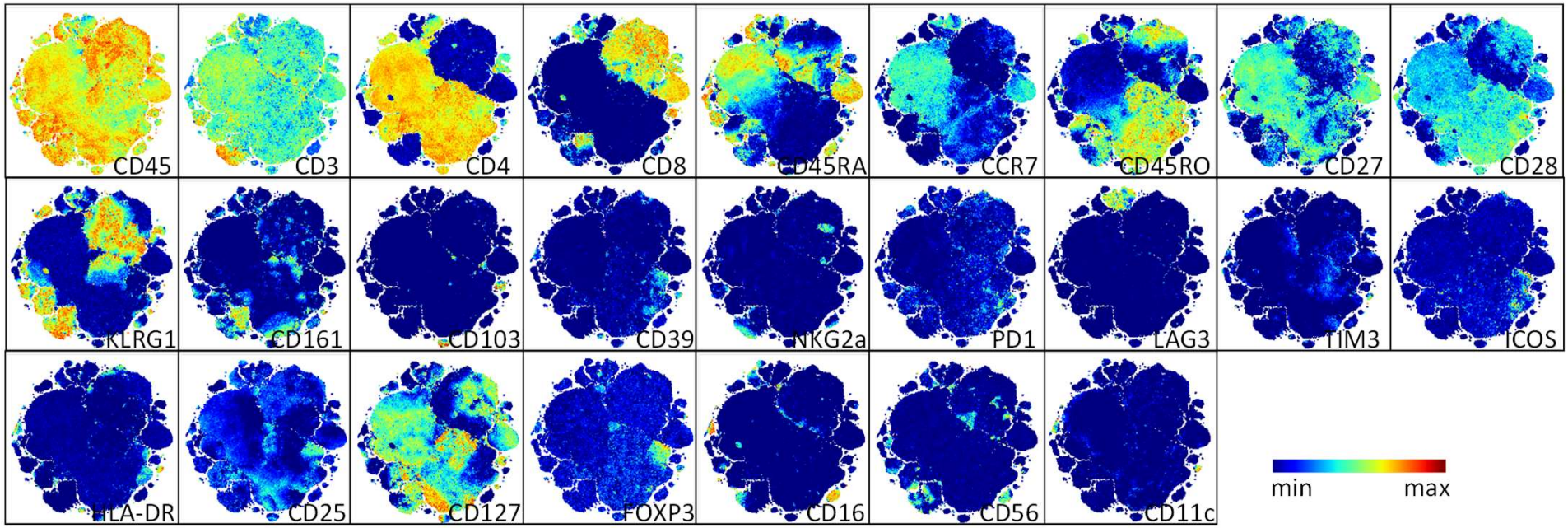



Figure S3. (A) OptSNE plots visualizing contour plots with the staining intensity of the individual markers used.

**Figure S3. Frequencies of CD3+ T cell populations between patients having response/no response to treatment**

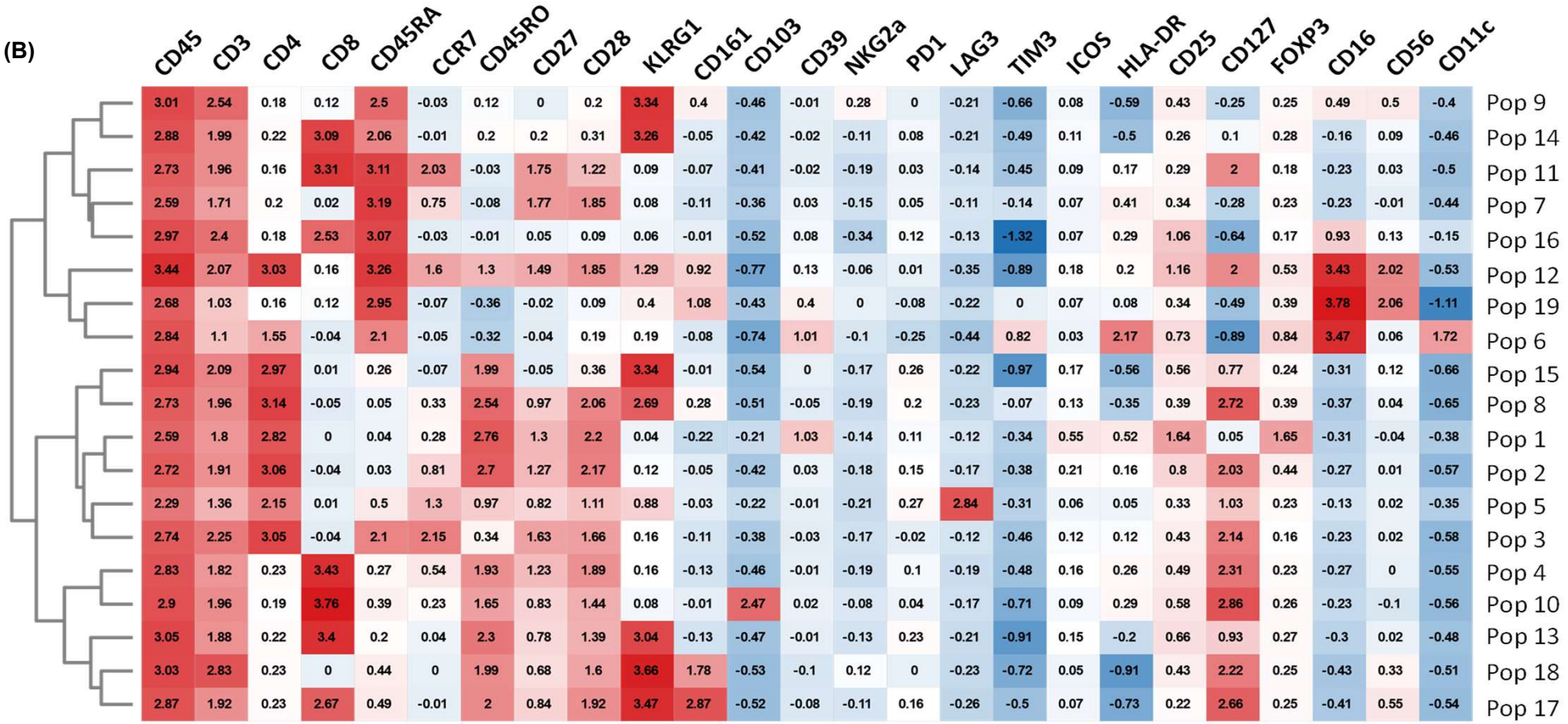



Figure S3. (B) Heat map (FlowSOM plugin output) of the relative fluorescent intensity for the markers associated with the identified T cell subpopulations.

**Figure S4. NK cell populations**

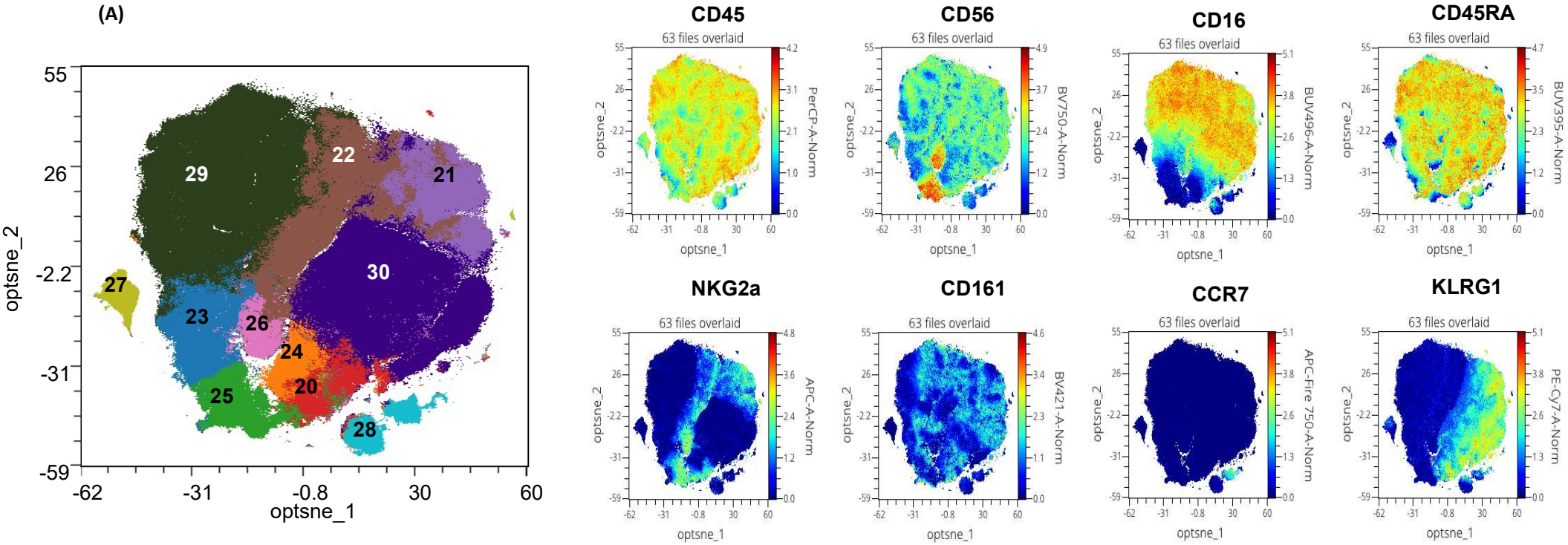



Figure S4. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for CD56+ NK cell markers. In total, 11 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right).

**Figure S4. NK cell populations**

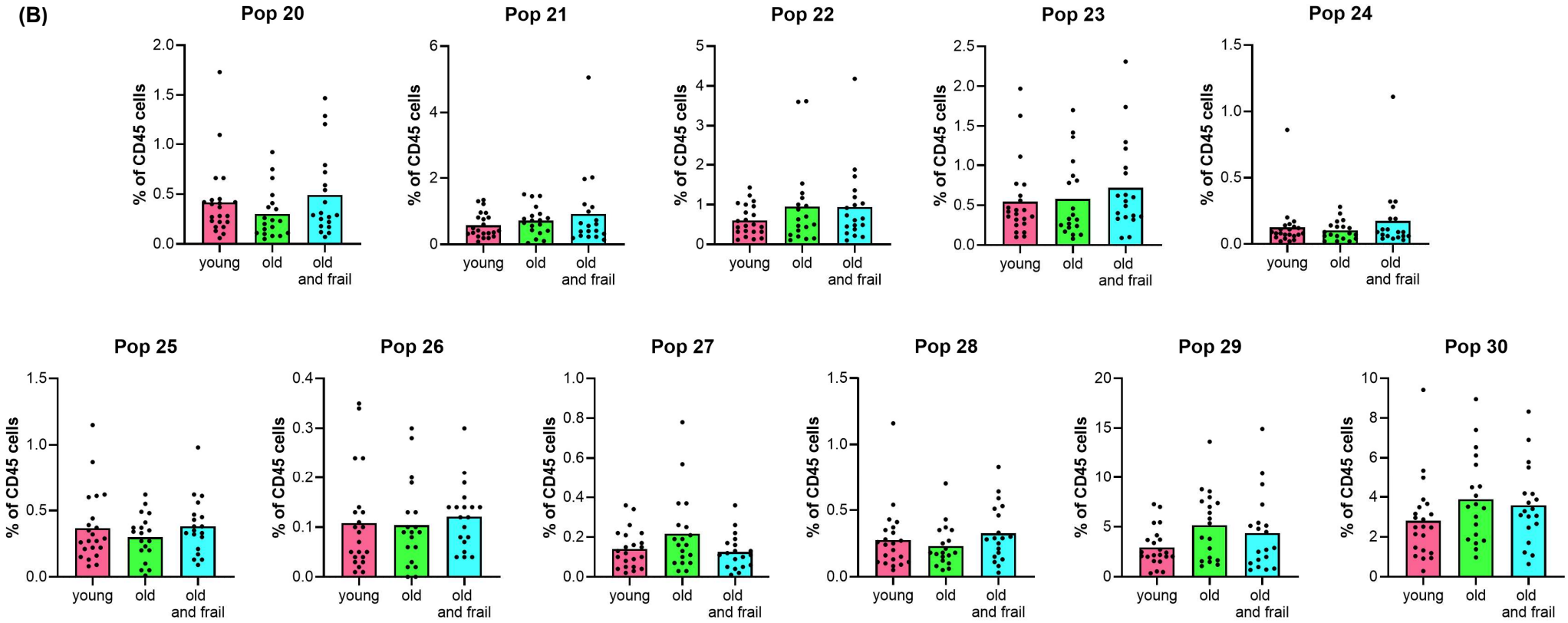
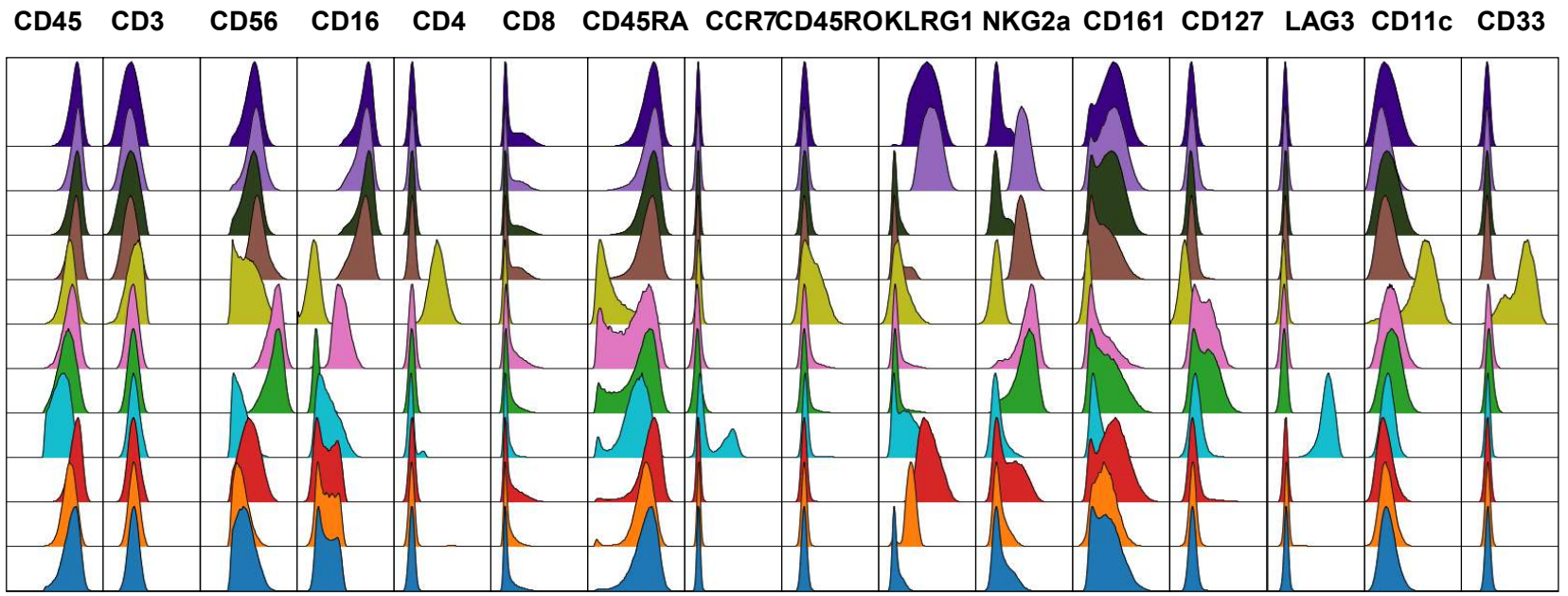



Figure S4. (B) Frequencies of NK cell populations in young, old and old-frail patient groups. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Kruskall-Wallis tests and $p<0.05$.

**(C)**

**Figure S4. NK cell populations**




| | |
|---|---|
| 20 | CD56+ CD45RA+ KLRG1+ CD161+ |
| 21 | CD45+ CD56+ CD16+ CD45RA+ KLRG1+ NKG2a+ CD161+ |
| 22 | CD45+ CD56+ CD16+ CD45RA+ KLRG1-NKG2a+ CD161+ |
| 23 | CD45+ CD56+CD16dim CD45RA+CD161+ |
| 24 | CD45+ CD56+CD16dim CD45RA+CD161+KLRG1+ |
| 25 | CD45+ CD56++ CD16-CD45RA+ NKG2a+ |
| 26 | CD45+ CD56++ CD16dim CD45RA+ NKG2a+ |
| 27 | CD45+ CD56+ CD4+ CD11c+ CD33+ |
| 28 | CD45+ CD56+CD16dim CD45RA+ LAG3+ |
| 29 | CD45+ CD56+ CD16+ CD45RA+KLRG1- |
| 30 | CD45+ CD56+ CD16+ CD45RA+ KLRG1+ CD161+ |

Figure S4. (C) Expression levels of each of the indicated markers is depicted for the individual cell populations.

**Figure S5. Myeloid cell populations**

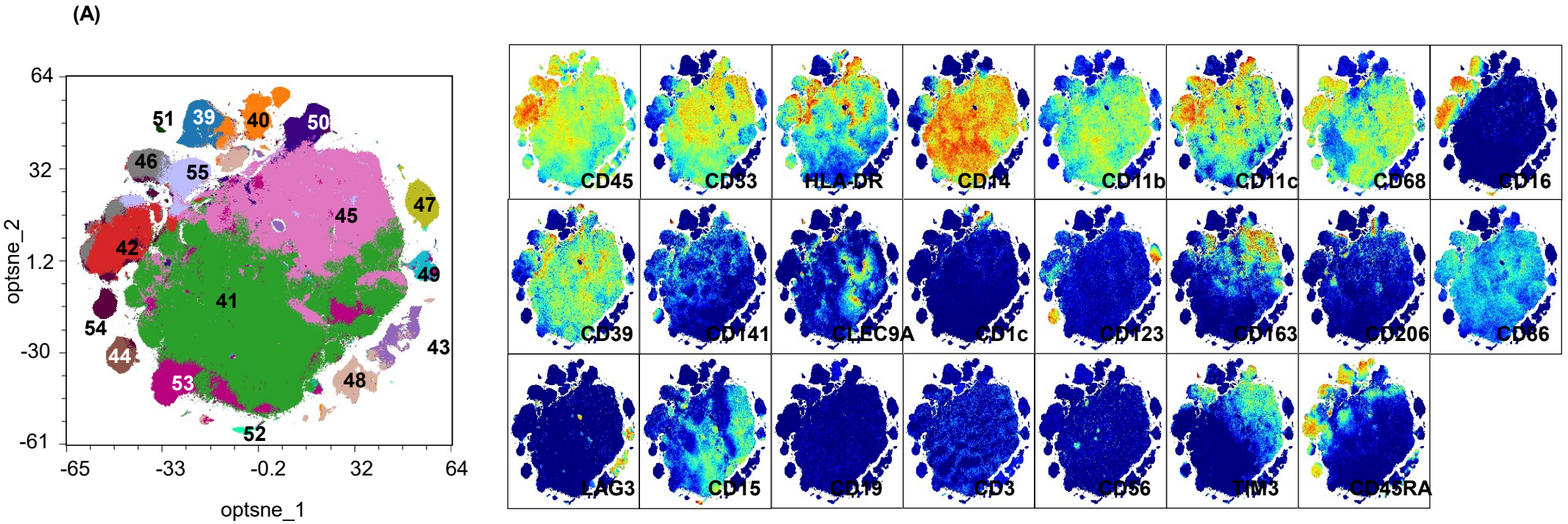



Figure S5. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for myeloid cell markers. In total, 17 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right).

**Figure S5. Myeloid cell populations**

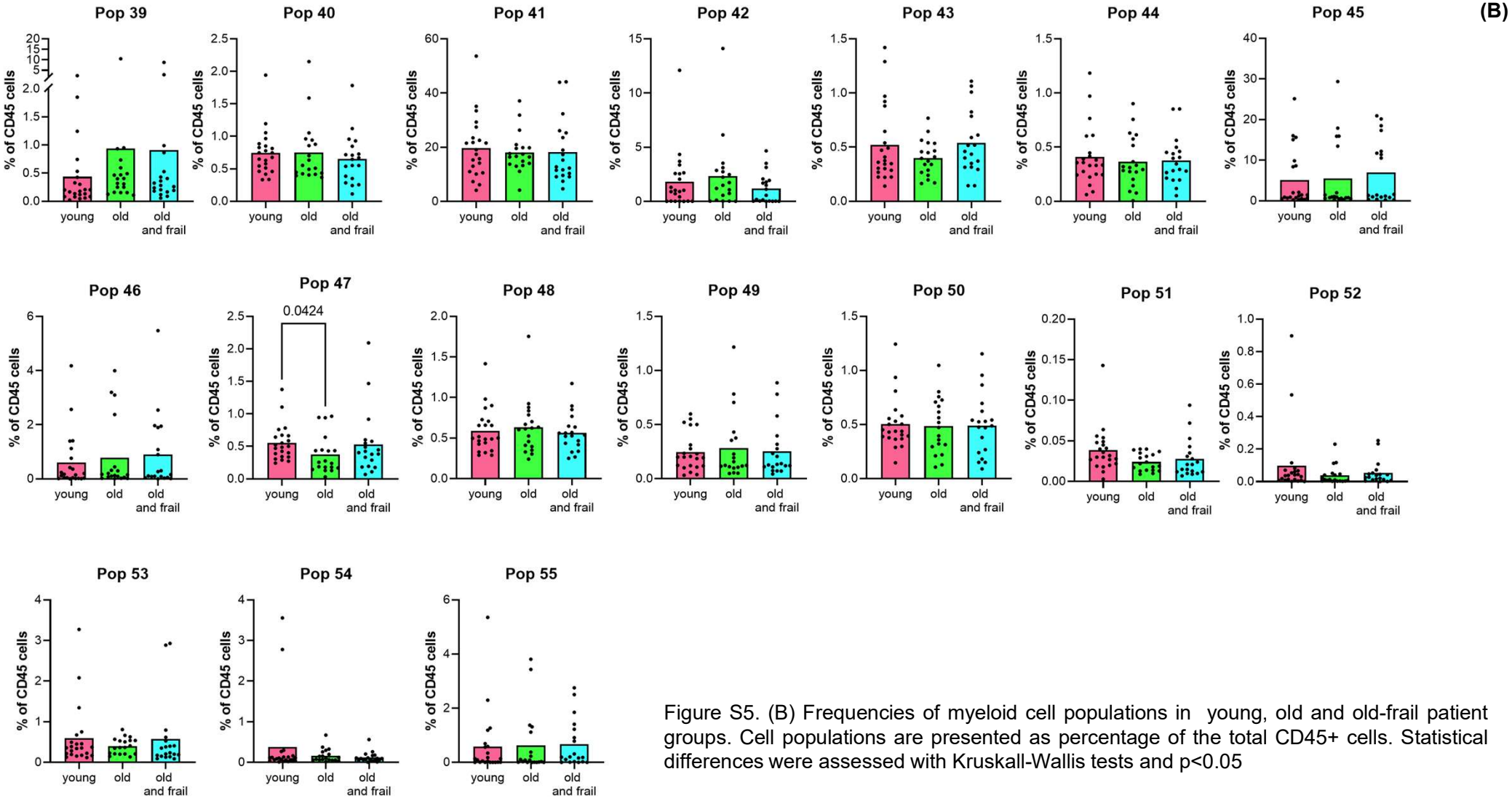
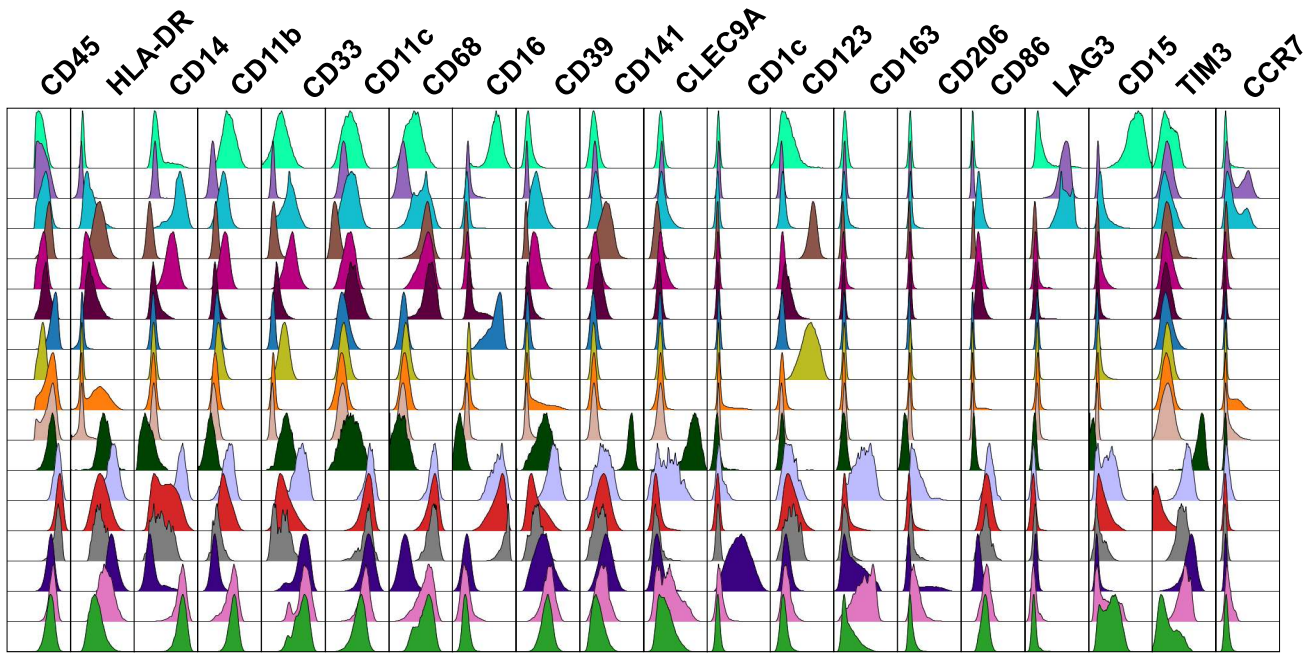



Figure S5. (B) Frequencies of myeloid cell populations in young, old and old-frail patient groups. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Kruskall-Wallis tests and $p<0.05$

(C)




| | |
|---|---|
| 39 | CD16+ |
| 40 | HLA-DR++ |
| 41 | HLA-DR+ CD68+CD14++ CD11b+ CD16- CD163- CD206- TIM3- |
| 42 | HLA-DR+ CD14dim CD16+ CD11c+CD86+ TIM3- |
| 43 | LAG3+ |
| 44 | HLA-DR+ CD14-CD11c- CD68+ CD123+ |
| 45 | HLA-DR+ CD68+CD14++ CD11b+ CD16- CD163+ CD206- TIM3+ |
| 46 | HLA-DR+ CD14dim/+CD11c+ CD16++ CD86+ TIM3+ |
| 47 | CD123++ |
| 48 | all neg |
| 49 | HLA-DR- CD14+ LAG3+ |
| 50 | HLA-DR+ CD14- CD68-CD1c+ CD163+/-TIM3+ |
| 51 | HLADR+ CD11cdim CD141+ CLEC9A+ |
| 52 | HLA-DR-CD15+ |
| 53 | HLA-DR- CD14+LAG3- |
| 54 | HLA-DRdim CD11cdim CD68+ CD45RA+ |
| 55 | HLA-DR+ CD14++ CD16+ CD163+ CD206dim/- TIM3+ |

**Figure S5. Myeloid cell populations**

Figure S5. (C) Expression levels of each of the indicated markers is depicted for the individual cell populations.

**Figure S6. B cell populations**

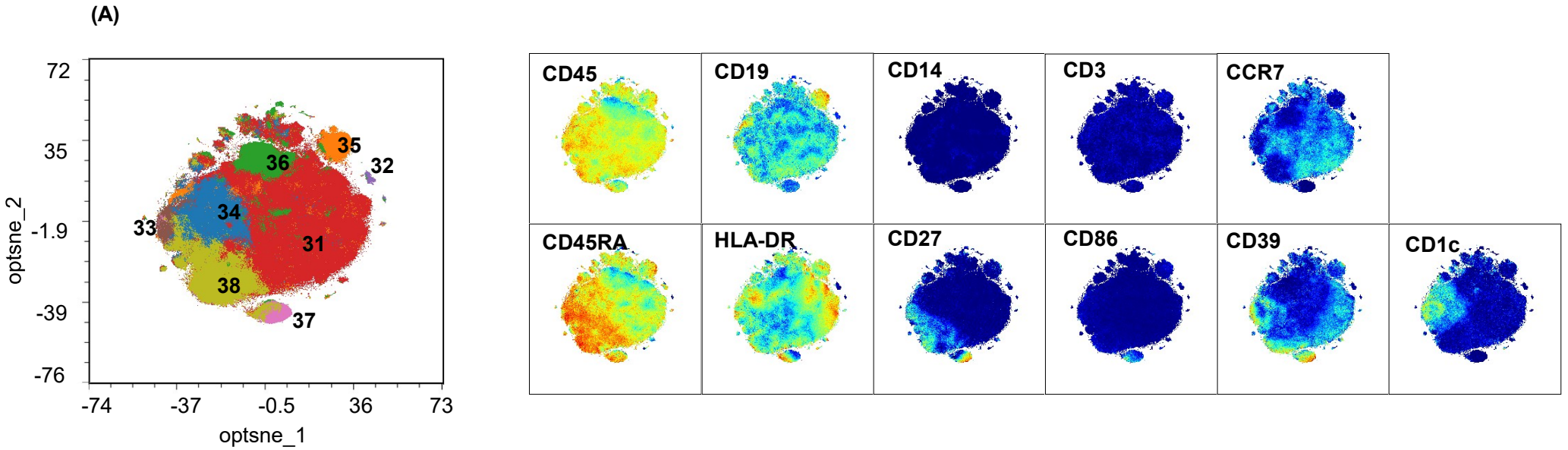



Figure S6. (A) Cluster partitions by FLOWSOM of PBMCs stained with antibodies for CD19+ B cell markers. In total, 8 different clusters were defined (left). OptSNE plots visualizing contour plots of the 3 patient groups (right).

**Figure S6. B cell populations**

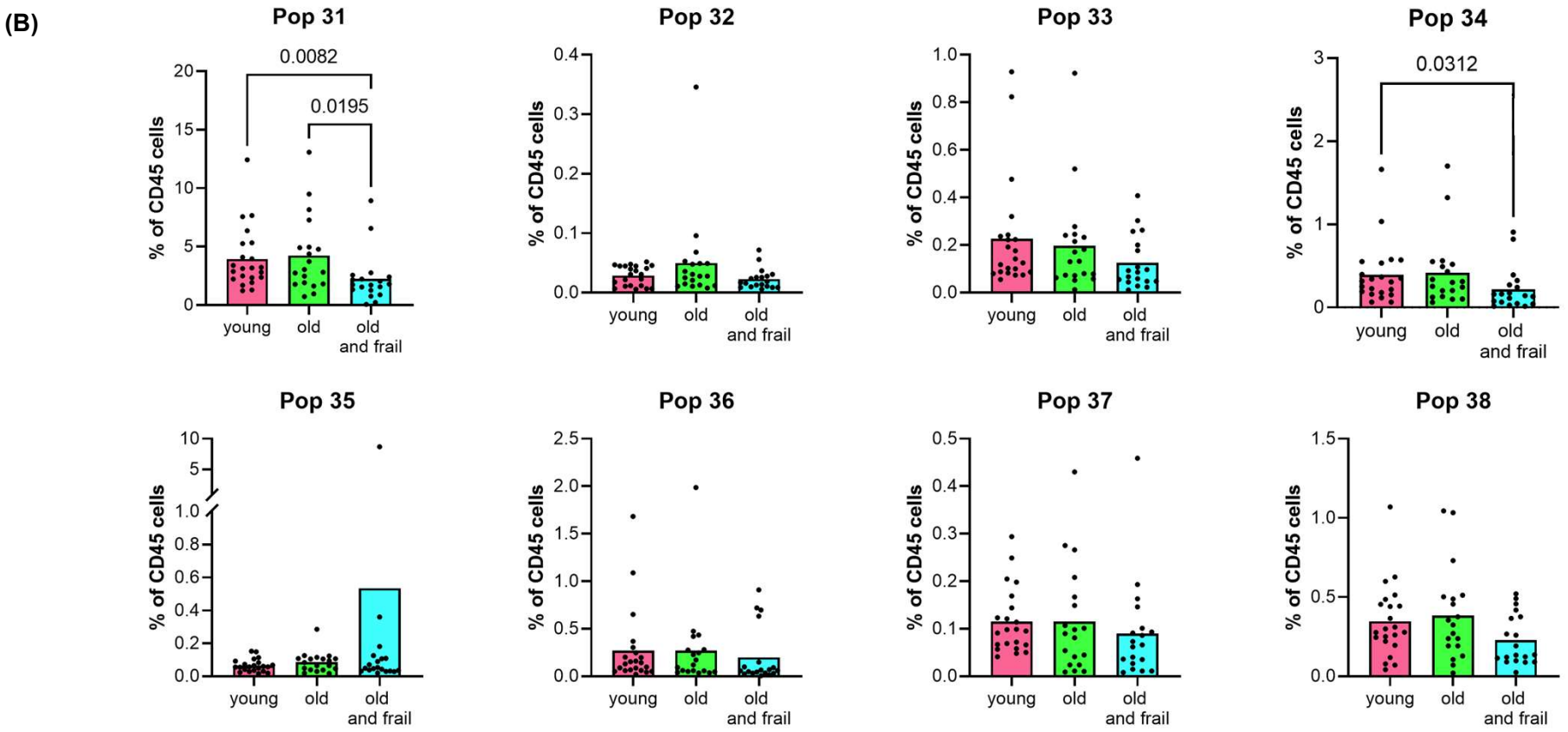
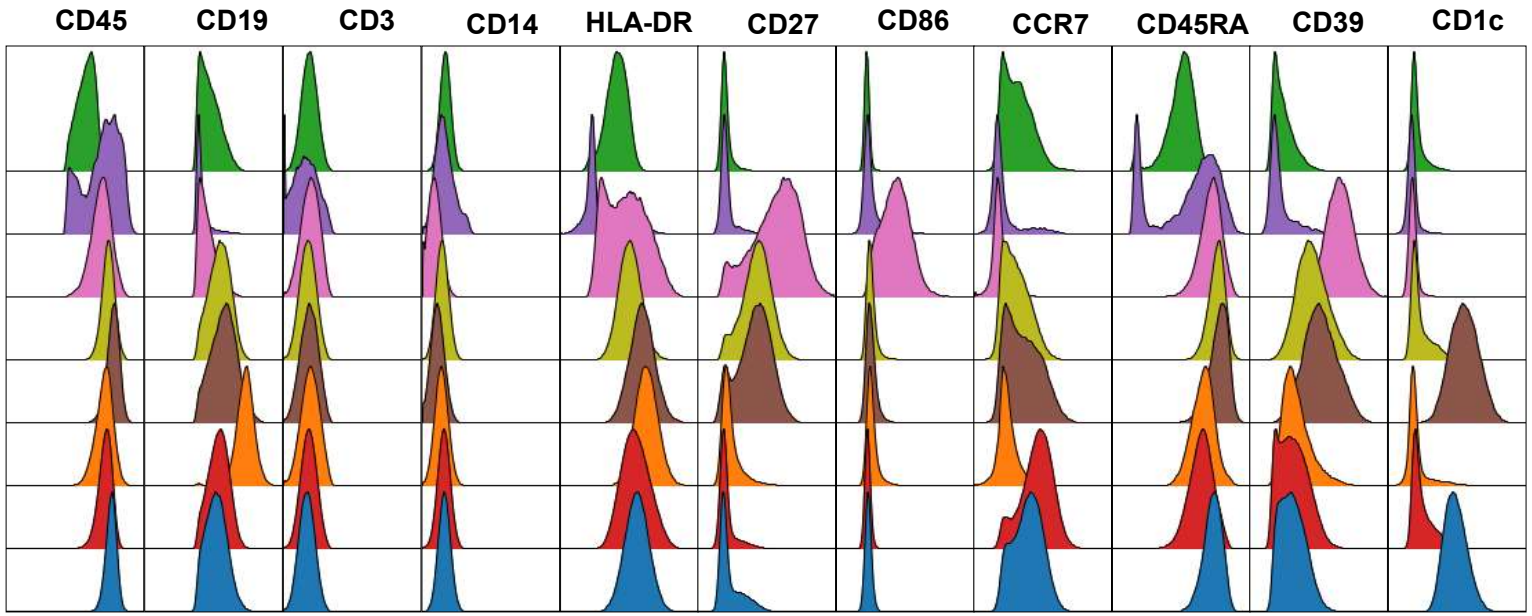



Figure S6. (B) Frequencies of B cell populations in young, old and old-frail patient groups. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Kruskall-Wallis tests and $p<0.05$.

**Figure S6. B cell populations**

(C)




| | |
|---|---|
| 31 | CD19+ HLADR+ CD27- CD86- CCR7+ CD45RA+ CD39dim |
| 32 | CD19dim HLADR- CD27- CD86- CCR7- CD45RA+/-CD39- |
| 33 | CD19+ HLADR+ CD27+ CD86- CCR7dim CD45RA+ CD39+ CD1Chi |
| 34 | CD19+ HLADR+ CD27- CD86- CCR7+ CD45RA+ CD39dim CD1c+ |
| 35 | CD19++ HLADR+ CD27- CD86- CCR7- CD45RA+ CD39- |
| 36 | CD19+ HLADR+ CD27- CD86- CCR7dim CD45RA+CD39dim |
| 37 | CD19dim HLADR+ CD27+ CD86+ CCR7- CD45RA+ CD39++ |
| 38 | CD19+ HLADR+ CD27+ CD86- CCR7dim CD45RA+ CD39+ |

Figure S6. (C) Expression levels of each of the indicated markers is depicted for the individual cell populations.

**Figure S7. Frequencies of CD3+ T cell populations between patients having response/no response to treatment (1/2)**

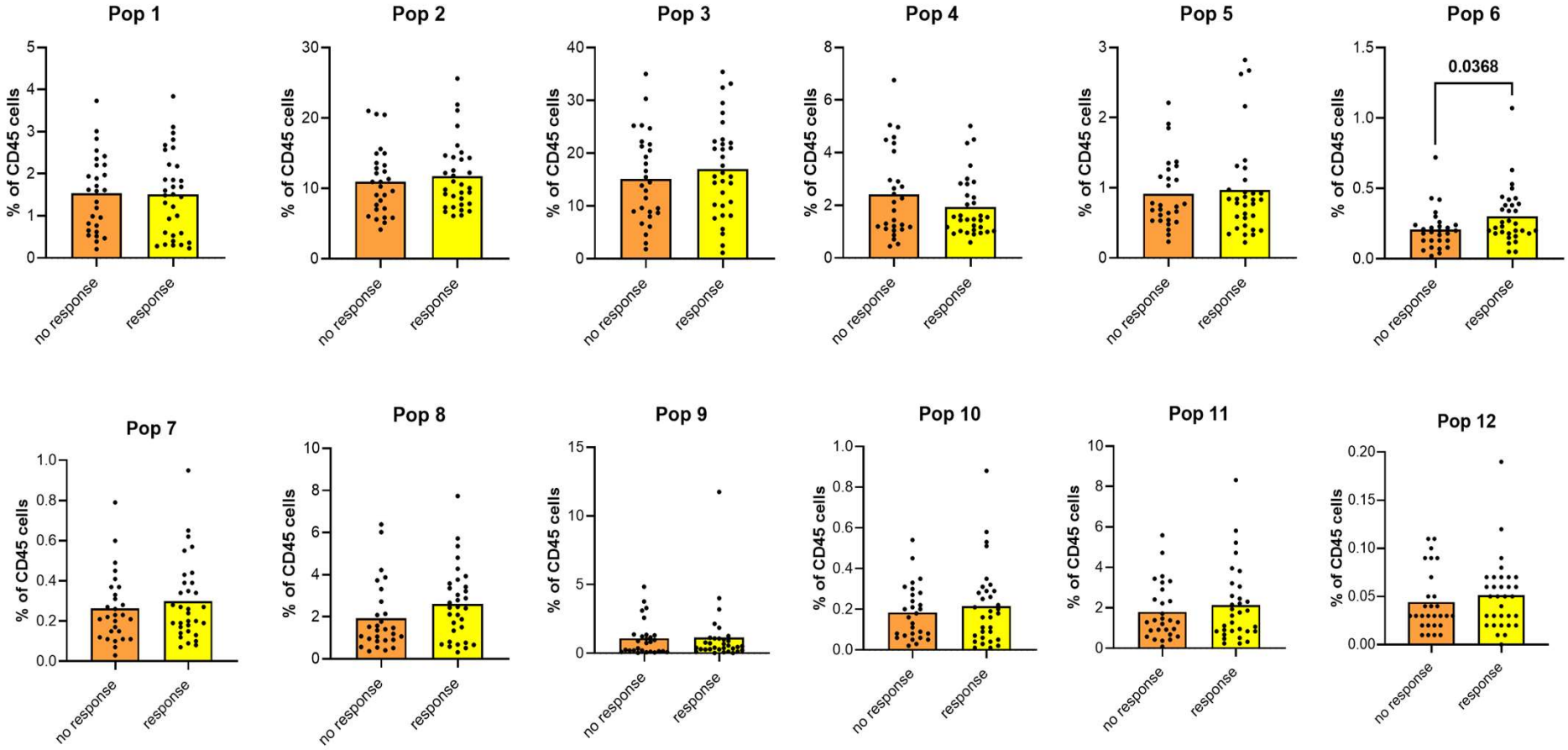



Figure S7. Frequencies of CD3+ T cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$. (1/2)

**Figure S7. Frequencies of CD3+ T cell populations between patients having response/no response to treatment (2/2)**

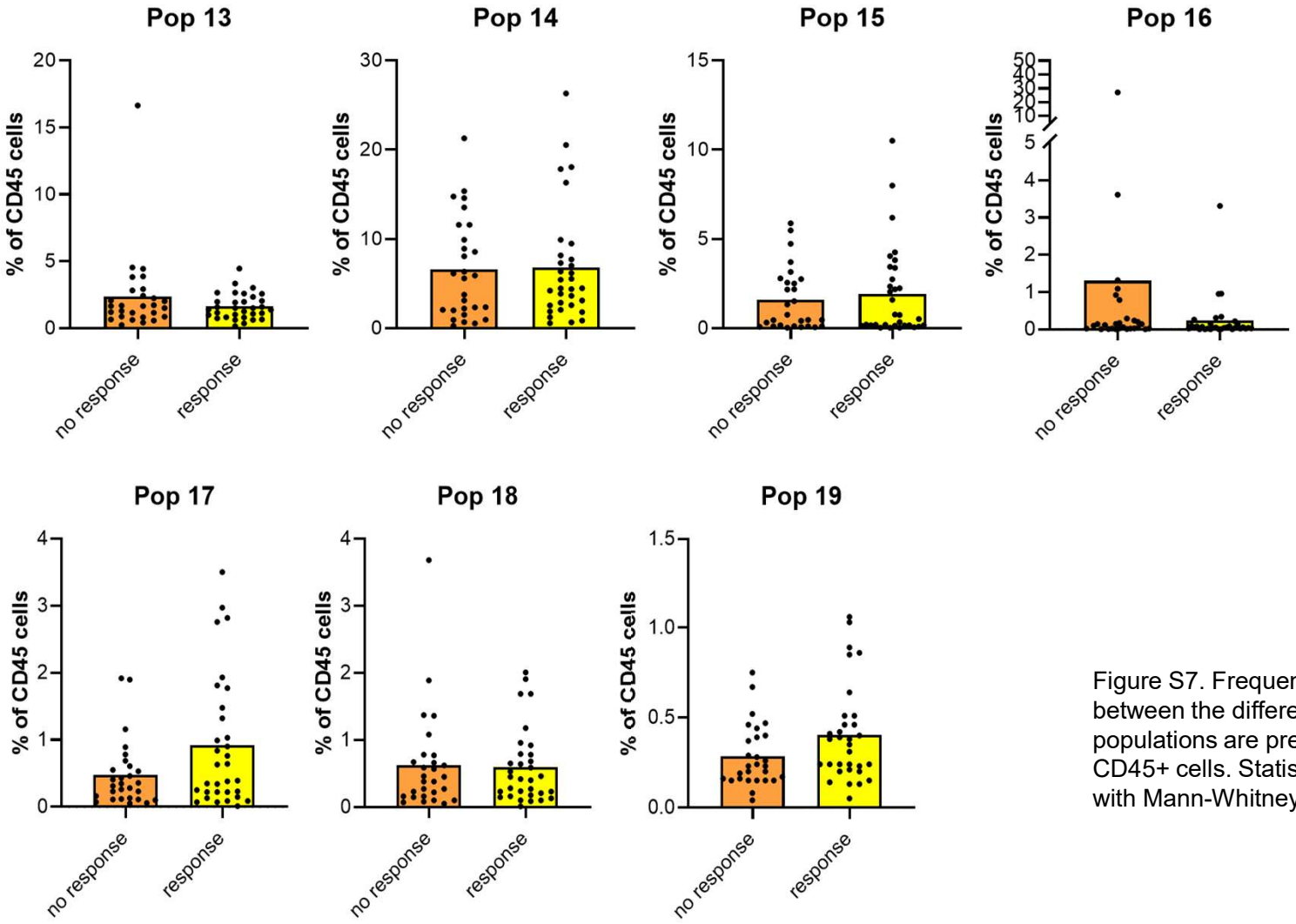



Figure S7. Frequencies of CD3+ T cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$. (2/2)

**Figure S8. Frequencies of NK cell populations between patients having response/no response to treatment**

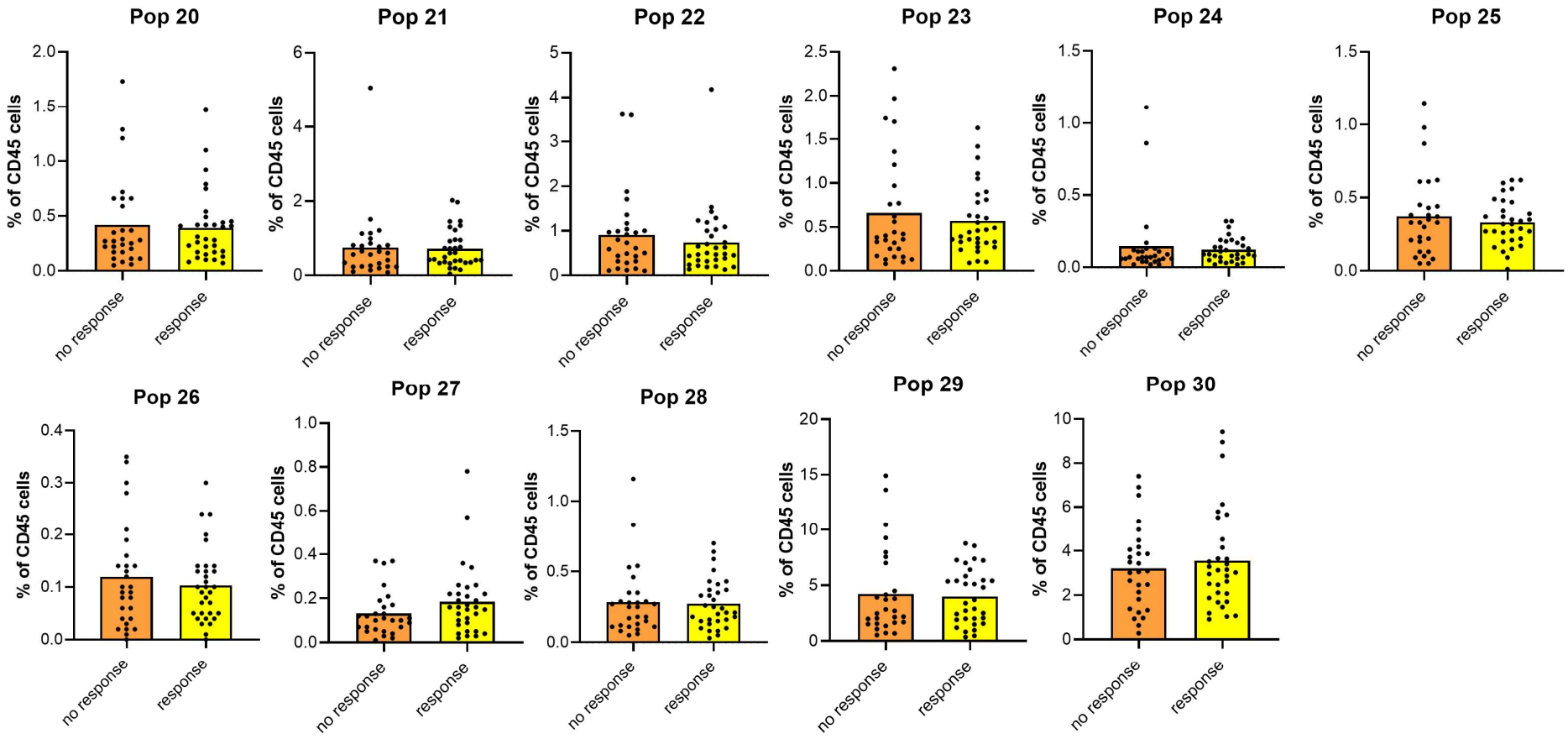



Figure S8. Frequencies of NK cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$.

**Figure S9. Frequencies of myeloid cell populations between patients having response/no response to treatment (1/2)**

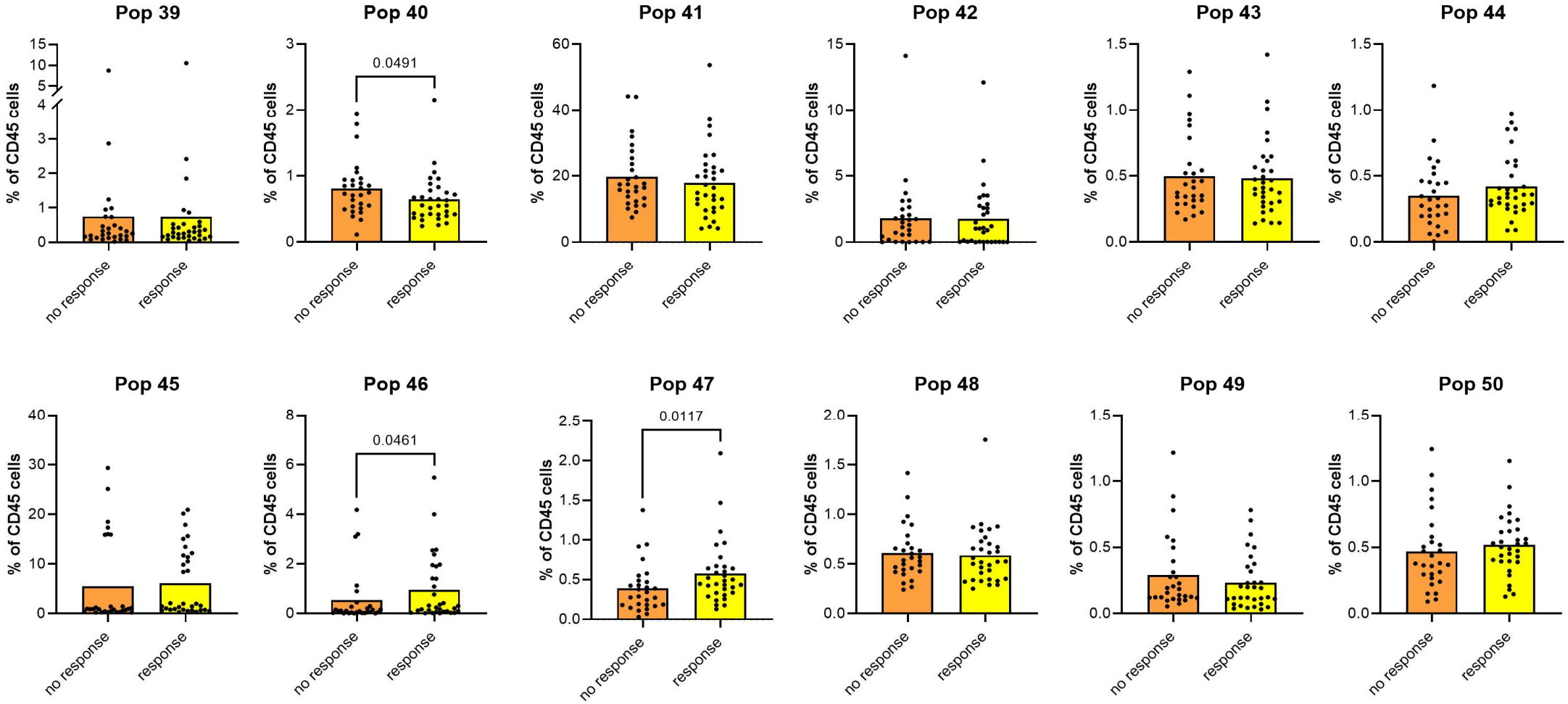



Figure S9. Frequencies of myeloid cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$. (1/2)

**Figure S9. Frequencies of myeloid cell populations between patients having response/no response to treatment (2/2)**

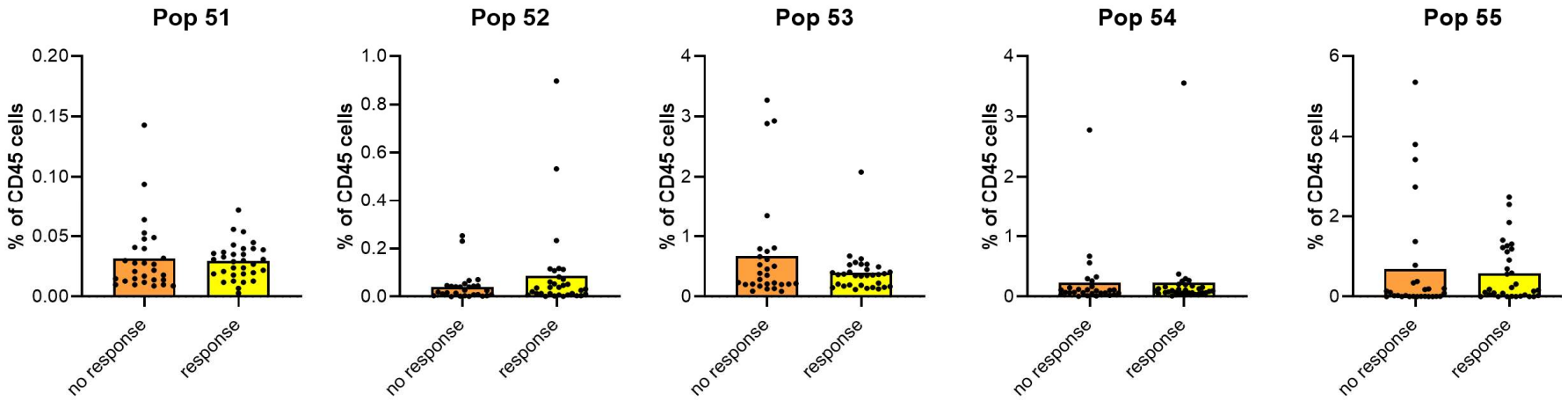



Figure S9. Frequencies of myeloid cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$. (2/2)

**Figure S10. Frequencies of B cell populations between patients having response/no response to treatment**

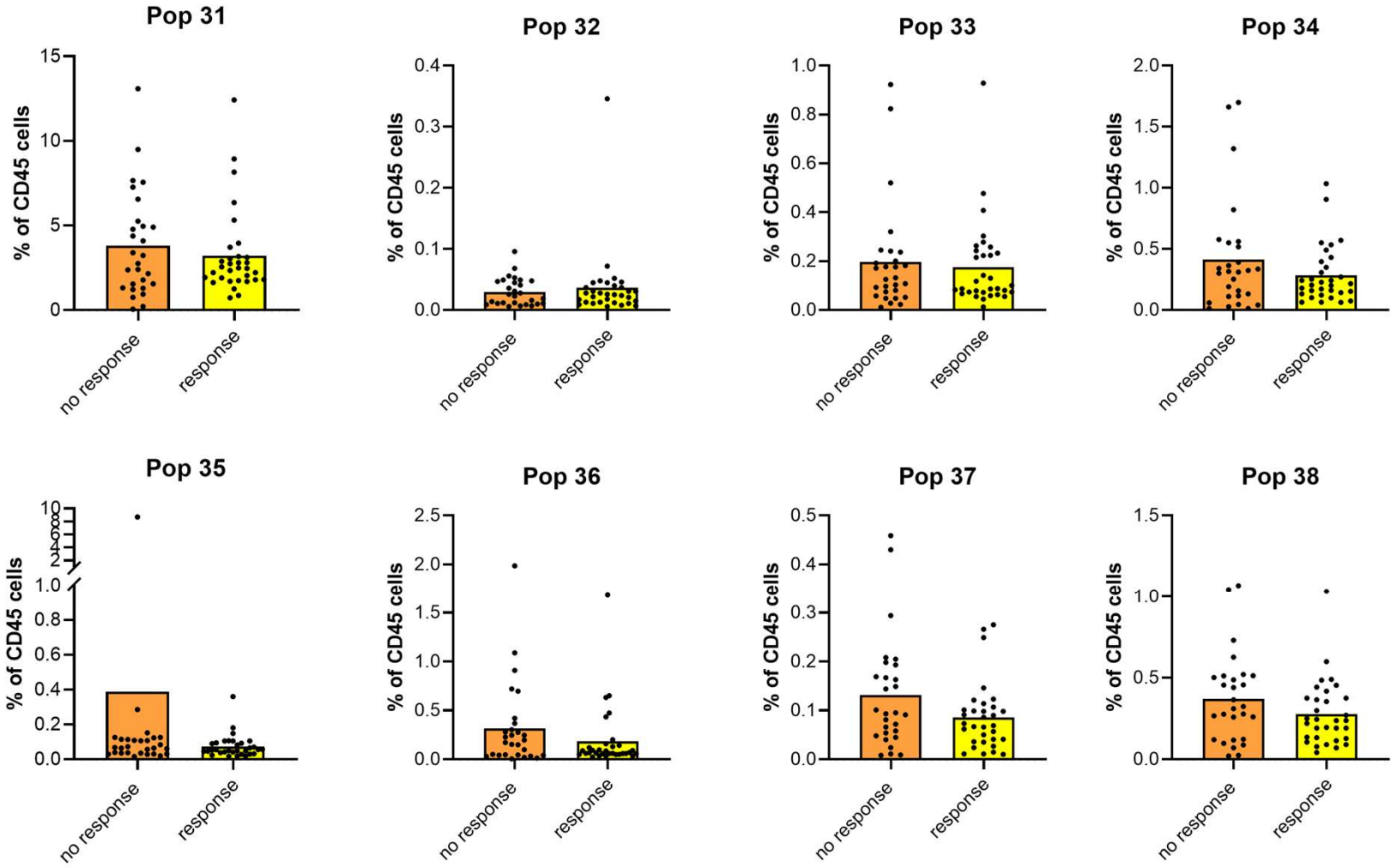



Figure S10. Frequencies of B cell populations between the different responses to treatment. Cell populations are presented as percentage of the total CD45+ cells. Statistical differences were assessed with Mann-Whitney tests and $p<0.05$.