

CD3-(CD56 or 16)+ natural killer cell distribution in blood from healthy adults and patients with ANCA-associated vasculitis

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CD3-(CD56 or 16)+ natural killer cell distribution in blood from healthy adults and patients with ANCA-associated vasculitis

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DECLARATIONS

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Consent for publication: Not applicable.

Availability of data and material: The datasets supporting the conclusions of this article are presented within the article and its additional file(s). Further datasets analyzed during the study are available from the corresponding authors on reasonable request.

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Authors' contributions: U.S., I.J., A.C.V. and J.T. were establishing and maintaining the biobank and mined data in vasculitis center 1. R.B. acquired data in vasculitis center 2. W.M., R.B. and N.V. were involved in data analysis and interpretation. W.M. and N.V. designed the study and took a leading role in writing the manuscript. All authors drafted the article and read and approved the final manuscript.

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ABSTRACT

Background Cytotoxic Natural Killer (NK) cells are an important target of new drugs entering the clinics, including checkpoint inhibitors and cell-depleting therapeutic antibodies. Still, basic blood NK cell parameters are poorly defined in healthy adults and in chronic inflammatory diseases like ANCA-associated vasculitis (AAV) which may alter the distribution of lymphocytes. The aims of this study were 1) to establish reference values of NK cell counts and percentages in healthy adults; 2) to describe these parameters in AAV; and 3) to investigate whether NK cell counts and percentages may be used as activity biomarker in the care of AAV patients, as suggested by a preceding study.

Methods CD3-(CD56 or 16)+ NK cell counts and percentages were determined in 120 healthy adults. Lymphocyte subset data from two German vasculitis centers were retrospectively analyzed (in total 407 measurements, including 201/49/157 measurements from 64/16/39 patients with granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), respectively).

Results CD3-(CD56 or 16)+ NK cell counts and percentages in healthy adults were highly variable, not Gaussian distributed and independent of age and sex. NK cell percentages ranged from 1.9 to 37.9% of lymphocytes, and were significantly more dispersed in AAV (0.3 to 57.6%). We further found that NK cell counts and percentages were different between AAV entities. However, during active disease, NK cell counts were consistently low in each AAV entity compared to healthy donors. NK cells were especially low in inactive EGPA. In 18% of EGPA patients we observed percentages of 1% or below which may be interpreted as temporary NK cell deficiency. Findings on differences between active and inactive GPA were discrepant between vasculitis centers.

Conclusions NK cell counts and percentages in blood are highly variable. This variability is further enhanced in systemic inflammatory diseases, and includes patients with temporary NK cell deficiency, in particular in EGPA. NK cell counts and percentages can presently not be recommended as biomarker in clinical care of AAV patients.

KEYWORDS

ANCA-associated vasculitis, natural killer cells

BACKGROUND

Anti-neutrophil cytoplasmatic antibody (ANCA)-associated vasculitis (AAV) is a rare systemic inflammatory condition affecting mainly small vessels [1, 2]. Despite some overlap, AAV comprise clinically, genetically and pathogenetically different entities. The most common entity is GPA, followed by MPA and EGPA, the latter of which is an especially rare condition. All three diseases are treated depending on activity, severity and the types of organs involved and often necessitate intense immunosuppression, including induction regimens with cyclophosphamide or the B cell-depleting anti-CD20 antibody rituximab [3].

While relatively specific autoantibodies - ANCAs with the two main sub-specificities anti-proteinase 3 (PR3) and anti-myeloperoxidase (MPO) - have some value in taking the diagnoses in AAV, disease activity and course cannot reliably be determined by laboratory tests. Treatment decisions are based on the physicians' judgments of disease activity and require substantial experience. Therefore, new activity biomarkers are needed for daily care in AAV [4]. In a preceding study, NK cell proportions in blood from patients with GPA were significantly increased in stable remission [5]. The usefulness of NK cells as activity biomarker

has not been investigated in other studies, and there are currently no data available on NK cells in MPA and EGPA.

Likewise, the role of NK cells in AAV is poorly understood. Based on their biologically known functions [6], NK cells may theoretically participate in autoimmune systemic inflammation by means of antibody dependent cell cytotoxicity (ADCC), by controlling other immune cells like CD4+ T lymphocytes [7], by secreting cytokines like interferon- γ or by killing neighbor cells expressing stress-induced ligands [8]. In our prior studies in GPA, we found that NK cells were mainly mature, functional cells with maintained cytotoxicity receptors, recognition of ligand-expressing target cells and ADCC [5, 9, 10]. However, we also observed several quantitative differences. For example, NK cells showed signs of activation, including the expression of CD69, CD54 and CCR5 and the down-regulation of CD16, in particular in active GPA.

In the last couple of years, NK cells became increasingly interesting as targets of cancer therapies, as recently summarized in *Nature* [11]. One example is the introduction of new checkpoint inhibitors that target innate immune cells including NK cells that are currently investigated in clinical trials. By virtue of the high expression of CD16 (Fc- γ -Receptor IIIa), NK cells can also be targeted via the Fc-part of therapeutic antibodies, which induces ADCC [6, 12]. NK cells thus play a role in the treatment with cell-depleting antibodies [13-16]. Accordingly, rituximab activates NK cells *in vivo* in patients with AAV [10]. A recent improvement of cell-depleting antibodies was achieved by engineering antibodies with an enhanced affinity to Fc receptors, such as the type 2 anti-CD20 antibody Obinutuzumab [17], which is superior to rituximab in the treatment of lymphomas [18]. Obinutuzumab is also a promising treatment strategy in systemic inflammatory diseases and has successfully passed a phase II trial in systemic lupus [19]. We have recently shown that Obinutuzumab is highly effective in depleting B cells and activating NK cells within PBMCs from GPA patients *in vitro*

[10]. However, formal proof of whether NK cell-targeting therapies aside rituximab work in systemic inflammatory diseases is pending.

The possible usefulness of NK cells as biomarker in AAV and the increased interest in NK cell-targeting therapies prompted us to investigate NK cells in AAV in more detail. When preparing this study, we frequently found the notion that NK cells comprise “about 5 to 10(or 15 or sometimes 20) percent of peripheral blood lymphocytes”. However, amazingly few state-of-the-art studies sustained these statements. In a study from 2004, “CD3-CD56+CD16+” NK cells from 51 healthy Caucasian individuals ranged from 51 to 652/ μ l, corresponding to 2 to 31% of lymphocytes [20]. A similar result was obtained in a representative Swiss cohort of 70 adults in which “CD3-/(CD16+/CD56+)” NK cells ranged from 77 to 427/ μ l, corresponding to 5.35 to 30.93% of lymphocytes [21]. Notably, the definition of NK cells varies between studies. While many antibody panels used in clinics to determine lymphocyte subsets by flow cytometry rely on commercial kits determining NK cells as “CD3 negative CD16/56 positive”, the consented definition of NK cells among immunologists is “CD3 negative and CD56 positive” [22]. In some studies, it remains unclear whether “CD16/56 positive” means CD16 *or* CD56 positive (e.g., both antibodies are linked to the same fluorochrome) or CD16 *and* CD56 positive (i.e., double-positive, meaning that CD56bright CD16negative NK cells are excluded) [20]. In addition, studies vary in whether other cell types are excluded from the NK cell gate. Today, CD14+ monocytes and CD19+ B cells are usually excluded next to CD3+ T cells prior to gate on CD56+ NK cells [22]. In particular in older studies, NK cells were determined by using only CD16 as single marker [23], or using two-color flow cytometry [21]. Analysis strategies of NK cells may further be variable based on whether they were determined in whole blood or in isolated peripheral blood mononucleated cells (PBMCs) after density gradient centrifugation. Finally, PBMCs can be used either freshly or after a freezing-thawing cycle. These analytical

differences may be responsible for the confusion about “normal” NK cell counts and percentages in peripheral blood. We did not find data on the statistical type of distribution of NK cell parameters (e.g. Gaussian). Re-analysis using state-of-the-art multicolor flow cytometry is therefore needed to establish standard ranges and distributions. To this end, we analyzed NK cell data from 120 healthy individuals in the present study. The method we used is a current diagnostic standard in German clinics. With the same protocol, we analyzed blood NK cell counts and percentages from patients with AAV.

PATIENTS AND METHODS

To test the potential use of CD3-(CD56 or CD16)+ NK cell percentages and counts as disease activity biomarker in ANCA-associated vasculitis, we retrospectively analyzed existing lymphocyte subset data from two German vasculitis centers [24]. Between 2011 and 2017 (vasculitis center 1) and 2016 and 2020 (vasculitis center 2), CD3-(CD56 or CD16)+ NK cells and matching Birmingham vasculitis activity scores (BVAS) were determined repeatedly from consenting patients that met current ACR classification criteria (ethics committee of Freiburg University, file no. 191/11, 46/04). In vasculitis center 1, we analyzed 360 measurements, including 151/50/158 measurements from 40/16/39 patients with GPA, microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), respectively. All measurements were included in the analysis, unless otherwise stated. Vasculitis center 2 contributed 50 measurements from 24 GPA patients, these results were analyzed separately (Figure 6 only). 120 healthy individuals served as controls, and were measured in the first vasculitis center. Further descriptive parameters are shown in table 1.

Vasculitis center	Entity	individuals, n	measurements, n	measurements per individual, mean	measurements per individual, range
1	HC	120	120	1	1
	GPA, total	40	151	3,8	1-8
	_inactive, BVAS =0	40	126	3,2	1-7
	_active, BVAS >0	15	25	1,7	0-3
	MPA, total	16	49	3,1	1-7
1	_inactive, BVAS =0	15	38	2,5	0-6
	_active, BVAS >0	8	11	1,4	0-3
	EGPA, total	39	157	4,0	1-9
	_inactive, BVAS =0	36	121	3,4	0-8
	_active, BVAS >0	21	36	1,7	0-4
	GPA, total	24	50	2,1	1-3
2	_inactive, BVAS =0	24	41	1,7	1-3
	_active, BVAS >0	9	9	1,0	0-1
1+2	all patients with AAV	119	407	3,4	nd

Table 1 Descriptive statistics. HC, healthy control; GPA, granulomatosis with polyangiitis; MPA microscopic polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; BVAS, Birmingham vasculitis activity score; AAV, ANCA-associated vasculitis.

Lymphocyte subpopulations phenotyping

Phenotyping of T-, B- and NK cells within the lymphocyte population was performed by a whole blood staining lyse-no wash protocol (Optilyse B, Beckman-Coulter) using six colour flow cytometry with the following fluorochrome-conjugated antibodies: BV421 anti-CD3 (clone UCHT1; Biolegend), APC anti-CD4 (clone SK3;Becton Dickinson), FITC anti-CD8 (clone B9.11; Beckman Coulter Immunotech), PE anti-CD16 (clone 3G8; Beckman Coulter Immunotech), PE-Cy7 anti-CD19 (clone J3-119; Beckman Coulter Immunotech), PerCP anti-CD45 (clone HI30; Biolegend), PE anti-CD56 (clone N901; Beckman Coulter Immunotech). Fixed antibody labelled cells were analyzed within 24 hours by flow cytometry (Navios; Beckman Coulter). Absolute cell counts were calculated using a two-platform method with leucocyte and lymphocyte counts determined by a hemocytometer. Flow cytometric data analysis was performed with the help of Kaluza Software 1.5a (Beckman Coulter). A representative gating strategy for definition of analyzed cell populations performed in vasculitis center 1 is described in figure 1.

RESULTS

Blood CD3-(CD56 or CD16)+ NK cell counts and percentages in healthy adults

To establish standard ranges under normal conditions, data from 120 healthy adults were analyzed (Figure 2). The youngest donor was 19, the oldest 71 years old (median 41.2 years).

NK cell counts ranged from 43/ μ l to 768/ μ l (median 180.5/ μ l). Percentages ranged from 1.9% to 37.9% of lymphocytes (median 11.05%) (Figure 2A). Neither counts nor percentages were Gaussian distributed (D'Agostino & Pearson omnibus normality test). In order to exclude gender-dependent differences, we compared males with females and observed no statistically significant differences (Figure 2B). Likewise, age did not show any tendency to influence NK cell counts and percentages (Spearman's $r = 0.12$ and 0.10 , respectively) (Figure 2C).

The distribution of NK cell counts and percentages in ANCA-associated vasculitis

Using the same analysis method, measurements in patients with AAV were performed in vasculitis center 1. Compared to healthy individuals, the distributions of NK cell counts and percentages was different in AAV, as determined by significant Kolmogorov-Smirnov tests and visualized by distribution histograms (Figure 3). In AAV, NK cell counts ranged from 1/ μ l to 687/ μ l (median 106/ μ l), and percentages ranged from 0.3% to 57.6% of lymphocytes (median 10.8%). The median absolute count was significantly lower in AAV than in HC, and the frequency distribution curves shifted towards low values, accordingly (Figure 3A). The median percentage of NK cells was not different from HC, but the frequency distribution curves shifted towards relatively more values on both extremes while values around the median were less frequent (Figure 3B).

One caveat in the analysis shown in figure 3 is that all measurements, meaning a variable number of multiple measurements per patients (see table 1), were included. This could have led to a bias if values in individual patients had been tightly similar. However, we observed a high intra-individual variability in patients with multiple measurements. Figure 3C shows example patients, each one patient with GPA, MPA and EGPA, respectively. It was therefore not possible to pick a single representative value per patient without causing other biases. In

an attempt to use one random measurement per patient, we grouped the first and last measurements performed in each patient, respectively, and observed similar distributions as with all measurements (supplementary figure 1). Together, these data show that the distributions of CD3-(CD56 or CD16)+ NK cell counts and percentages in AAV patients were different from that in healthy individuals, with a tendency towards more extreme values.

We next analyzed the three AAV entities separately. We found that NK cell counts and percentages were significantly different (Figure 4). The most prominent differences were the relatively low counts and percentages in EGPA. We observed that 13 measurements (8.2%) in EGPA corresponding to 7 different patients (18%) revealed a percentage of 1% of lymphocytes or below.

NK cells in relationship to AAV activity

A previous study found that NK cell percentages may be used as disease activity biomarker in the most common form of AAV, granulomatosis with polyangiitis (GPA) [5]. In order to control this finding, we defined AAV activity based on the Birmingham vasculitis activity scores (BVAS). BVAS=0 defined inactive and BVAS>0 active disease. BVAS reflects the presence of clinical symptoms and is therefore relevant for treatment decisions.

After sorting the data from vasculitis center 1 according to AAV entity and activity, we found that NK cell counts were

- significantly lower in all active AAVs compared to healthy controls,
- significantly lower in inactive GPA and EGPA compared to healthy controls,
- not significantly different between active and inactive AAVs (Figure 5).

NK cell percentages were

- significantly higher in inactive MPA compared to healthy controls,
- significantly lower in inactive EGPA compared to healthy controls,
- not significantly different between active and inactive AAVs (Figure 6).

In EGPA, both counts and percentages were decreased compared to healthy controls, especially in inactive disease (Figures 5C and 6C).

In contrast to the previous study [5], we did not observe a difference between active and inactive GPA in vasculitis center 1. As the definition and gating strategy of NK cells varied between the two studies, we next examined data from GPA patients from another vasculitis center (center 2 in the present study), which used a similar gating strategy to that used in vasculitis center 1. Similar to the data from vasculitis center 1, NK cell counts and percentages were highly variable in GPA patients from vasculitis center 2 and ranged from 8/ μ l to 718/ μ l (median 171.5/ μ l) and from 1.3% to 46.9% (median 14.85%), respectively (Figure 7). NK cell counts were significantly higher in inactive GPA. Percentages tended to be increased in inactive GPA, without reaching significance.

DISCUSSION

Given the paucity of state-of-the-art studies describing NK cell counts and percentages under normal conditions, we here analyzed data from 120 healthy adults. To the best of our knowledge, this is the largest study on normal NK cell counts and percentages so far. Another study comprising 253 healthy people counted 82 to 594 NK cells/ μ l blood (mean 253/ μ l), but percentages among lymphocytes were not provided [26]. In contrast to previous suggestions, we did not find age or sex dependent differences within this adult cohort. Our data instead

confirm the heterogeneous distribution and variability of NK cells in blood, and add the information that NK cells are non-normally distributed.

This NK cell variability was further increased in patients with inflammatory disease. In AAV, the frequency distributions of NK cell counts were shifted towards more extremely low values, and the frequency distribution of NK cell percentages was shifted towards both extremes, meaning that very high and very low percentages can be encountered in patients with AAV. This circumstance should be considered when planning trials with therapeutic antibodies that bind with high affinity to Fc_YRIIIA (such as Obinutuzumab), or when established NK cell-targeting therapeutics are administered in order to treat cancers in patients with systemic inflammatory diseases. So far, it is unknown whether NK cell counts or percentages affect the outcome of NK cell-based therapeutics or cancer incidence.

Possible reasons for the increased variability of NK cells in systemic inflammatory disease are unknown; however, activation and altered migration may play a role in GPA, as corresponding surface proteins like ICAM-1 or CCR5 are differentially expressed [9]. Also, therapeutics like glucocorticoids, immunosuppressants or rituximab may shift lymphocyte populations. However, this descriptive study was not designed to elicit underlying reasons for altered NK cell parameters.

The second aim of the study was to describe and compare NK cell parameters in all three entities of AAV. No data were available for MPA and EGPA so far. The type of retrospective analysis of data from AAV patients we used has several limitations. One is the diverging numbers of measurements per patient. Individual patients might be over- or under-represented. However, NK cell parameters were often variable within individual patients (not shown), so that it was impossible to pick out one measurement per patient without causing

biases. Patients' histories, (pre-)treatments and further characteristics like duration of remission and ANCA subtype were not analyzed. Therefore, potential differences in subgroups could not be clarified. However, the strengths of this study lie in the huge number of measurements - it is the largest published cohort on NK cell counts and percentages in ANCA-associated vasculitis - and in its independency from study conditions. Based on our data, we conclude that NK cells are differently distributed in AAV compared to healthy adults, with a tendency towards more extreme values. Overall, the lowest counts and percentages were found in EGPA. Some AAV patients had NK cell percentages below 1%, which may be interpreted as a secondary, numerical NK cell deficiency in analogy to the definition of primary NK cell deficiency. The latter is defined by the inherited persistent lack of NK cells (<1%) in absence of other NK cytopenia-inducing factors (and alternatively by the lack of NK cell cytotoxicity against K562 target cells) [25, 27]. Symptoms occur in childhood and are mainly caused by Herpes virus infections. Whether low NK cells predispose to viral infections and/or tumors in AAV has not been investigated. However, in our previous study, we showed that NK cell percentages correlated with the cytotoxicity against K562 target cells [9], indicating that extremely low NK cells may be associated with a reduced clearance of NK cell targets.

The third aim of the present study was to find out whether NK cell percentages and/or counts can be used to distinguish active from inactive GPA. While recommendations prefer a strict definition of "remission" in clinical trials - requiring a detailed evaluation of the patient (>6 months absence of symptoms, absence of increased immunosuppression, low and stable doses of prednisone) - [28], we here used an approach based solely on the presence or absence of symptoms (BVAS=0 vs. BVAS>0). This approach was supposed to have the highest clinical practicability, especially with respect to treatment decisions which are based on the actual presence of symptoms. In contrast to the previous study [5], in vasculitis center 1, NK

cell counts and percentages were not significantly different between active and inactive disease (BVAS >0 vs. BVAS =0). However, in vasculitis center 2, we observed increased counts in inactive GPA, a finding similar to the previous study [5]. These discrepancies in GPA may be explained by several factors, including different definitions and gating strategies of NK cells between the pilot study and the present study, different study populations between the vasculitis centers, possible regional treatment habits, diverging immunosuppressive therapy, and different clinicians judging activity (BVAS). It is also known that environmental factors have an impact on AAV [29]. However, we explicitly waived these factors that are, in their complexity, impossible to be evaluated in retrospective analyses. The results therefore allow the statement that in routine clinical care, i.e. in an undefined cohort as met by treating physicians, CD3-(CD56 or 16)+ NK cells are currently not helpful to exclude or include disease activity. Noteworthy, the data presented here do not exclude a potential utility of other NK cell parameters like the expression of surface molecules as biomarkers [9].

CONCLUSION

In conclusion, NK cells are highly variable in healthy adults, especially in patients with systemic inflammatory disease like AAV. Despite differences compared to healthy controls, CD3-(CD56 or 16)+ NK cell counts and percentages in AAV patients can presently not be recommended as disease activity marker in "real life" clinical practice.

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FIGURES AND LEGENDS

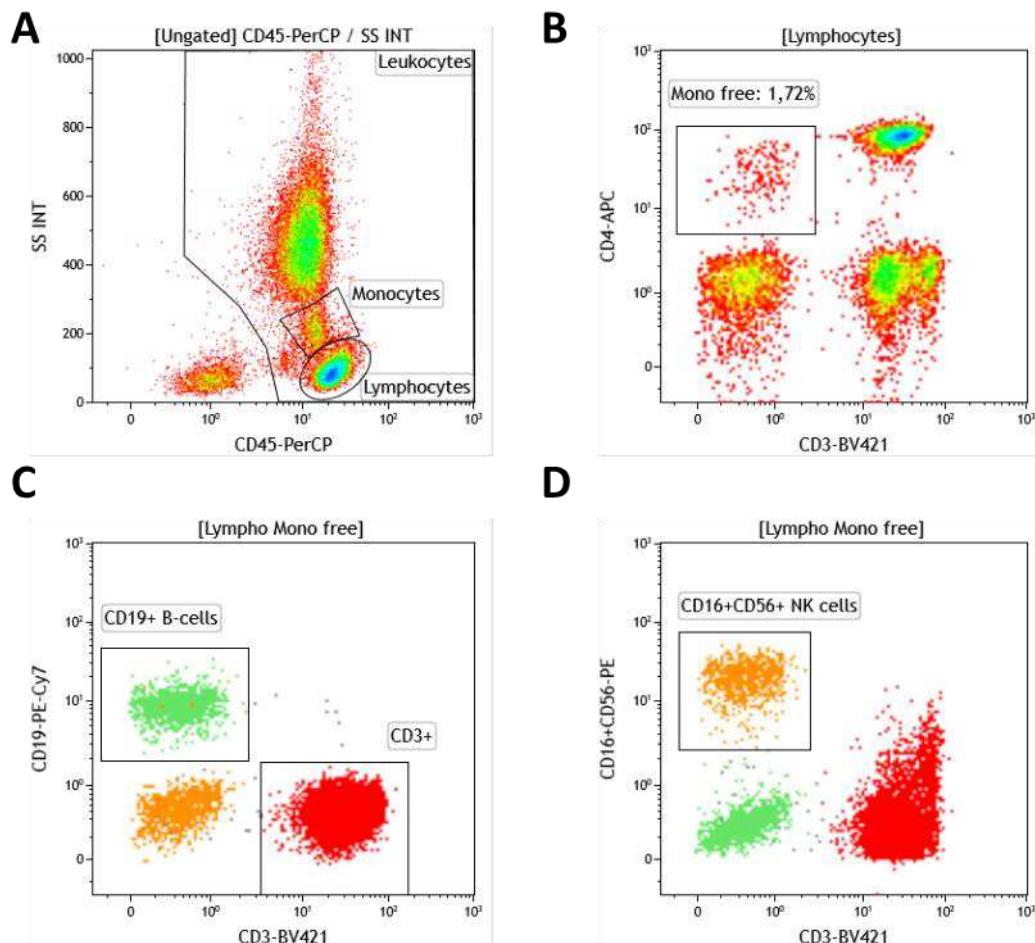


Figure 1: Gating strategy for NK cells in vasculitis center 1. **a** Definition of leucocyte subpopulations of monocytes and lymphocytes by CD45 versus SSC staining. **b** Exclusion of contaminating monocytes in the lymphocyte subpopulation by gating out CD3-CD4dim cells **c** Identification of CD19+ B-cells and CD3+ T-cells by CD3 versus CD19 staining. **d** Identification CD16+ or CD56+NK-cells by co-staining of CD16 and CD56 versus CD3.

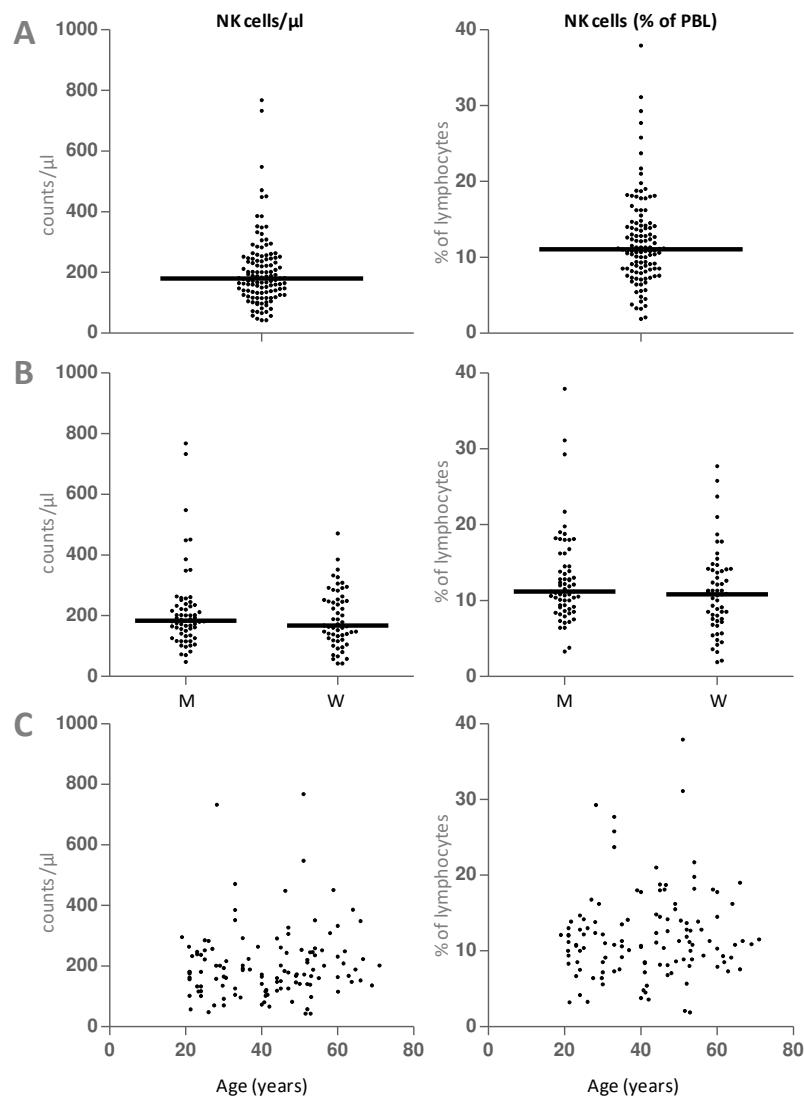


Figure 2. CD3-(CD56 or CD16)+ NK cell counts and percentages in 120 healthy human adults. In **b**, men (M, n=63) and women (W, n=57) are plotted separately. In **c**, counts and percentages are shown in relationship to age. Data derived from vasculitis center 1.

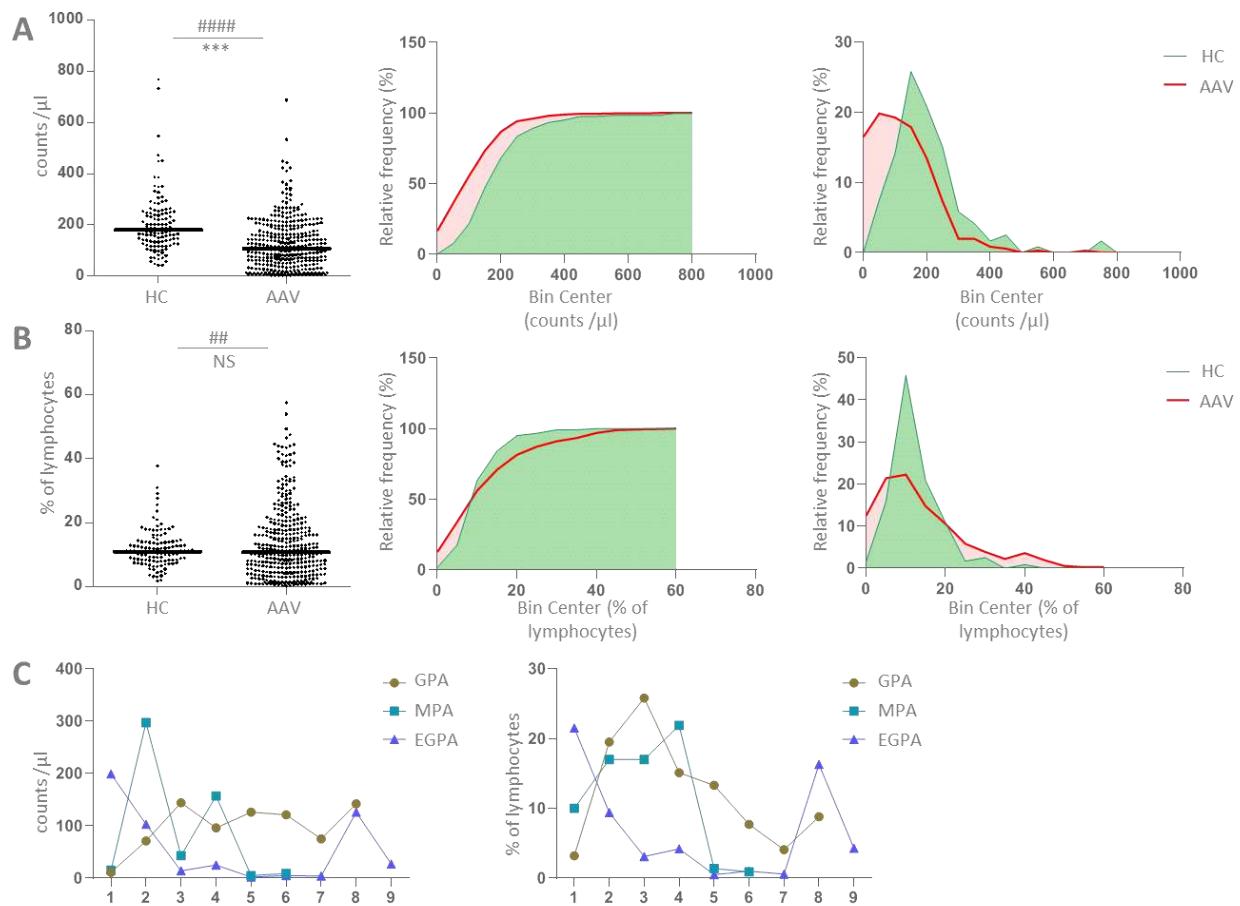


Figure 3. The distribution of NK cell counts and percentages in AAV. **a, b** HC, n=120 healthy controls; AAV, n=357 measurements in 95 AAV patients. The Kolmogorov-Smirnov test revealed different distributions of NK cell counts (a) and percentages (b) in AAV compared to HC (****, p<0.0001; ##, p=0.0018). The medians were compared using Mann Whitney test (***, p<0.0001; NS, not significant). The middle graphs show the cumulative relative frequencies and the right graphs the actual relative frequencies as histograms, respectively; the bin width was 50 counts/μl and 5%, respectively. In c, the course of three patients with GPA, MPA and EGPA over several consecutive measurements (1 to 9) are shown to demonstrate examples of intra-individual variability of NK cell counts (left) and percentages (right). All data derived from vasculitis center 1.

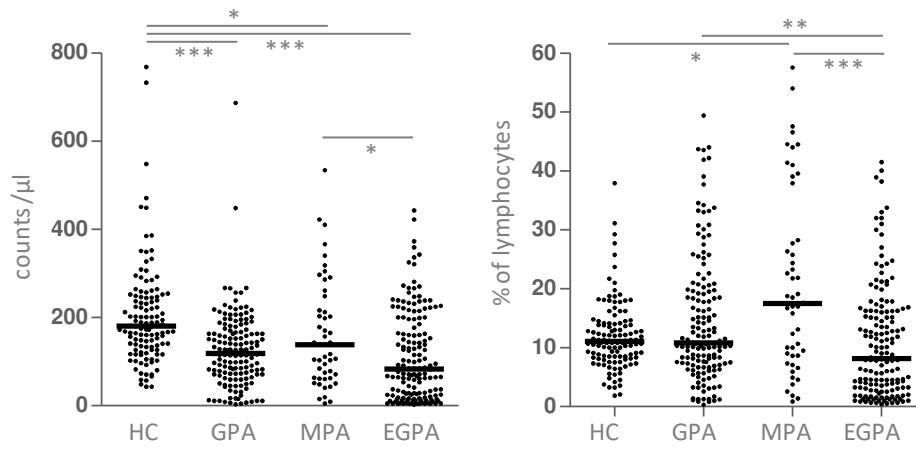


Figure 4. NK cell counts and percentages in GPA, MPA and EGPA. HC, n=120 healthy controls; GPA, n=251 measurements in 40 patients; MPA, n=49 measurements in 16 patients; EGPA, n=157 measurements in 39 patients. The Kruskal Wallis test was significant for both counts and percentages ($p<0.0001$, respectively). Dunn's post tests were significant where indicated in graphs. Data derived from vasculitis center 1.

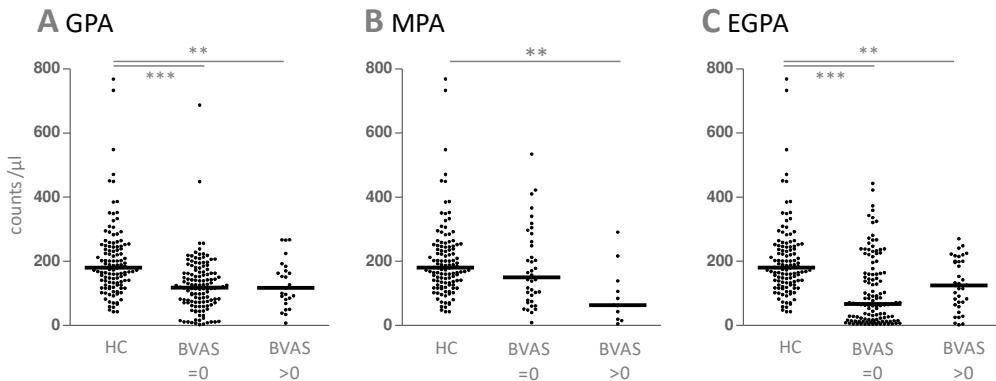


Figure 5. NK cell counts in relationship to disease activity. **a** granulomatosis with polyangiitis (GPA), **b** microscopic polyangiitis (MPA) and **c** eosinophilic granulomatosis with polyangiitis (EGPA). The Birmingham Vasculitis Activity Score (BVAS) was used to differentiate active (>0) and inactive ($=0$) AAVs. An exploratory statistical analysis was performed using Kruskal-Wallis test ($p<0.0001$; $=0.0023$; <0.0001 in GPA; MPA; EGPA, respectively). Dunn's post tests were significant as indicated by stars. All data derived from vasculitis center 1.

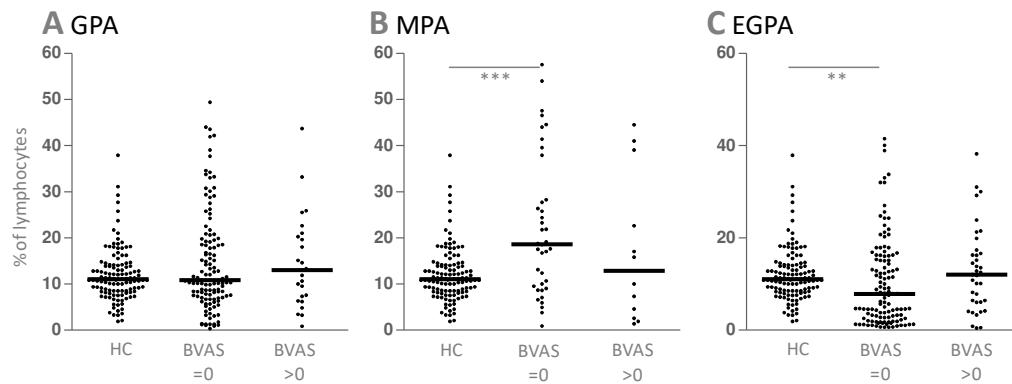


Figure 6. NK cell percentages in relationship to AAV activity. a GPA, b MPA and c EGPA. Kruskal-Wallis tests were not significant in the case of GPA, but significant in MPA and EGPA ($p=0.0012$ and $p=0.0020$, respectively). Dunn's post tests were significant where indicated. Data derived from vasculitis center 1.

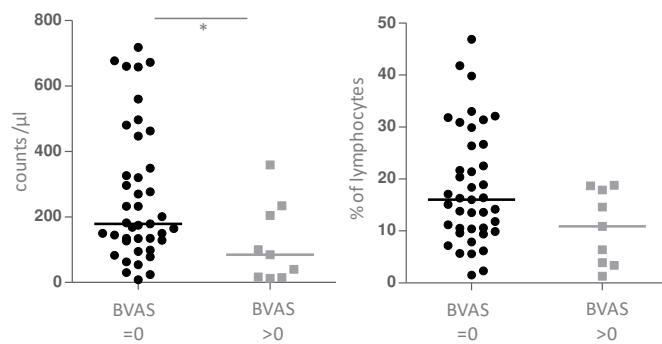
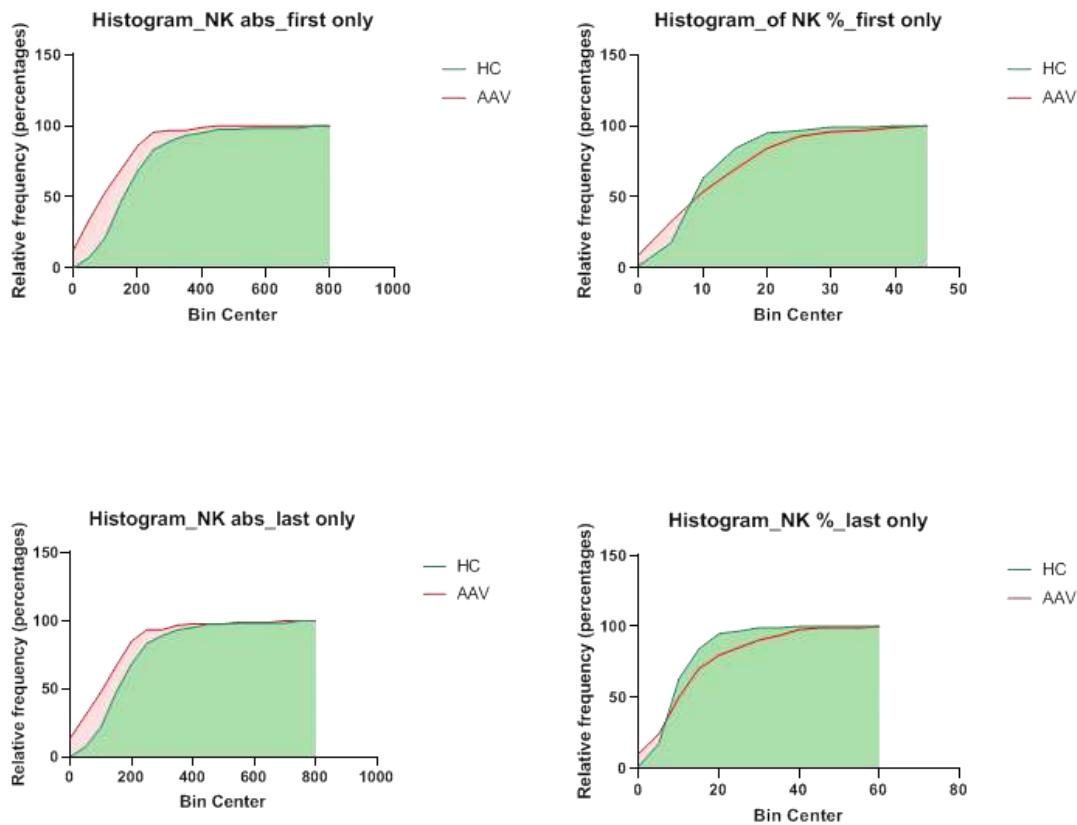


Figure 7. NK cell counts and percentages from GPA patients from vasculitis center 2. BVAS=0, n=41; BVAS>0, n=9. Statistical analysis was performed using Mann Whitney test (*, $p<0.05$). All data derived from vasculitis center 2.



Supplementary figure 1. The distribution of NK cell counts and percentages in AAV (one measurement/patient). HC, n=120 healthy controls; Upper left, n=93 first measurements of absolute NK cell counts in patients with ANCA-associated vasculitis (AAV). Upper right, n=95 first measurements of NK cell percentages in blood lymphocytes. Lower row, n=94 last measurements of NK counts and percentages, respectively. In each graph of this figure, the Kolmogorov-Smirnov tests were significant. Data derived from vasculitis center 1.

Figures

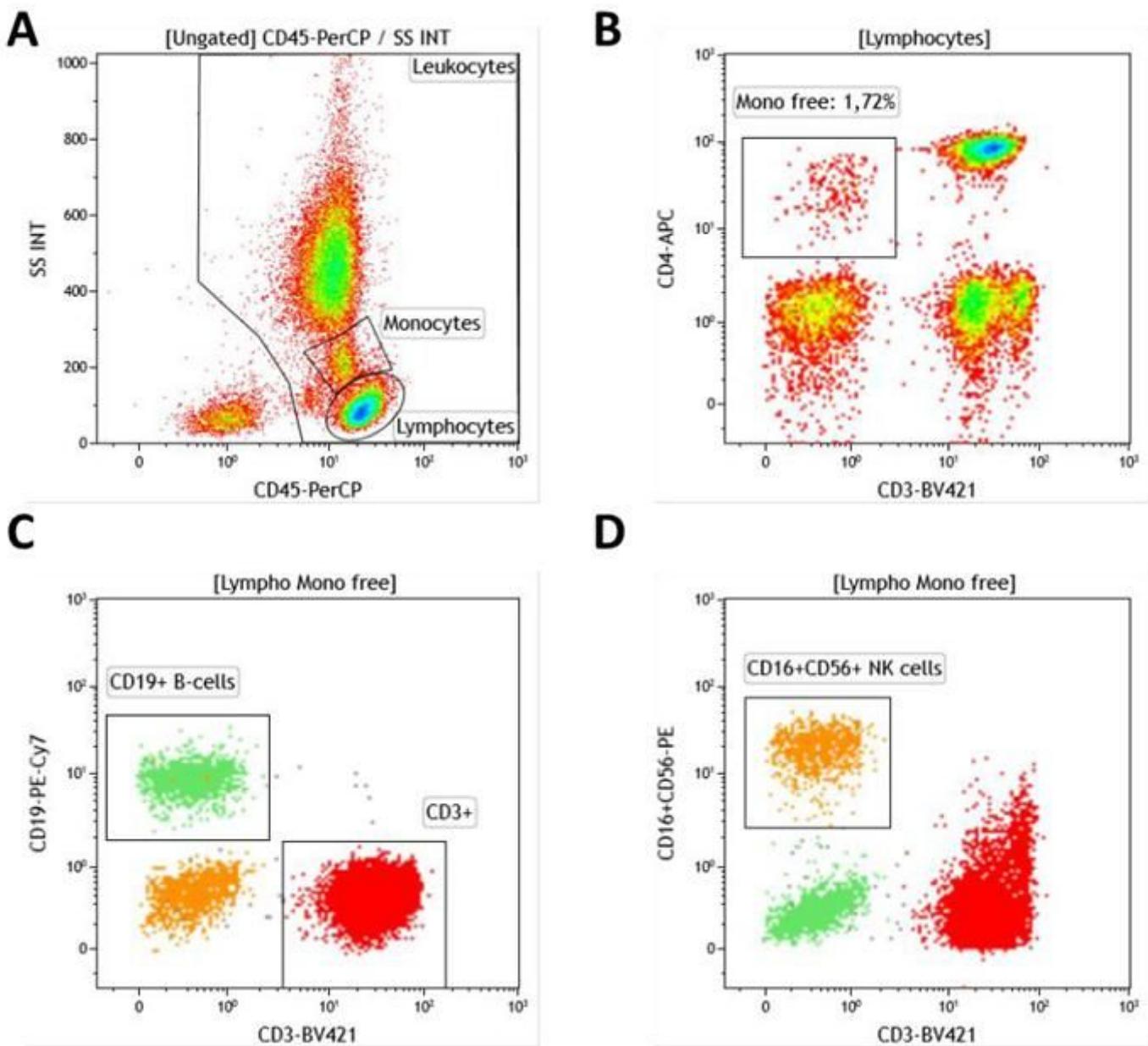


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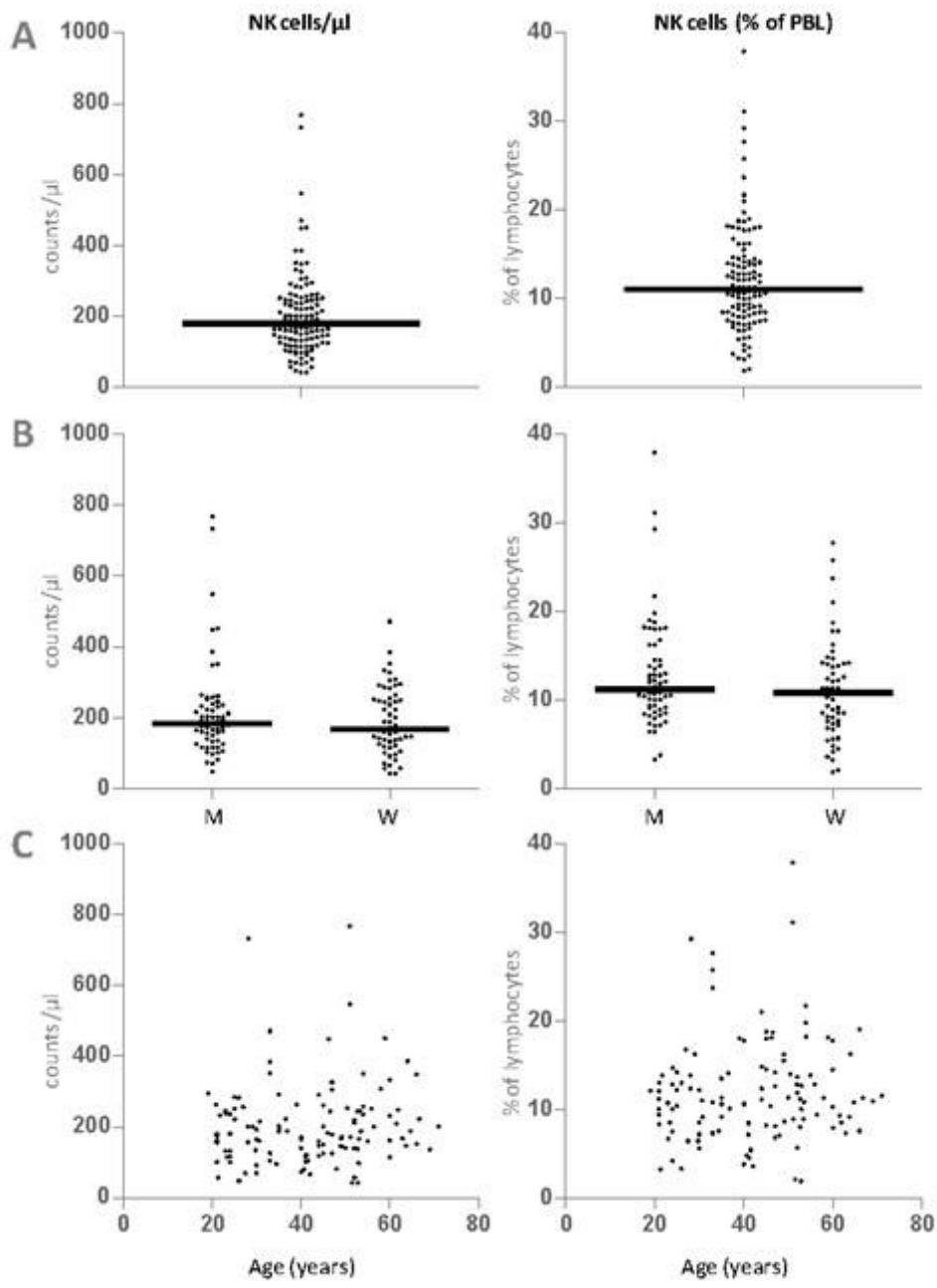


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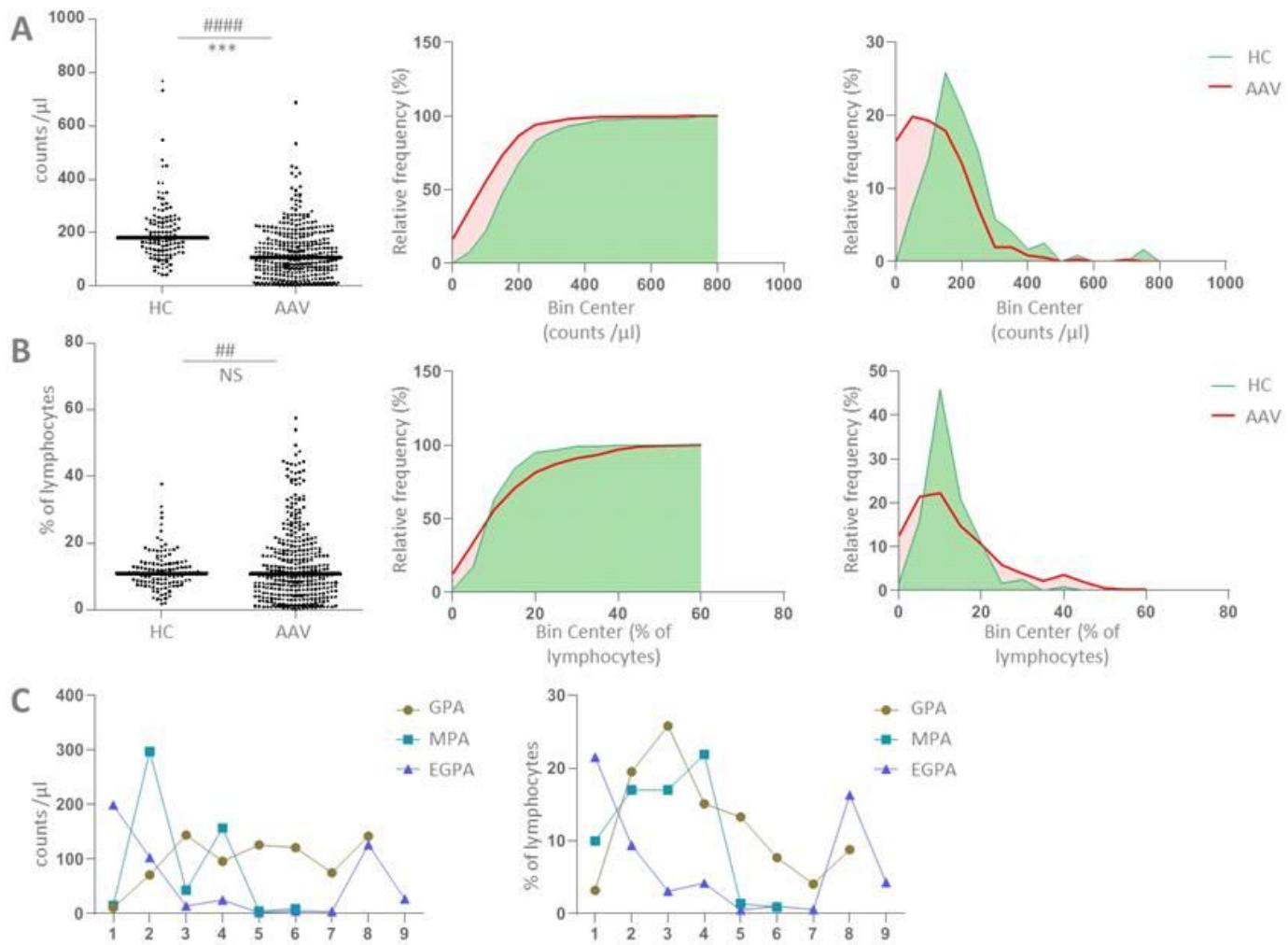


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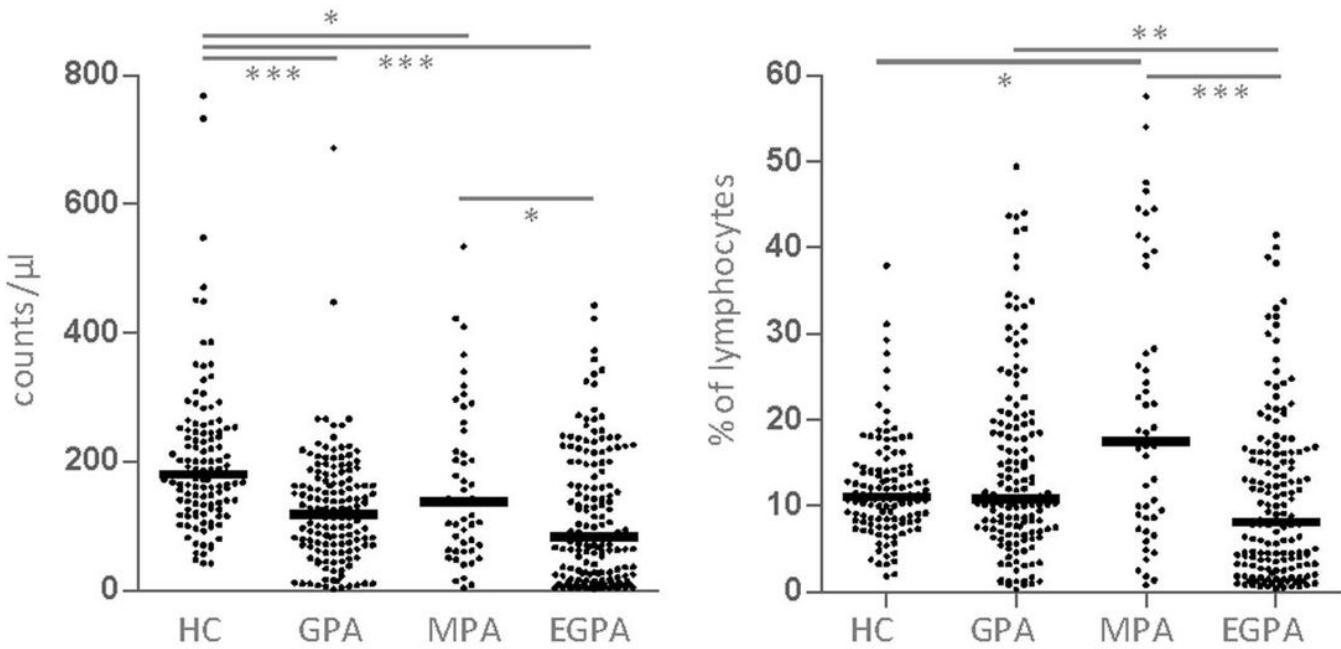


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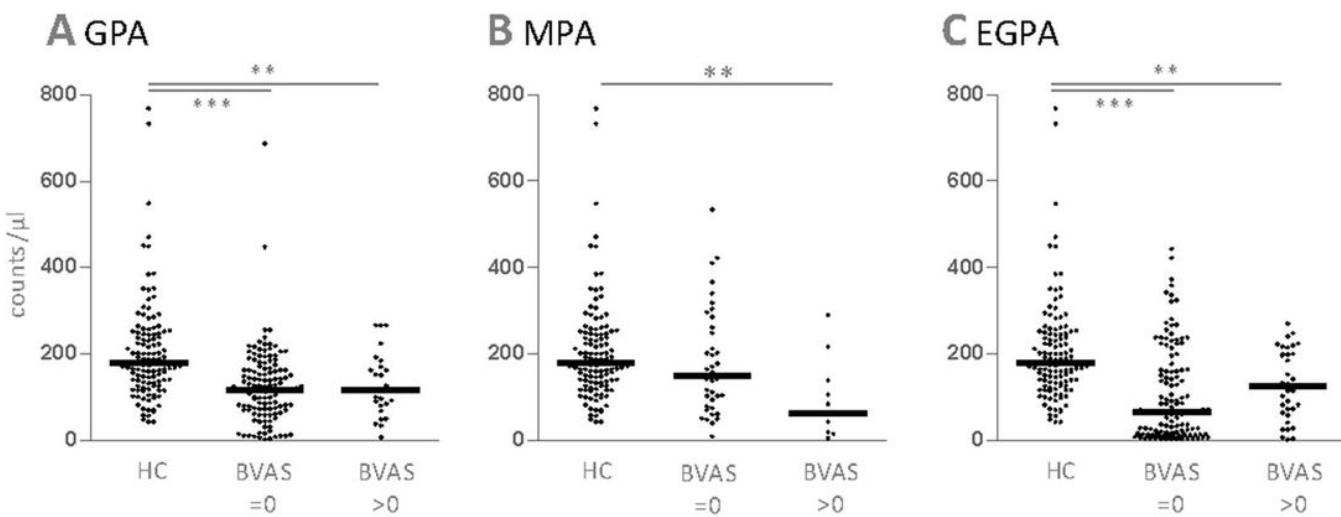


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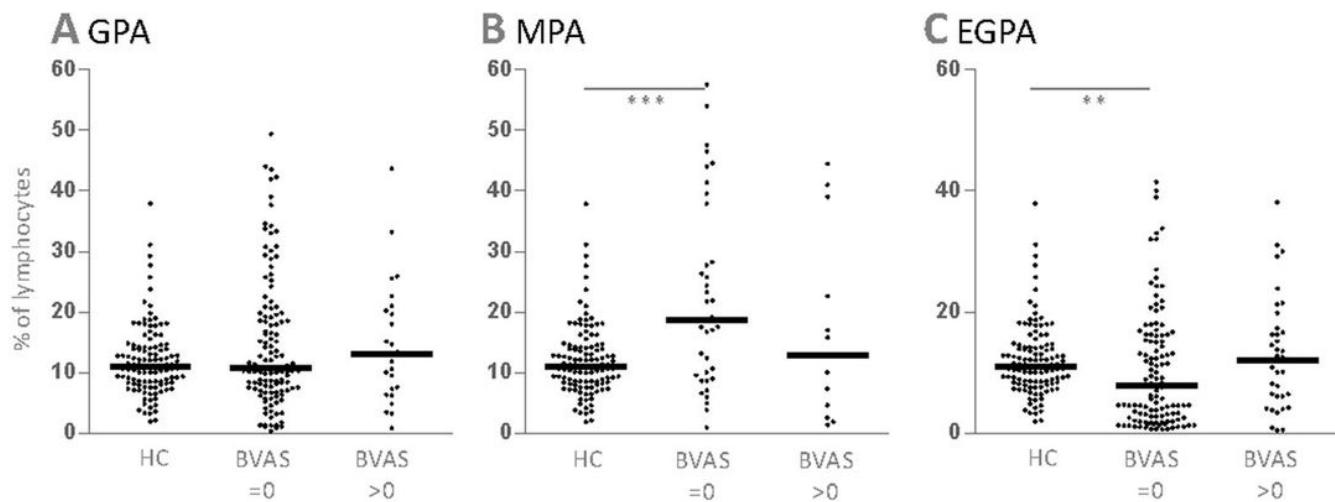


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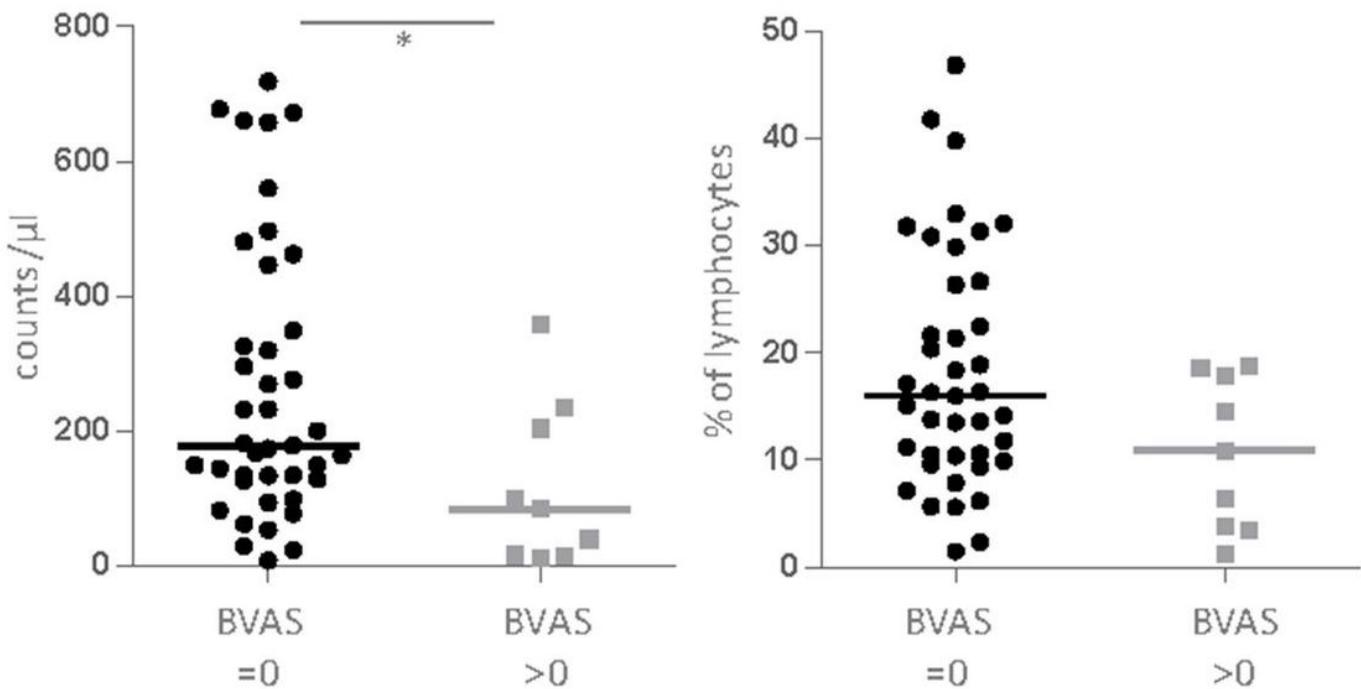


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