Arch Toxicol (2016) 90:2481–2495
DOI 10.1007/s00204-016-1809-5

IMMUNOTOXICOLOGY

Measuring the immune system: a comprehensive approach for the analysis of immune functions in humans

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Received: 17 June 2016 / Accepted: 4 August 2016 / Published online: 13 August 2016
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Abstract The immune system is essential to provide protection from infections and cancer. Disturbances in immune function can therefore directly affect the health of the affected individual. Many extrinsic and intrinsic factors such as exposure to chemicals, stress, nutrition and age have been reported to influence the immune system. These influences can affect various components of the immune system, and we are just beginning to understand the causalities of these changes. To investigate such disturbances, it is therefore essential to analyze the different components of the immune system in a comprehensive fashion. Here, we demonstrate such an approach which provides information about total number of leukocytes, detailed quantitative and qualitative changes in the composition of lymphocyte subsets, cytokine levels in serum and functional properties of T cells, NK cells and monocytes. Using samples from a cohort of 24 healthy volunteers, we demonstrate the feasibility of our approach to detect changes in immune functions.

Keywords Immunophenotyping · Functional analysis · Cytokines · Human

Electronic supplementary material The online version of this article (doi:10.1007/s00204-016-1809-5) contains supplementary material, which is available to authorized users.

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Introduction

The immune system provides protection from infections and transformed cells and is therefore essential for human health. It is distributed over the entire body, and cells of the immune system can be found in essentially every organ. The mobility of these cells, their dynamic interactions and their ability to change their phenotype and function during an immune response are just some of the hallmarks that distinguish the immune system from other organs in the body.

The importance and the essential function of the immune system to maintain health becomes clear by the pathologies which can be observed in individuals suffering from defects in the immune system. Many primary immune deficiencies (PIDs) result in the decrease or loss of specific immune cell types such as T, B or NK cells in SCIDs (Chinn and Shearer 2015), resulting in greatly enhanced susceptibility to various infections. However, there are also immune defects without any significant changes in cell count or percentage. These diseases are caused by defective cell functions, such as the lack of specific Ig subclasses due to B cell defects in several CVIDs (Saikia and Gupta 2016) or HLH caused by cytotoxic lymphocytes carrying mutations in their cytotoxic machinery (Filipovich and Chandrakasan 2015). The comprehensive analysis of immune function is a valuable tool in the diagnosis of these PIDs (Boldt et al. 2014).

The function of the immune system can also be influenced by multiple endogenous and exogenous factors such as gender and age (Giefing-Kroll et al. 2015), exposure to toxic compounds (Casey et al. 2015), stress (Webster Marketon and Glaser 2008), exercise (Walsh et al. 2011), nutrition (Veldhoen and Ferreira 2015), smoking (Huttunen et al. 2011) or infections (Doitsh and Greene 2016; Farooq and Bergmann-Leitner 2015; Weltevrede et al. 2016) with

sometimes dramatic consequences for the health of the affected individual.

During aging, the proportion of immature lymphocytes decreases while the percentage of cells expressing markers for activation and maturation was shown to increase (Al-Attar et al. 2016; Comans-Bitter et al. 1997; Hannet et al. 1992). As a consequence, success of vaccination decreases with aging, while susceptibility to infection is increased (Giefing-Kroll et al. 2015). Males seem to be more susceptible to many infectious diseases and cancer. After menopause, these effects get lost due to altered sex steroid-hormone levels. Receptors for sex hormones like estrogens, progesterone and androgens are expressed on many cells of the innate and adaptive immune system. As a consequence, immune function is influenced by alterations in sex hormone levels during maturation, pregnancy or during aging and immune senescence (Al-Attar et al. 2016; Giefing-Kroll et al. 2015).

Stress is another important modulator of the immune response. The effects are mainly mediated by the HPA axis and/or the sympathetic nervous system which control the release of stress hormones such as glucocorticoids and catecholamines (Scanzano and Cosentino 2015; Webster Marketon and Glaser 2008). Acute stress primarily induces the production of pro-inflammatory cytokines and the redistribution of neutrophils and NK cells to initiate the so-called flight or fight response. In contrast, chronic stress dampens immune reactions like T cell proliferation and NK cell cytotoxicity, and accelerates immune senescence (Segerstrom and Miller 2004). Further, sensitivity of immune cells to glucocorticoid stimulation is modulated by the chronic exposition to stress hormones (Rohleder et al. 2003).

Exposure to toxic compounds can also affect the immune system. For example, Bisphenol A, Phthalates and Dibutyltin are plasticizing agents that are known to affect function of NK cells, T cells and macrophages (Celada and Whalen 2014; Robinson and Miller 2015). Cigarette smoke contains the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which was shown to inhibit the cytotoxic functions of macrophages in the lung (Proulx et al. 2007).

Compared to the alterations of the immune system observed in genetically caused primary immune deficiencies, the effects of the extrinsic and intrinsic factors discussed above on the immune system are usually much more moderate. This can provide a challenge when trying to define the changes in immune function caused by factors such as aging, stress or exposure to chemicals. Therefore, broad analysis of immune parameters is necessary to gain reliable data about immune function. Here, we describe a comprehensive approach which provides information about total number of leukocytes, quantitative and qualitative changes in the composition of lymphocyte subsets, cytokine levels in serum and functional properties of T cells, NK cells and monocytes.

Materials and methods

Cells

Unless stated otherwise, all media and supplements were purchased from Thermo Fisher Scientific. The human MHC-I deficient erythroleukaemic cell line K562 was maintained in IMDM containing 10 % FCS and 1 % penicillin/streptomycin at 37 °C in a humidified 5 % CO_2 incubator. Cells were grown to mid-log phase prior to use as targets in cytotoxicity assays.

Peripheral venous blood was collected in heparinized tubes and serum monovettes (Sarstedt, Germany) from healthy donors. Each subject gave written informed consent for participation in the study after the nature and possible consequences of the studies had been fully explained. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll density gradient centrifugation (PAN-Biotech, Germany). Cells were washed twice with PBS. At least 10×10^6 cells per aliquot were resuspended in 1 ml FCS supplemented with 10 % DMSO and stored at −170 °C for 1–6 months.

For functional analyses, PBMC were thawed and cultured over night at a density of $1.5–3 \times 10^6$ cells/ml in IMDM supplemented with 10 % human serum, 1 % non-essential amino acids, 1 % sodium pyruvate, 1 % penicillin/streptomycin at 37 °C in a humidified 5 % CO_2 incubator.

Serum cytokine levels

Serum monovettes were stored for 30 min at RT to allow clotting, and then centrifuged. Serum supernatant was harvested and centrifuged at 10,000×*g* to remove platelets and aggregates. Samples were aliquoted and stored at −80 °C. Cytokine levels in serum were measured using the Bio-Plex Pro Human Cytokine 17-Plex Panel (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions.

Glucocorticoid sensitivity assay/ELISA

Heparinized whole blood samples were stimulated for 3 h at 37 °C with 1 µg/ml LPS and increasing amounts of Dexamethasone, ranging from 0 to 10,000 nM. Then, samples were centrifuged and plasma supernatant was aliquoted and stored at −80 °C. Production of IL-6 and TNF-α was measured using the ELISA MAX™ Deluxe Sets from

BioLegend according to manufacturer's instructions. Data were analyzed for TOP and BOTTOM plateaus of cytokine concentration and EC_{50} of Dexamethasone using the nonlinear regression tools of the GraphPad Prism software.

Absolute cell count

To determine the absolute number of leukocyte populations, 50 µl fresh heparinized blood sample was added to TruCount tubes (BD Biosciences) and incubated for 20 min at RT with the indicated antibodies (Table 1). Samples were subjected to erythrocyte lysis using FACS Lysing Solution (BD Biosciences, Heidelberg, Germany) and were measured on a BD LSRFortessa (BD Biosciences). Data were analyzed using FlowJo Software (FlowJo LLC, USA). Absolute cell number in blood samples was calculated as

$$\frac{\#\,\text{events in cell gate}}{\#\,\text{events in bead gate}} \times \frac{\#\,\text{beads per test}}{\text{test volume}} = \text{cells per µl blood}$$

Multicolor flow cytometry

Five different panels were set up to analyze the samples for (1) general leukocyte overview, (2) B cell subpopulations, (3) T cell memory, Treg and homing markers, (4) NK activation and memory, and T cell activation and (5) activating NK cell receptors. All antibodies were individually titrated to determine the optimal dilution. All antibodies and dilutions are listed in Table 1. PBMC were used immediately after thawing and were kept on ice during the staining procedure. For each panel, 0.2×10^6 cells were stained with the indicated antibody cocktails for 20 min at 4 °C in the dark and then washed with FACS buffer (PBS/2 % FCS). Cells were resuspended in 150 µl FACS buffer and kept on ice until analysis at the same day on a BD LSRFortessa. Data were analyzed using the FlowJo software (FlowJo LLC, USA).

Degranulation assay

To determine the degranulation of NK cells in response to stimulation with tumor cells 2×10^5 PBMC in 200 µl assay medium (IMDM with 10 % FCS and 1 % penicillin/streptomycin) were dispensed in Falcon 2054 tubes. Then, 5 µl PE-Cy5-conjugated anti-CD107a mAb or isotype control was added and cells were incubated with 2×10^5 K562 target cells for 3 h at 37 °C in a humidified 5 % CO_2 incubator. Control samples were incubated without target cells to detect spontaneous degranulation. Thereafter, samples were stained on ice with FITC-conjugated anti-CD3 and PE-conjugated anti-NKp46 mAbs, followed by flow cytometric analysis on a BD LSRFortessa. Data were analyzed using FlowJo software.

^{51}Cr release assay

K562 target cells were labeled in 100 µl assay medium (IMDM with 10 % FCS and 1 % penicillin/streptomycin) with 100 µCi ^{51}Cr (Hartmann Analytic, Braunschweig, Germany) for 1 h at 37 °C in a humidified 5 % CO_2 incubator. Cells were washed twice and resuspended at 5×10^4 cells/ml in assay medium. Five-thousand target cells per well were used in the assay. PBMC were distributed on a U-bottom 96-well plate and mixed with labeled target cells at different effector-to-target ratios typically ranging from 6.25/1 to 50/1 per well. Maximum ^{51}Cr release was determined by incubating target cells in 1 % Triton X-100. For spontaneous release, targets were incubated without effectors in assay medium alone. All samples were performed in triplicates. Plates were incubated for 4 h at 37 °C, and supernatant was harvested. ^{51}Cr release was measured in a gamma counter. Percent specific release was calculated as

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100 = \%\ \text{specific lysis}$$

T cell proliferation

PBMC were loaded with 0.5 µM CFSE, washed and resuspended in IMDM with 10 % FCS and 1 % penicillin/streptomycin. Cells were cultured for 6 days with or without 4 µg/ml PHA-P. T cells were then stained with AF647-labeled anti-CD3 antibody, and cells were analyzed for CSFE dilution in CD3 positive events. T cell proliferation was analyzed using the Proliferation analysis platform of the FlowJo software.

Results

To establish our approach for the comprehensive analysis of immune functions, we analyzed samples from 24 healthy volunteers. First we analyzed leukocyte numbers in peripheral blood, as these can change during infections, upon environmental influences or during aging, due to homing into tissues, altered cell survival or changes in development. We used TruCOUNT™ Tubes to assess the total number of lymphocytes and monocytes in whole blood via FACS (Fig. 1). Beads, monocytes and lymphocytes were identified by SSC and fluorescence or CD45 characteristics. Beads were subgated in a second fluorescence channel to eliminate contaminating events. Staining for CD3 and CD56 was used to identify T cells, NK cells and $CD56^+$ T cells in the lymphocyte gate. B cells were defined as $CD19^+$ $CD3^-$ lymphocytes. Absolute number

Table 1 Panel description, antigens, antibody clones and coupled fluorochromes, distributors and Ab dilution used to stain 0.2×10^6 PBMC are listed

Panel	Antigen	Clone	Fluorochrome	Company	Dilution 1/*x*
Lymphocytes/monocytes	CD19	HIB19	BV421	BD Horizon™	200
	CD3	UCHT1	BV510	BD Horizon™	400
	CD16	3G8	FITC	BD Pharmingen™	200
	HLA-DR	G46-6	PerCP-Cy5.5	BD Horizon™	100
	CD14	MφP9	PE	BD Pharmingen™	500
	CD64	10.1	PE-Cy7	BD Pharmingen™	200
	CD56	B159	APC	BD Pharmingen™	50
	CD45	HI30	AF700	BD Pharmingen™	500
B cells	CD19	HIB19	BV421	BD Horizon™	200
	CD138	MI15	BV510	BD Horizon™	100
	IgD	IA6-2	FITC	BD Pharmingen™	50
	CD27	M-T271	PerCP-Cy5.5	BD Pharmingen™	100
	CD21	B-ly4	PE	BD Pharmingen™	200
	CD38	HIT2	PE-Cy7	BD Pharmingen™	100
	IgM	G20-127	APC	BD Pharmingen™	50
	CD45	HI30	AF700	BD Pharmingen™	500
NK + T cells + Treg + memory + homing	CD62L	DREG-56	BV421	BD Horizon™	200
	CD3	UCHT1	BV510	BD Horizon™	400
	CD8	RPA-T8	FITC	BD Pharmingen™	200
	CD28	CD28.2	PerCP-Cy5.5	BD Pharmingen™	100
	CD197	150503	PE	BD Pharmingen™	100
	CD127	HIL-7R-M21	PE-CF594	BD Pharmingen™	100
	CD25	M-A251	PE-Cy7	BD Pharmingen™	100
	CD56	B159	APC	BD Pharmingen™	50
	CD45RA	HI100	AF700	BD Pharmingen™	400
	CD4	RPA-T4	APC-H7	BD Pharmingen™	400
NK + T activation + memory	KLRG1	2F1	BV421	BD Horizon™	400
	CD3	UCHT1	BV510	BD Horizon™	400
	NKG2C	134591	AF488	R&D Systems	100
	CD56	B159	PerCP-Cy5.5	BD Pharmingen™	50
	CD57	NK-1	PE	BD Pharmingen™	800
	CD25	M-A251	PE-Cy7	BD Pharmingen™	100
	GITR	621	APC	BioLegend	100
	CD69	FN50	APC-H7	BD Pharmingen™	100
NK receptors	CD56	B159	BV421	BD Pharmingen™	50
	CD3	UCHT1	BV510	BD Horizon™	400
	DNAM-1	DX11	FITC	BD Pharmingen™	50
	NKp44	p44-8.1	PE	BD Pharmingen™	200
	2B4	C1.7	PE-Cy5.5	Beckman Coulter	200
	NKp46	9E2/Nkp46	PE-Cy7	BD Pharmingen™	100
	NKp30	p30-15	AF647	BD Pharmingen™	200
	NKG2D	149810	AF700	R&D Systems	100
TruCount	CD19	HIB19	FITC	BD Pharmingen™	25
	CD56	B159	PE	BD Pharmingen™	25
	CD3	SK7	PerCP	BD	25
	CD45	HI30	APC	BD Pharmingen™	25

Table 1 continued

Panel	Antigen	Clone	Fluorochrome	Company	Dilution 1/*x*
Degranulation	CD107a	H4A3	PE/Cy5	BD Pharmingen™	67
	CD3	HIT3a	FITC	BioLegend	100
	NKp46	9E2	PE	BioLegend	100
Proliferation	CD3	HIT3a	AF647	BioLegend	200

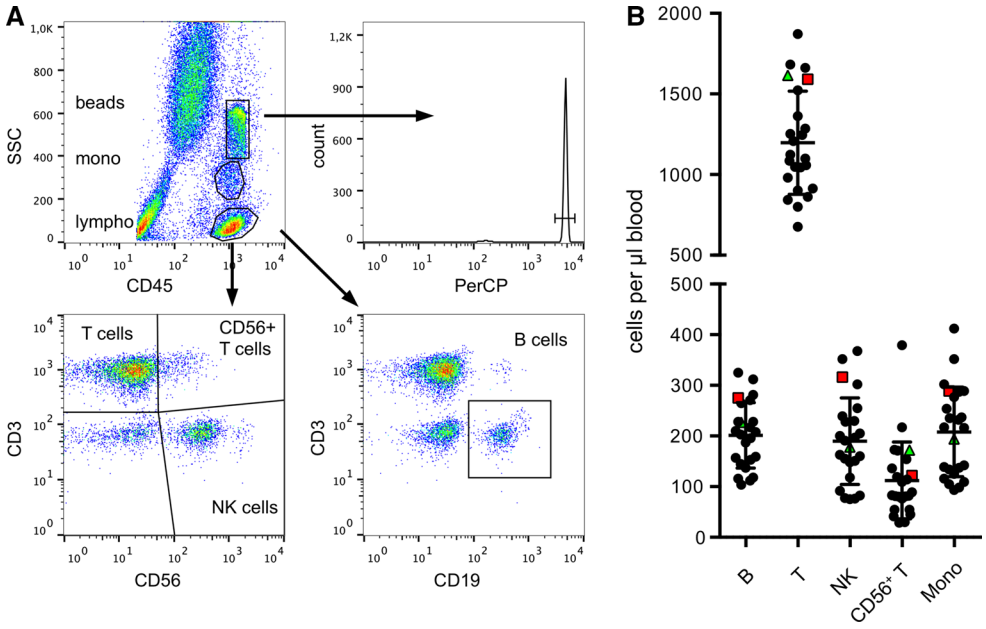

Fig. 1 Determination of total cell count in whole blood using BD TruCount™ Tubes. **a** Lymphocytes, monocytes and beads are identified by CD45 or fluorescence and SSC features. Beads are gated in a second fluorescence channel to exclude contaminating events. T cells ($CD3^+$), NK cells ($CD56^+$), CD56pos T cells ($CD56^+$, $CD3^+$) and B cells ($CD19^+$) are determined in the lymphocyte gate. **b** Total cell count was calculated as described in materials and methods. Mean ± SD with data from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

of cells was calculated by using the known concentration of beads in the sample as internal standard. Absolute numbers of analyzed leukocyte populations show great variability; however, all values lie within the range of published data (Comans-Bitter et al. 1997; Hannet et al. 1992).

To provide detailed information of various leukocyte subpopulations, we created five different antibody panels for multicolor FACS analysis with up to ten different fluorescently labeled antibodies per panel (Table 1). Panel 1 (leukocyte overview) gives an overview over the general lymphocyte subsets and common monocyte subpopulations. Lymphocytes and monocytes were identified by CD45 expression and SSC features. Gating strategy and statistics for all 24 healthy donors are shown in Fig. 2. We found 63.2 ± 9.9 % lymphocytes and 18.3 ± 6.0 % monocytes in thawed PBMC samples. Classical monocytes were identified as $CD14^+$ and $CD64^+$. As expected for healthy donors, classical monocytes were found at a high percentage (84.5 ± 5.8). Additionally, the expression level of HLA-DR was determined on all $CD14^+$ monocytes. B cells were subgated from the lymphocyte gate based on the expression of CD19 (12.7 ± 3.5 %), T cells (67.8 ± 7.4 %), NK cells (9.7 ± 3.9 %) and $CD56^+$/$CD3^+$ cells (5.05 ± 4.3 %) cells were identified by their expression of CD3 and/or CD56 (Fig. 2e). Among NK cells, 92.0 ± 4.9 % were $CD56^{dim}$ and $CD16^+$.

Panel 2 (B cell subsets) was designed to quantify common B cell subsets and developmental stages, which were shown to be affected in various diseases (Boldt et al. 2014; Kaminski et al. 2012a). Gating strategy and statistics are shown in Fig. 3. B cells were identified by CD45/CD19 expression (Fig. 3a). Immature (3.5 ± 1.8 %), naive (67.6 ± 10.9 %), memory (26.3 ± 10.3 %) and $CD21^{low}$ B cells (2.6 ± 1.2 %) were gated based on the expression of CD21 and CD27 (Fig. 3b, g). Memory B cells can be further divided into class-switched (49.8 ± 12.2 %) and non-switched cells (36.1 ± 11.5 %) by staining for IgM and IgD (Fig. 3c). Activated $CD21^{low}$/$CD38^{low}$ B cells were shown to be related to several immune defects (Thorarinsdottir et al. 2015). In our healthy donors, only low amounts of these cells were found (2.8 ± 1.1 %) (Fig. 3d). The combination of CD38 and IgM allows the discrimination between immature transitional B cells ($CD38^{++}$, IgM^+) (2.3 ± 1.4 %) and plasmablasts (2.5 ± 1.1 %), which do not express IgM (Fig. 3e). Residual plasma cells in peripheral blood can be separated from plasmablasts by $CD138^{+++}$ (Fig. 3f).

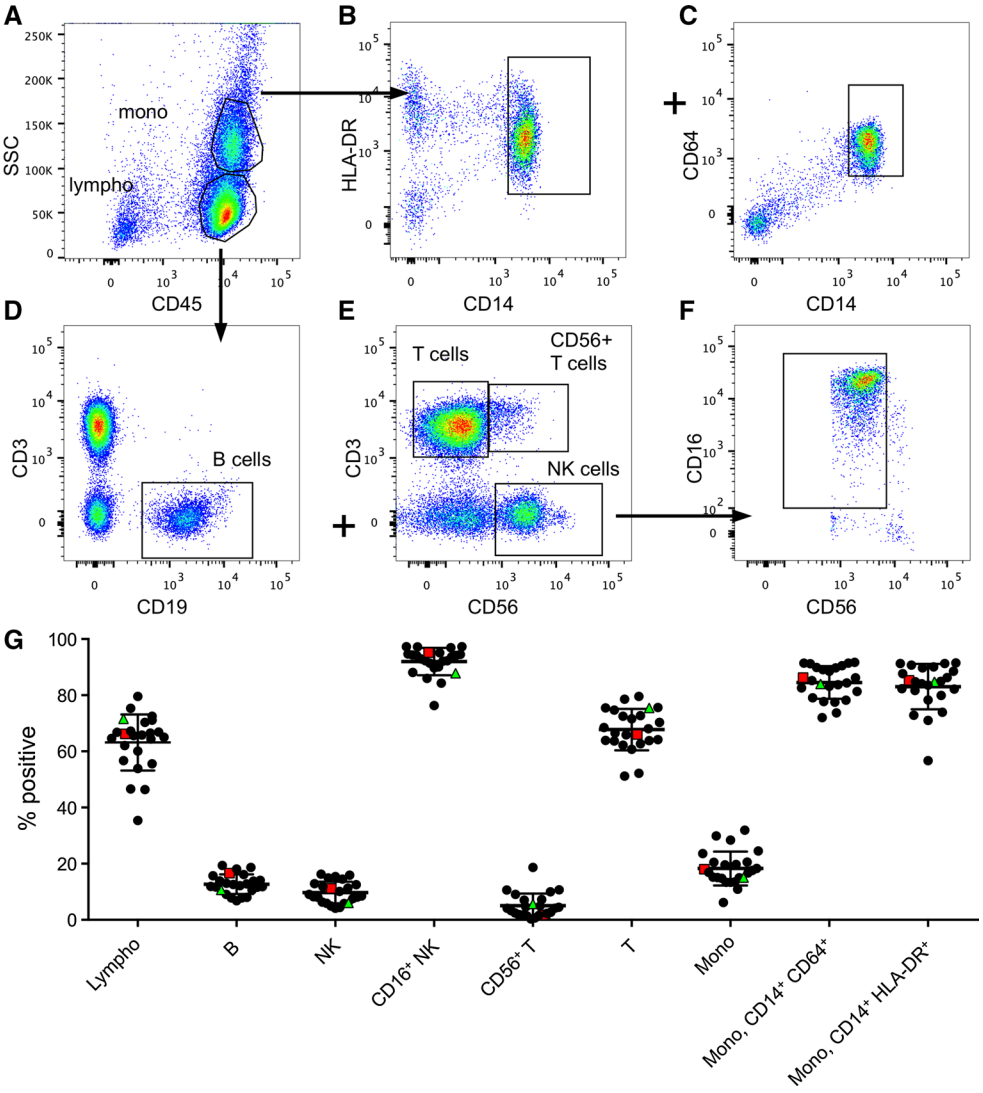

Fig. 2 General overview of lymphocyte and monocyte subpopulations. **a** Lymphocytes and monocytes are identified by CD45 and SSC characteristics. Based on CD14 gating, HLA-DR$^+$ (**b**) and classical CD64$^+$ (**c**) monocytes can be identified. Lymphocytes were further subgated to identify B cells (CD19$^+$) (**d**), T cells (CD3$^+$), CD56pos T cells (CD56$^+$, CD3$^+$), NK cells (CD56$^+$) (**e**) and CD16 positive CD56^{dim} NK cells (**f**). **g** Mean ± SD with data from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

The gating strategy and statistics for panel 3 (T cell subsets) is shown in Fig. 4. T cells were subgated from the lymphocyte gate and separated from NK cells as CD3$^+$ CD56$^-$ events (Fig. 4a). CD4 and CD8 co-staining (Fig. 4c) allowed the discrimination of CD4$^+$ T helper and CD8$^+$ cytotoxic T cells. In accordance with the literature, we found 71.7 ± 8.1 % CD4$^+$ and 22.7 ± 8.2 % CD8$^+$ T cells. Further, immunoregulatory double-negative T cells (Hillhouse and Lesage 2013) were quantified. In our study, only very few of these cells were found (4.7 ± 3.6 %). Within the CD4$^+$ and CD8$^+$ subsets, CD45RA was used to separate naive CD45RA$^+$ (50.6 ± 8.2 % for CD4$^+$, 60.7 ± 8.4 % for CD8$^+$) from memory cells. Based on the expression of CD62L (L-selectin) or CD197 (CCR7), memory T cells can be further subdivided into central and effector memory cells (Fig. 4d, e, i) (Sallusto et al. 1999). CD28 acts as costimulatory receptor for the TCR. In particular, CD4$^+$ CD28$^-$ T cells have been correlated with immunosenescence and might play a role in rheumatoid arthritis and other inflammatory diseases (Mou et al. 2014). In our cohort, we found 0.92 ± 0.75 % CD4$^+$ and 15.8 ± 8.7 % CD8$^+$ T cells lacking CD28 expression (Fig. 4f, g). Regulatory T cells (T_{reg}) (6.2 ± 1.4 %) were identified as CD4$^+$, CD25^{++}, CD127^{low} (Mahnke et al. 2013) (Fig. 4h). Since CD3 and CD56 were used to identify T cells, we were also able to analyze CD3$^-$ CD56$^+$ NK cells in the same sample for the expression of the homing markers CD62L and CD197 (Fig. 4b). CD62L was found on 43.4 ± 11.6 % of all NK cells. CD197 is exclusively expressed on CD56^{bright} NK cells (2.7 ± 1.3 %).

Alterations in immune function are often accompanied by changes in the activation status of immune cells or result in altered maturation (Hannet et al. 1992). Panel 4 (NK and T cell activation and maturation) was designed to monitor the expression of selected markers for NK cell and T cell maturation and activation. Gating strategy and

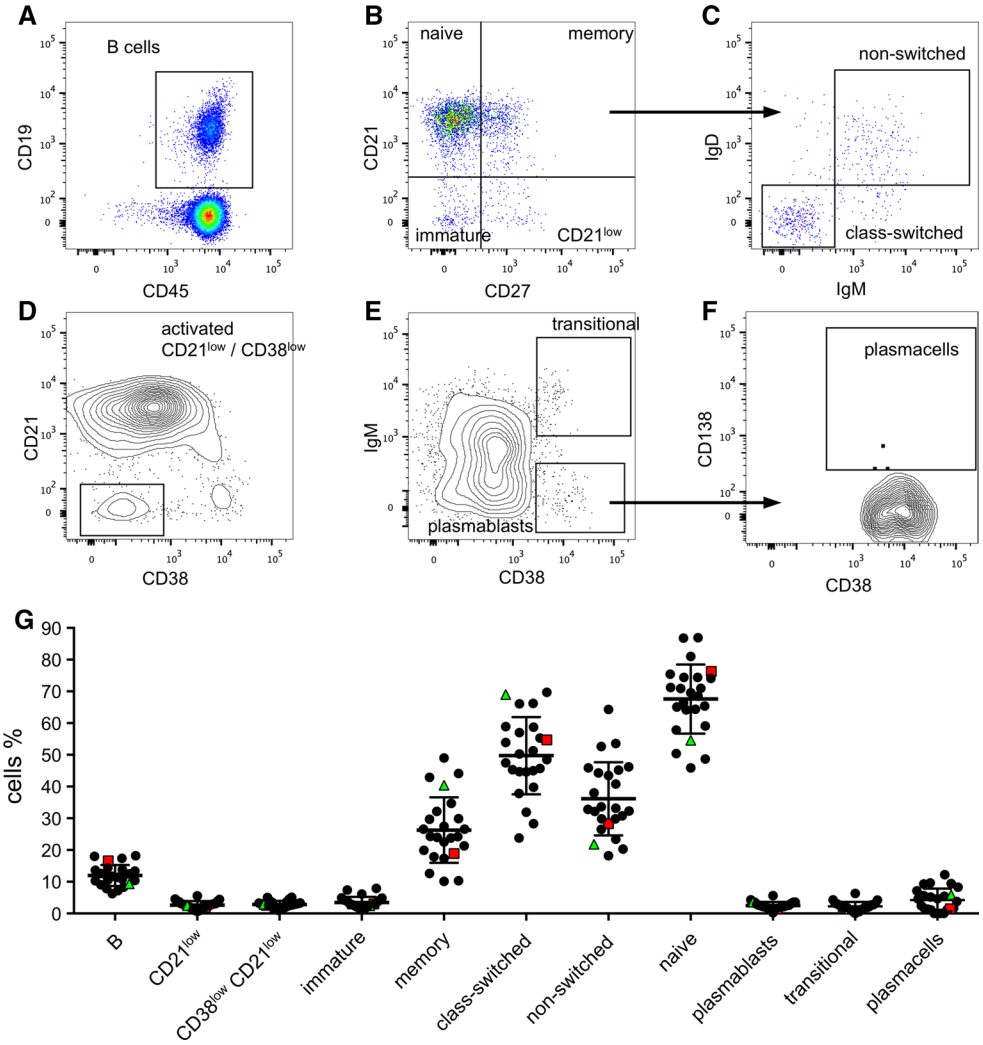

Fig. 3 B cell subpopulations. **a** B cells were identified by CD45 and CD19 expression. **b** Immature, naive, memory and $CD21^{low}$ B cells were identified by CD21/CD27 subgating, and class-switched versus non-switched memory B cells were separated by IgM and IgD expression based on the memory B cell gate (**c**). **d** Activated $CD21^{low}$ $CD38^{low}$ B cells were identified by CD21 and CD38 staining from the B cell gate. **e** $CD38^{+++}$ transitional B cells and plasmablasts were discriminated by the expression level of IgM, and plasma cells ($CD138^{+}$) were identified by further subgating from plasmablasts (**f**). **g** Mean ± SD of data from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

statistics are shown in Fig. 5. NK cells and T cells were subgated from lymphocytes by their expression of CD3 and CD56 (Fig. 5a). CD57 (62.6 ± 11.4 %) and KLRG1 (45.5 ± 14.9 %) have been correlated with the late stages of NK cell maturation and are also found on senescent T cells (Geiger and Sun 2016; Kared et al. 2016) (CD57: 10.9 ± 4.7 %; KLRG1: 12.5 ± 5.2 %). The activating receptor NKG2C (20.5 ± 8.7 %) has been associated with CMV reactivation and NK cell memory (Cerwenka and Lanier 2016). CD69 and GITR are upregulated on activated cells (Clausen et al. 2003; Li et al. 2003) and are only weakly expressed on the resting cells studied here (Fig. 5d). CD25 is expressed on activated NK and T cells, but is also found on T_{reg} and memory T cells (Andre et al. 2004; Letourneau et al. 2009) (Fig. 5d).

NK cell activation often results in modulation of receptor expression (Sandusky et al. 2006). Therefore, panel 5 (NK receptors) was designed to monitor expression levels of a variety of activating NK cell receptors. NCRs such as NKp30 (MFI 847 ± 310) and NKp46 (MFI 718 ± 188) are expressed at varying levels on all NK cells (Fig. 6b, c). In contrast, NKp44 is upregulated upon activation and is nearly absent on the resting cells studied here (Fig. 6b). DNAM-1 was recently described to be involved in the generation of NK cell memory in the mouse (Nabekura et al. 2014) and was expressed on all NK cells, although at varying levels (MFI 1097 ± 406). 2B4 (MFI 449 ± 102) and NKG2D (MFI 769 ± 135) are more uniformly expressed on all NK cells. The panel also enables the analysis of receptor expression on T cells. While NCRs are exclusively expressed on NK cells, different T cell subsets expressed also DNAM-1 (33.1 ± 8.6 %), NKG2D (28.9 ± 7.3 %) and 2B4 (10.2 ± 4.3 %) (Fig. 6d, e).

Not only the number of immune cells and the composition of subsets can be affected, but also the function of immune cells can be influenced by extrinsic and intrinsic factors. We therefore established test systems to measure functional T, NK and monocyte responses. T cell proliferation was measured by a flow cytometry-based CFSE dilution assay. PBMC were loaded with CFSE and cultured in

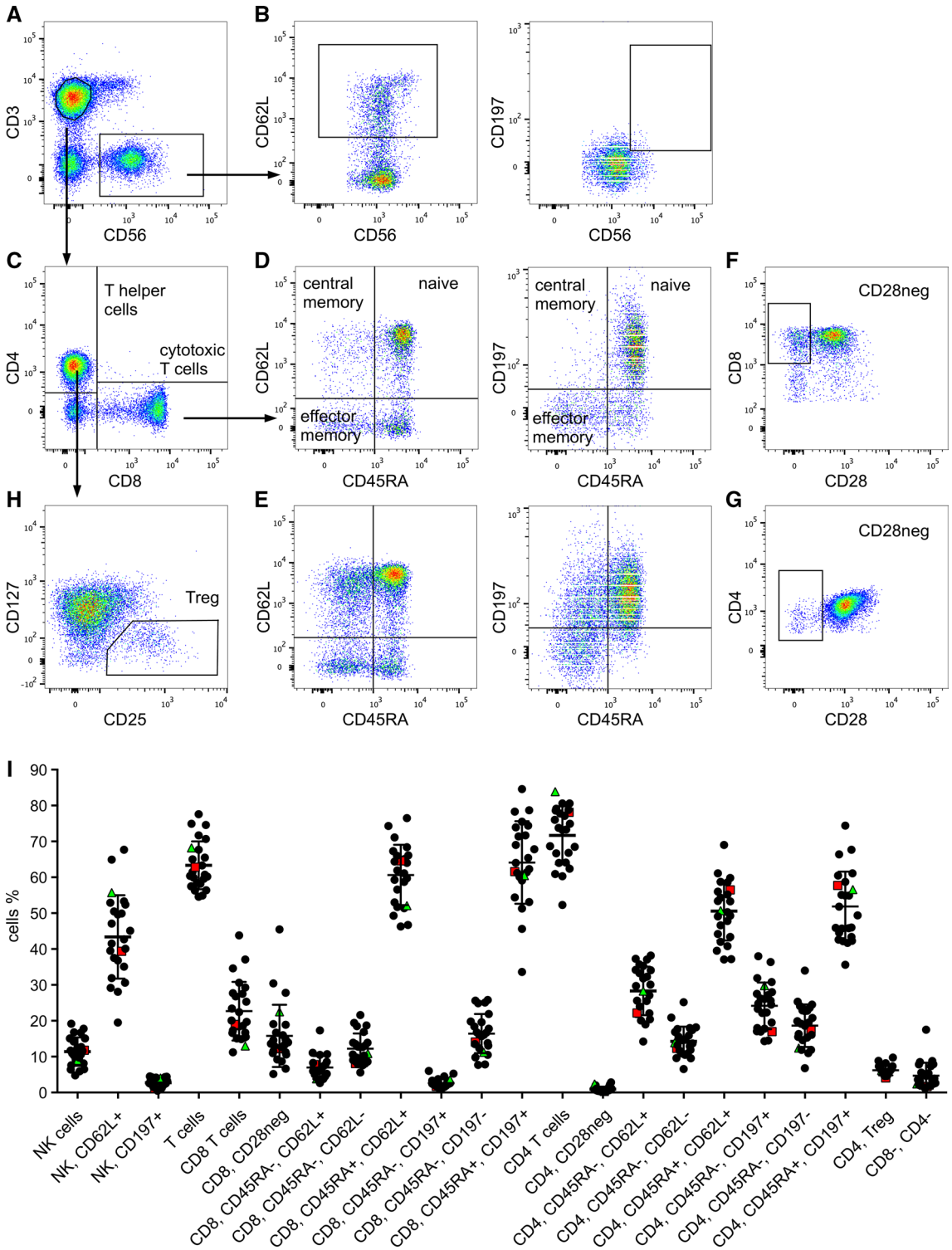

Fig. 4 T cell subpopulations. **a** NK and T cells were separated by CD3 and CD56 expression. **b** Expression of homing markers CD62L (L-selectin) and CD197 (CCR7) on NK cells. **c** T helper cells and cytotoxic T cells were identified by CD4 and CD8 expression. Naive, central and effector memory cells were separated from CD8 (**d**) and CD4 (**e**) T cells by CD45RA and CD62L or CD197 staining. **f**, **g** CD28 negative CD8 and CD4 T cells. **h** $CD4^+$ regulatory T cells (T_{reg}) can be identified by $CD25^{++}$ and $CD127^{low}$ expression. **i** Mean ± SD of data from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

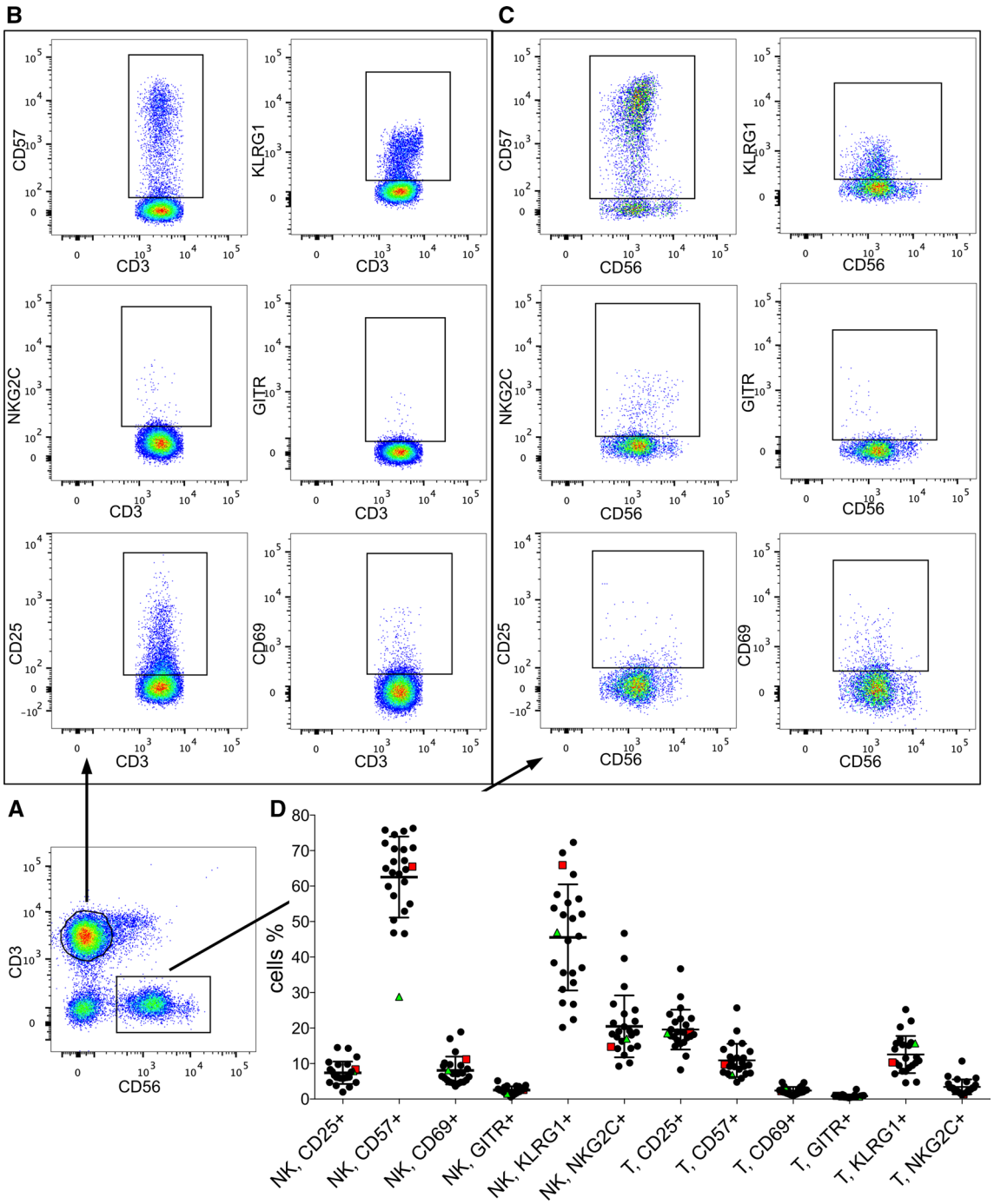

Fig. 5 NK and T cell activation and maturation. **a** NK and T cells were separated by CD3 and CD56 expression. T cells (**b**) and NK cells (**c**) were analyzed for the expression of maturation markers CD57, NKG2C and KLRG1, and the activation markers CD25, CD69, and GITR. **d** Mean ± SD of data from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

the absence or presence of PHA-P for 6 days. T cells were identified by CD3 staining, and samples were analyzed for CSFE dilution in CD3 positive events (Fig. 7a). T cell proliferation was calculated using the proliferation analysis platform of the FlowJo software. Data are usually presented as % divided precursors. However, further analyses can help to interpret the data in more detail. The cell division index gives the average number of cell divisions that one cell in the initial population has undergone. The cell division index includes also cells which never divided. Only

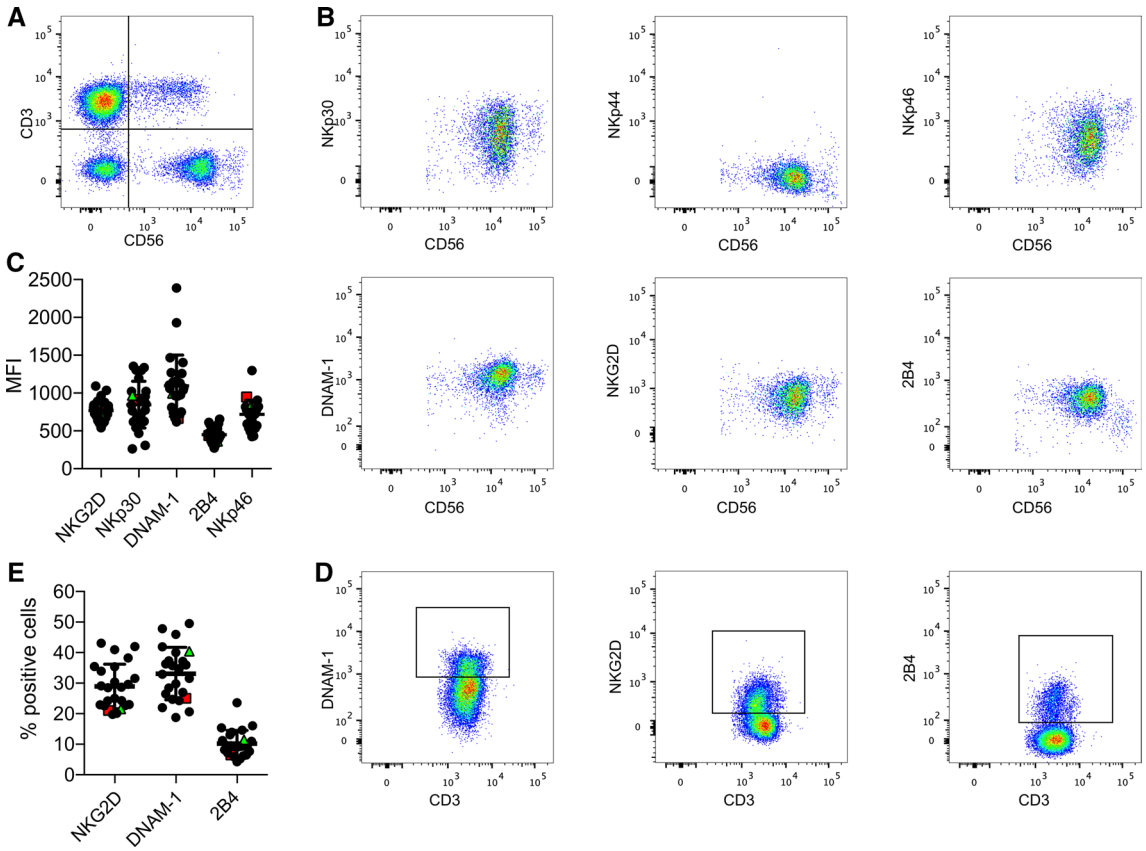

Fig. 6 NK cell receptors. **a** NK and T cells were separated by CD3 and CD56 expression. **b** Expression levels of activating NK cell receptors were determined on NK cells. **c** Scatter plots, mean ± SD of MFI of NK receptors from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. **d** T cells positive for DNAM-1, NKG2D and 2B4 were identified. **e** Mean ± SD of 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

dividing cells are considered in the proliferation index. The proliferation index gives the total number of divisions divided by the number of cells that actually had undergone division (Fig. 7a).

NK cells recognize and kill infected or transformed cells. Activated NK cells form a tight contact with the target cell. They polarize lytic granules toward the immunological synapse and release cytolytic proteins toward the target cell. Thereby, they present CD107a at the cell surface (Orange 2008). NK cell cytotoxicity was analyzed in two ways. NK cell degranulation toward tumor target cells was detected by up-regulation of cell surface CD107a by flow cytometry (Fig. 7b, d). Lysis of target cells by increasing numbers of NK cells was determined in a standard 4 h ^{51}Cr release assay (Fig. 7c, d). Both methods showed the low cytotoxic activity of resting NK cells, but also demonstrated the high variability between donors (Fig. 7d).

Upon triggering with LPS, monocytes produce proinflammatory cytokines like IL-6 and TNF-α. Glucocorticoids inhibit monocyte function, but chronic triggering of glucocorticoid receptors induces adaptation and reduced glucocorticoid sensitivity. To determine monocyte function and glucocorticoid sensitivity, whole blood samples were stimulated with LPS in the presence of increasing amounts of the synthetic glucocorticoid Dexamethasone. Production of IL-6 and TNF-α was measured and data were analyzed for top and bottom plateaus of cytokine concentration and logEC_{50} of Dexamethasone (Fig. 7e, f). While logEC_{50} was more stable (IL-6: −7.49 ± 0.2 M; TNF-α: −7.55 ± 0.2 M), top plateaus (IL-6: 21.357 ± 10.176 pg/ml; TNF-α: 2.732 ± 1.189 pg/ml) and bottom plateaus (IL-6: 4.560 ± 2.500 pg/ml; TNF-α: 483 ± 241 pg/ml) showed high variability among donors (Fig. 7f).

Cytokine levels in serum illustrate the immune status with regard to a more pro- or anti-inflammatory milieu. In the healthy subjects studied here, most analytes were below detection range. However, stronger expressed MIP-1β (193.3 ± 62.4 pg/ml) or the proinflammatory cytokine IFN-γ (109.6 ± 48.8 pg/ml) was highly variable. Further, some donors were found to express high amounts of proinflammatory cytokines such as IL-2, IL-6 and IL-12 (Fig. 8, donor #5, squares) or slightly increased concentration of

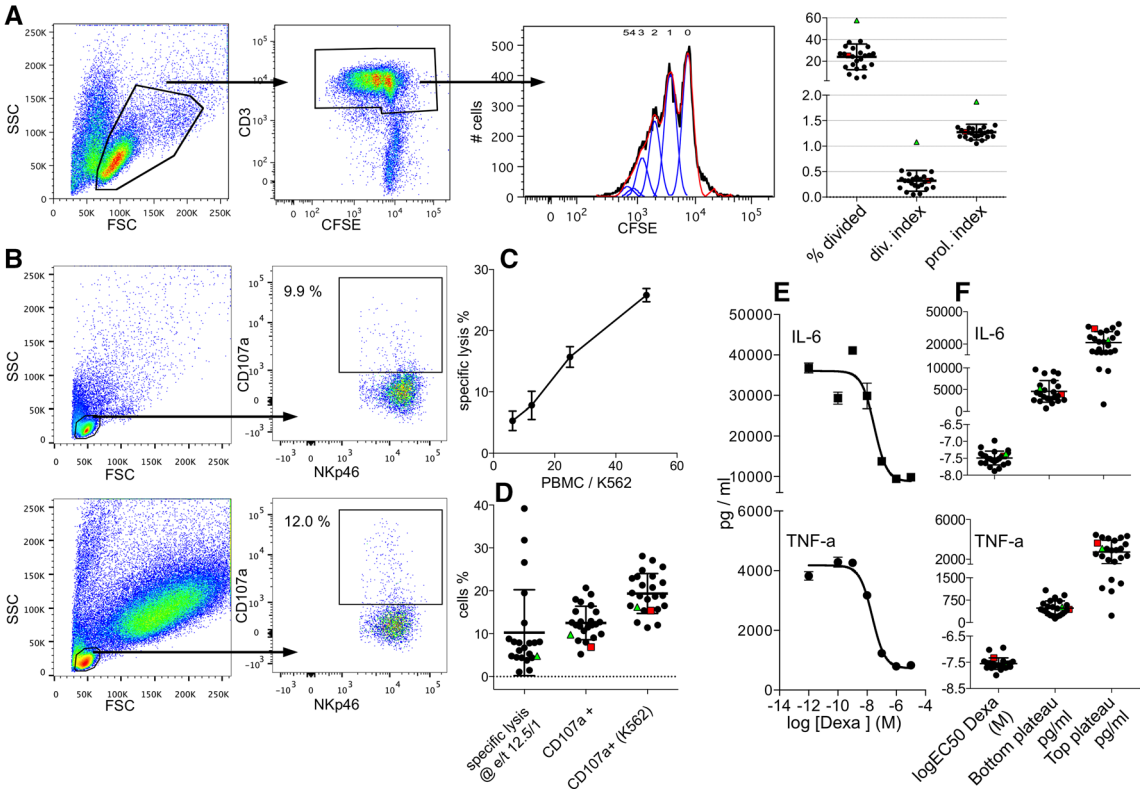

Fig. 7 Functional analyses. **a** PBMC were loaded with CFSE and stimulated with PHA-P for 6 days. T cell proliferation was analyzed by flow cytometry as described in materials and methods. Gating strategy for one representative experiment and summary for 24 donors is shown. **b** NK cell degranulation was determined in the absence (*upper row*) or presence (*lower row*) of K562 target cells as described in materials and methods. Degranulated NK cells were identified as $NKp46^{+}$ $CD107a^{+}$. One representative experiment is shown. **c** NK cell cytotoxicity was determined by a standard 4 h ^{51}Cr release assay. One representative experiment is shown. **d** Mean ± SD of data for NK cell degranulation and ^{51}Cr release from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. **e** Glucocorticoid sensitivity assay to analyze monocyte function was performed as described in materials and methods. Whole blood samples were stimulated with LPS and increasing amounts of Dexamethasone. Production of TNF-α and IL-6 was determined by ELISA. One representative experiment is shown. **f** Mean ± SD of data for monocyte glucocorticoid sensitivity from 24 healthy donors are shown. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

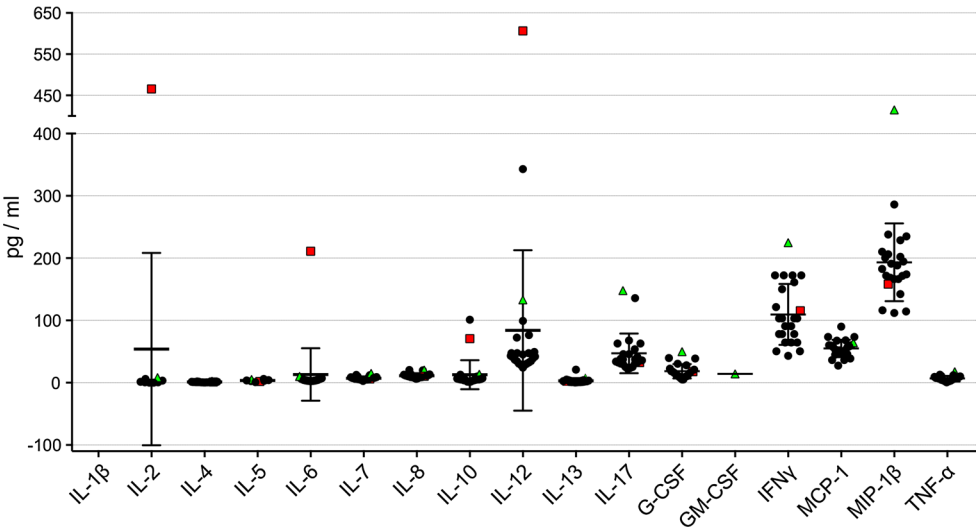

Fig. 8 Cytokine levels in serum were measured using the Bio-Plex Pro Human Cytokine 17-Plex Panel (Bio-Rad Laboratories, Inc.). Data are shown as mean ± SD from 24 healthy donors. *Red squares* and *green triangles* represent data from two individuals. Raw data for all 24 individuals are included in the electronic supplement (color figure online)

IL-17, IFN-γ and MIP-1β (Fig. 8, donor #16, triangles), which might hint to an early immune response to infection. To investigate this further, we analyzed the other immune parameters of these two donors obtained in the analyses described above. In accordance with the elevated pro-inflammatory cytokine levels, we found increased numbers of B, T and NK cells in donor #5 and increased T cell numbers in donor #16 (Fig. 1). However, when we performed functional assays, only T cell proliferation of donor #16 was enhanced, while we found no functional differences in the other assays (Fig. 7). Further, despite elevated pro-inflammatory cytokines, no significant differences became apparent when we analyzed lymphocyte subpopulations or markers for maturation and activation. These findings underline the need for a broad and comprehensive analysis of immune function to identify isolated effects that might be missed otherwise.

Discussion

In this study we describe a range of methods for the comprehensive analysis of immune cell function. We provide six antibody panels to evaluate absolute numbers and distribution of lymphocyte subsets by multicolor flow cytometry. Further, we applied functional assays to measure key functions of T cells, NK cells and monocytes. These methods require only small amounts of samples with less than 0.5 ml serum, 4 ml of fresh whole blood and 5×10^6 PBMC (which can easily be obtained from 10 ml of whole blood). This amount of sample can usually be obtained within a few minutes by an experienced nurse or a physician. Additionally, the sample analysis is not very complex, enabling the handling of up to ten different samples in parallel. Therefore, our approach allows for a fast and economical analysis of a great number of samples.

A variety of multicolor panels for comprehensive immunophenotyping has already been published. These panels were designed for clinical applications such as the diagnosis of PIDs (Boldt et al. 2014) or myelodysplastic syndromes (Porwit and Rajab 2015), or monitoring of patients after transplantation (Streitz et al. 2013). Furthermore, a great number of multicolor panels were developed to study phenotype and function of specific cell types (Roederer and Tarnok 2010). With our panels we aim to analyze the most common lymphocyte subpopulations in clinically healthy individuals. We are particularly interested in alterations in subset composition and the expression of markers for activation or maturation of lymphocytes.

We established the TruCount panel and panel one to get a general overview over the number and composition of lymphocyte and monocyte subsets. Changes in the proportion of NK, B or T cells as well as monocytes are easily detectable and might provide first hints toward changes in cell development and survival or altered migration patterns.

The B cell panel (2) was designed to illustrate the most common B cell subsets. This panel will give information about the differentiation from naive B cells to memory subsets and will additionally allow the identification of transitional and $CD21^{low}$ B cells, which have been correlated with inflammatory and autoimmune diseases (Kaminski et al. 2012b; Thorarinsdottir et al. 2015).

The T cell panel (3) allows the analysis of CD4 and CD8 T cells. The CD4/CD8 ratio was shown to change during HIV infection (Bellissimo et al. 2016), but also due to chronic stress (Segerstrom and Miller 2004) and in immunosenescence (Pera et al. 2015). This panel also allows the distinction of naive and memory subsets within $CD4^+$ and $CD8^+$ cells by using the marker CD45RA together with CD197 (CCR7) or CD62L. CD28 is a co-receptor for the TCR, and the loss of CD28 expression during aging has been correlated with immunosenescence. In particular, $CD4^+$ $CD28^-$ T cells were shown to play a role in rheumatoid arthritis and other inflammatory diseases (Mou et al. 2014). Similarly, $CD25^{++}$ and $CD127^{low}$ T_{reg} are involved in a variety of inflammatory and autoimmune diseases and play a role in tumor immunity (Chen et al. 2016). T_{reg} number or function might also be affected during aging (Jagger et al. 2014) or by sex hormones (Nie et al. 2015). In addition, analysis of CD62L and CCR7 on NK cells gives information about NK cell maturation. Further, expression of CD62L on NK cells and migration of these cells was shown to be affected by acute stress (Dhabhar et al. 2012).

Activation and maturation of NK and T cells was analyzed in panel four. CD25, CD69 and GITR are known to be upregulated during activation (Clausen et al. 2003; Li et al. 2003) and were therefore used to identify activated NK and T cells. GITR expression was shown to be regulated during parasite infection (Costa et al. 2016) and infectious diseases (Li et al. 2003) and is involved in the regulation of T_{reg} function (Chen et al. 2016). Increased expression of CD69 was shown to correlate with high NK cell cytotoxicity, while up-regulation of CD25 may predict NK cell proliferative potential in breast cancer patients (Clausen et al. 2003). Little is known about the function of the inhibitory receptor KLRG1 on T and NK cells. KLRG1 might be involved in the control of chronic virus infection (Bigley et al. 2015; Ouyang et al. 2003; Wang et al. 2013). The receptor is expressed on memory T cells (Marcolino et al. 2004) and has further been linked to immunosenescence in T cells and marks cells with reduced proliferative potential (Voehringer et al. 2002). Accordingly, expression of KLRG1 on T cells increases during aging (Ouyang et al. 2003). In contrast, KLRG1 expression is highest on $CD56^{dim}$ NK cells in young adults and is decreased in the elderly (Hayhoe et al. 2010). CD57 identifies terminally

differentiated T cells and mature NK cells and was found to be upregulated in different cancers, chronic viral infections and during aging (Kared et al. 2016). Like CD57, the activating receptor NKG2C has been associated with CMV reactivation and NK cell memory (Cerwenka and Lanier 2016).

Panel five was set up to analyze the expression of activating receptors on NK cells. NK cell function is regulated by modulation of activating receptor expression (Sandusky et al. 2006). Expression levels of several activating NK receptors such as 2B4, DNAM-1, NKp30 and NKG2D were shown to be modulated in different leukemias (Baier et al. 2013). NKp30 and NKp46 are up-regulated in chronic HCV infection (De Maria et al. 2007). In contrast, expression of these receptors is down-modulated during infection with HIV (De Maria et al. 2003) or mycobacteria (Bozzano et al. 2009). Moreover, expression of NKp30 and NKp46 changes during aging (Almeida-Oliveira et al. 2011). In addition, several activating NK cell receptors were found to regulate also T cell function (Gilfillan et al. 2008; McMahon and Raulet 2001; Waggoner and Kumar 2012).

Lymphocyte functions such as T cell proliferation and NK cell cytotoxicity are affected by different factors including stress, age, infections and cancer (Doitsh and Greene 2016; Giefing-Kroll et al. 2015; Pitt et al. 2016; Webster Marketon and Glaser 2008). Therefore, the assessment of functional features, together with the cytokine levels in serum, can give important information about the current immune status of an individual. The response of monocytes to pathogens is modulated by a pro-inflammatory milieu, which leads to pre-activation and increased cytokine production in chronic inflammatory diseases (Leirisalo-Repo et al. 1995). Pro-inflammatory immune reactions are counter-regulated by the activation of glucocorticoid receptors, which are expressed on all immune cells (Gotovac et al. 2003). Levels of the glucocorticoid Cortisol are under the control of the HPA axis and can be chronically increased due to stress (Miller et al. 2014), sleep-deprivation (Buckley and Schatzberg 2005) or obesity (Vicennati et al. 2014). Sustained triggering of glucocorticoid receptors finally changes their signaling and therefore leads to decreased glucocorticoid sensitivity (Rohleder et al. 2003), which can be easily measured in monocytes by the assay described in our study. Determination of cytokine levels produced by monocytes, together with the EC_{50} for glucocorticoid treatment thus can provide quantitative information about the effects of psychological strain, metabolic influences (obesity) or inflammation.

Most immune phenotyping approaches were developed for clinical settings such as the analysis of PIDs, during transplantation or chronic infections. In these situations, one can often detect major changes in immune cell numbers, phenotype or function. In contrast to this, the immune modulatory effects of factors such as age, stress or exposure to chemicals are expected to be much smaller. It is therefore challenging to determine the immune status of clinically healthy individuals. This is further complicated by the fact that immune parameters show quite some variability among healthy individuals. Therefore, the data reported here can also be used to establish a baseline for the measured immune parameters in healthy individuals. To determine immune modulatory effects, one needs a comprehensive analysis of many immune parameters as demonstrated here. All of our 24 volunteers reported to be healthy. Nevertheless, we could detect signs of an early low-level infection in two of these individuals which resulted in changes in several parameters. This demonstrates that our analysis can be helpful to determine the changes in the immune system induced by factors such as age, stress, nutrition, exposure to chemicals or other internal and external factors.

Acknowledgments We would like to thank all volunteers who participated in this study and we gratefully acknowledge the help of Linda Drenkelforth and Sabine Wingert with sample preparation. We are grateful to Christine Falk and Kerstin Daemen for their help with the Bio-Plex cytokine measurements. Our work is supported by funding of the Leibniz Association (SAW-2013-IfADo-2) and the Deutsche Forschungsgemeinschaft (DFG, WA-1552/5-1).

Compliance with ethical standards

Ethical approval All procedures performed in this study were approved by the institutional ethics committee and were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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