Effects of aging on human leukocytes (part II): immunophenotyping of adaptive immune B and T cell subsets

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Abstract Immunosenescence results from a continuous deterioration of immune responses resulting in a decreased response to vaccines. A well-described age-related alteration of the immune system is the decrease of de novo generation of T and B cells. In addition, the accumulation of memory cells and loss of diversity in antigen specificities resulting from a lifetime of exposure to pathogens has also been described. However, the effect of aging on subsets of $\gamma\delta\text{TCR}^+$ T cells and Tregs has been poorly described, and the efficacy of the recall response to common persistent infections in the elderly

remains obscure. Here, we investigated alterations in the subpopulations of the B and T cells among 24 healthy young (aged 19–30) and 26 healthy elderly (aged 53–67) individuals. The analysis was performed by flow cytometry using freshly collected peripheral blood. $\gamma\delta\text{TCR}^+$ T cells were overall decreased, while $\text{CD4}^+\text{CD8}^-$ cells among $\gamma\delta\text{TCR}^+$ T cells were increased in the elderly. $\text{Helios}^+\text{Foxp3}^+$ and $\text{Helios}^-\text{Foxp3}^+$ Treg cells were unaffected with age. Recent thymic emigrants, based on CD31 expression, were decreased among the $\text{Helios}^+\text{Foxp3}^+$, but not the $\text{Helios}^-\text{Foxp3}^+$ cell populations. We observed a decrease in Adenovirus-specific CD4^+ and CD8^+ T cells and an increase in CMV-specific CD4^+ T cells in the elderly. Similarly, $\text{INF}\gamma^+\text{TNF}\alpha^+$ double-positive cells were decreased among activated T cells after Adenovirus stimulation but increased after CMV stimulation. The data presented here indicate that $\gamma\delta\text{TCR}^+$ T cells might stabilize B cells, and functional senescence might dominate at higher ages than those studied here.

Ulrik Stervbo and Cecilia Bozzetti contributed equally to this work.

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Abbreviations

TCR	T cell receptor
Tregs	regulatory T cells
CM	central memory
EM	effector memory
TEMRA	terminally differentiated effector memory
RTE	recent thymic emigrants

Introduction

The effectiveness of the adaptive immune system decreases with age, resulting in an increased burden on the health care systems in societies with an increasing proportion of mature population (Walford 1964; Weng 2006; National Institute on Aging 2011). This decrease in effectiveness of the immune system reflects degeneration on many levels of the development and function of lymphoid cells; a decreased potential for lymphoid differentiation of hematopoietic stem cells diminishes the generation of immune cells (Pang et al. 2011). Additionally, continuous thymic involution gradually decreases the generation of new T cells (Simpson et al. 1975). Moreover, the functionality of existing cells decreases, which partly reflects the increased activation threshold of T cells (Li et al. 2012). The age-dependent decline in the effectiveness of the immune system increases the risk for severe infections in the elderly population (Nikolich-Zugich 2008), lowers the efficacy of vaccines (Haynes and Swain 2006; Nichol et al. 2007; Osterholm et al. 2012), and increases the risk for the development of autoimmune disorders and cancer (Prelog 2006; Derhovanessian et al. 2008; Foster et al. 2011).

Due to homeostatic proliferation, naïve T cells might increase with age, despite the decreased thymic output (Appay and Sauce 2014). However, the diversity of the naïve T cell compartment decreases with age. Deep sequencing of the complementarity-determining region 3 of the T cell receptor (TCR)- β chain from healthy donors revealed that the fraction of expanded clones increases with age (Britanova et al. 2014). Similarly, spectratyping of the TCRVB genes revealed the expansion of particular segments (Pennesi et al. 2001). Life-long exposure to pathogens results in an age-dependent increase in antigen-experienced T cells (Vescovini et al. 2014). Particularly, chronic infections, such as CMV and EBV, induce the age-dependent expansion of memory cells (Stowe et al. 2007), further decreasing the T cell receptor repertoire size, making the aging immune system vulnerable to infections.

Thymic involution not only affects CD4⁺ and CD8⁺ T cells but also $\gamma\delta$ TCR⁺ T cells. This T cell subset is less well studied than T and B cells, but similarities to what is known for $\alpha\beta$ TCR⁺ T cells remain clear: the repertoire of $\gamma\delta$ TCR⁺ T cells and the cell counts decrease with age (Giachino et al. 1994; Colonna-Romano et al. 2002; Argentati et al. 2002; Re et al. 2005; Andreu-Ballester

et al. 2012). The Treg population increases with age (Gregg et al. 2005; Vukmanovic-Stejic et al. 2006; Lages et al. 2008; Agius et al. 2009; Booth et al. 2010), although the internal composition changes from a naïve to a memory phenotype (Gregg et al. 2005; Booth et al. 2010).

B cells are differently affected with age than T cells. Although the total numbers of mature B cells decrease with age (reviewed in Frasca et al. 2011), conflicting reports on the ratio of naïve and memory cells exists (Kolar et al. 2006; Caraux et al. 2010; Bulati et al. 2011; Frasca et al. 2011). Furthermore, a decrease in class-switched B cells and decrease in affinity antibodies has been observed in the elderly (Frasca et al. 2008). The decrease in humoral protection links B cells, similar to T cells, with immunosenescence (Bulati et al. 2011; Frasca et al. 2011).

To characterize the composition of cell subsets of the immune system in young and elderly human populations, blood was drawn from healthy individuals aged 19 to 67 years and analyzed through flow cytometry. Peripheral blood mononucleated cells (PBMCs) were stained for lineage-specific markers for the identification of adaptive B and T cell subsets. The composition of the innate immune cells of the same donors has been examined elsewhere (Stervbo et al. 2015). Here, we report that alterations of subpopulations of $\gamma\delta$ TCR⁺ T cells are present in the elderly, and Helios-negative regulatory T cells (Tregs), in contrast to Helios⁺ Treg, are unaffected by the age-dependent reduction in thymic output.

Materials and methods

Study cohort

The samples were obtained in the fall of 2011 from the PRIMAGE-study cohort at the Berlin-Brandenburg Center for Regenerative Therapies, Charité – Universitätsmedizin Berlin. The study was approved by the ethics board of the Charité (approval number EA 1/175/11). All study participants gave their written informed consent prior to enrollment for the study. A total of 50 donors were recruited, with no minors under the age of 18 participating. A total of 50 donors were recruited and provided informed consent from the local ethical committee at the Charité - Universitätsmedizin. All study participants were healthy, at the age of 19–

67 years. Individuals who were receiving immunomodulatory therapy, had hemoglobin value less 12 g/dl, or were pregnant were excluded from the study. The cohort made of 24 young donors (19–30 years, 12 females, 12 males) and 26 elderly donors (53–67 years, 16 females, 10 males), summarized in Supplementary Table 1. From each donor, a total of 100 ml blood was drawn using 10 ml Lithium-Heparin Vacutainers (BD Biosciences) and processed immediately.

Preparation of PBMCs

Peripheral blood mononucleated cells (PBMCs) were prepared from whole blood by using Leucosept-Tubes (Cellstar) according to the manufacturer's instructions. Briefly, separation tubes were prepared with 15-ml Ficoll-Paque Plus (GE Healthcare) followed by the addition of blood pre-diluted in PBS (Gibco) at a 1:1 ratio. Tubes were centrifuged at 800 g for 15 min at room temperature, and the PBMC-enriched layer was washed twice with PBS.

Reactivity of T cells

1×10^6 cells were stimulated with PepTivator AdV5 Hexon peptide pool (1 μ g/ml; Miltenyi Biotec), *Aspergillus fumigatus* Lysate (40 μ g/ml; Miltenyi Biotec), PepTivator CMV pp65 (1 μ g/ml; Miltenyi Biotec) or pooled PepTivator EBV BZLF1, and PepTivator EBV EBNA (1 μ g/ml; Miltenyi Biotec) for 6 h under standard conditions (37 °C, 5 % CO₂, saturated humidity) in RPMI-1640 (PAA), supplemented with 10 % (v/v) human AB-serum (Lonza), 100 U/ml penicillin (Biochrom), 0.1 mg/ml streptomycin (Biochrom), and 1 μ g/ml anti-CD28 (Clone: CD28.2; BD Biosciences). The last 2 h of stimulation were done in the presence of 5 μ g/ml Brefeldin-A (Sigma).

Antibodies and staining procedure

Isolated PBMCs were stained with optimal dilutions of CCR7-A488 (Clone: TG8/CCR7; BioLegend), CD27-APC (Clone: M-T271; Miltenyi Biotec), CD3-VioBlue (Clone: BW264/56; Miltenyi Biotec), CD31-PE (Clone: AC128; Miltenyi Biotec), CD4-APC-H7 (Clone: RPA-T4; BD Biosciences), CD45RA-EF 605 (Clone: HI100; eBioscience), CD45RO-ef650 (Clone: UCHL1; eBioscience), CD8-PE-VioV770 (Clone: BW135/80; Miltenyi Biotec), and CD62L-PerCp-ef710 (Clone:

DREG-56; eBioscience) for the analysis of the CD4⁺ and CD8⁺ T cell compartments.

Tregs were captured with CD3-PerCp (Clone: BW264/56; Miltenyi Biotec), CD4-APC-H7 (Clone: RPA-T4; BD Biosciences), CD8-PE-Vio770 (Clone: BW135/80; Miltenyi Biotec), Helios-A647 (Clone: 22 F6; BioLegend), FoxP3-A488 (Clone: 259D/C7; BD Biosciences), CD25-BV421 (Clone: 3C7; BioLegend), CD45RA-ef605 (Clone: HI100; eBioscience), CD45RO-A700 (Clone: UCHL1; BioLegend), and CD31-PE (Clone: AC128; Miltenyi Biotec), with exclusion of dead cells by LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen).

Analysis of B cells included a dump channel consisting of CD14-APC-Vio770 (Clone: T $\ddot{U}$ K4; Miltenyi Biotec), CD3-APC-H7 (Clone: SK7; BD Biosciences), and LIVE/DEAD Fixable Near-IR Dead Cell Stain (Invitrogen) and discrimination of naïve and memory B cells by CD19-PE-Vio770 (Clone: LT19; Miltenyi Biotec), CD27-APC (Clone: M-T271; Miltenyi Biotec), IgD-V450 (Clone: IA6-2; BD Biosciences).

Analysis of $\gamma\delta$ TCR⁺ T cells was performed with CD3-APC-H7 (Clone: SK7; BD Biosciences), $\gamma\delta$ TCR-APC (Clone: B1; BD Biosciences), CD4-PerCp (Clone: VIT4; Miltenyi Biotec), CD8-V500 (Clone: SK1; BD Biosciences), CD38-PE (Clone: IB6; Miltenyi Biotec), Ki67-V450 (Clone: B56; BD Biosciences), CD45RA-PeCy7 (Clone: HI100; BioLegend), and CCR7-A488 (Clone: 4B12; BioLegend).

Activated T cells were analyzed with CD4-APCH7 (Clone: RPA-T4; BD Biosciences), CD8-PerCp (Clone: BW135/80; Miltenyi Biotec), CD40L-VioBlue (Clone: 5C8; Miltenyi Biotec), INF- γ -FITC (Clone: 45–15; Miltenyi Biotec), IL-17-PE (Clone: BL168; BioLegend), IL-2-APC (Clone: MQ1-17H12; BD Biosciences), TNF α -PE-Vio770 (Clone: CA2; Miltenyi Biotec), and a dump channel of CD14-VioGreen (Clone: T $\ddot{U}$ K4; Miltenyi Biotec) and CD20-VioGreen (Clone: LT20; Miltenyi Biotec) with dead cell exclusion by LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen).

The surface staining was performed in PBS containing 10^7 cells/ml for 15 min at room temperature. Prior to antibody staining, unspecific antibody binding was blocked with Beriglobin (ZLB Behring) at a final concentration of 1 mg/ml.

Foxp3 was stained according to the manufacturer's directions. Briefly, surface molecules were stained as described above and dead cells were marked with LIVE/DEAD Fixable Aqua Dead Cell Stain

(Invitrogen) as described below. Cells were fixed using the supplied fixation/permeabilization buffer at 4 °C for 60 min. After washing, fixed cells were resuspended in Foxp3-staining buffer (anti-Foxp3 in fixation/permeabilization buffer), and incubated at 4 °C for 60 min.

Antigen specific T cells were identified by expression of CD40L or INF γ . Stimulated cells were stained for surface molecules as described above and dead cells were marked with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) as described below. Stained cells were fixed with the formaldehyde containing FACS-lysing-solution (BD Biosciences) for 10 min at room temperature. After permeabilization with FACS-perm-solution (BD Biosciences) for 10 min at room temperature, CD40L and INF γ were stained intra-cellular for 30 min at room temperature in FACS-perm-solution. Ki67 was stained in a similar manner in naïve cells.

Dead cells were excluded from staining panels not involving fixation by addition of PI (BD Biosciences) or DAPI (BD Biosciences) prior to sample acquisition. For the activated T cell and the Foxp3 staining panels, dead cells were marked with LIVE/DEAD Fixable Aqua Dead Cell Stain (Invitrogen) at a final concentration of 1 μ g/ml, after surface stain and prior to fixation.

B cells, $\gamma\delta$ TCR $^+$ T cells, and activated T cells were analyzed on a MACS Quant (Miltenyi Biotec) flow cytometer equipped with blue 488-, red 633-, and violet 405-nm lasers. CD4 $^+$ /CD8 $^+$ T cells and Tregs were acquired on a BD LSR-II (BD Biosciences) flow cytometer equipped with blue 488-, yellow-green 561-, red 640-, and violet 405-nm lasers.

QC was performed daily on each flow cytometer using SPHERO Rainbow Calibration Particles (BD Biosciences). No adjustment to the PMT voltage was required between the runs. A compensation matrix for each staining panel was established the day before the first batch of donors using single antibody stains.

Statistical analysis

FACS data was analyzed using FlowJo version 9.6.4 (Tree Star), and statistical analysis was performed using R, version 2.15 (R Core Team 2012). p values <0.05 were considered as significant. Plots were generated with the R-package ggplot2 (Wickham 2009). Network diagrams were constructed using Cytoscape, version 2.8 (Smoot et al. 2011).

Results

PBMCs were isolated from the peripheral blood of 24 young (aged 19–30 years) and 26 elderly (aged 53–67 years) healthy donors as previously described (Stervbo et al. 2015). The cells were analyzed for naïve and memory populations of B cells, CD4 $^+$ and CD8 $^+$ T cells, and $\gamma\delta$ TCR $^+$ T cells using flow cytometry. The mean frequencies, absolute cell counts and standard deviations of all of the populations analyzed, along with p values for comparative analyses, are summarized in Supplementary Table 2 and 3.

Naïve and memory B cells have similar levels in young and elderly individuals

There are several conflicting reports concerning the effects of aging on the composition of CD19 $^+$ B cells (Colonna-Romano et al. 2003, 2009; Chong et al. 2005; Shi et al. 2005). We therefore assessed the effect of aging on CD27 $^-$ IgD $^+$ naïve and CD27 $^+$ IgD $^{low/-}$ memory B cells (Supplementary Fig. 1a). No significant differences in the percentage or cell counts of naïve or memory B cells were observed between young and elderly individuals (Fig. 1a; b cell cluster). Together, these results demonstrate that the composition of the B cell compartment is unaltered between young and elderly individuals, although an increase in the memory compartment was observed with respect to frequencies and absolute cell counts (Fig. 1b, c; Supplementary Fig. 3).

CM and TEMRA, but not EM, are increased in the elderly

Next, we analyzed the global composition of the T cell lineage in terms of CD3 $^+$ cells, $\gamma\delta$ TCR $^+$ T cells, CD4 $^+$ T cells and CD8 $^+$ T cells (Fig. 1a; the clusters $\gamma\delta$ TCR $^+$ T

Fig. 1 Global view of all analyzed populations. Each circle represents a population, and the arrow indicates the relationship between populations. Diamond shape indicates live cells. **a** Cell fraction (left half circle) or cell count (right half circle). White indicates no difference; the fill color indicates significant decrease or increase in the elderly compared to the young. Difference tested by Wilcoxon rank-sum test, p values <0.05 were considered significant. **b** Fraction of cells normalized per cell population. **c** Estimated cells/ μ l normalized per cell population. Bold circle borders indicate population with significant difference

cells, CD4⁺ T cells, CD8⁺ T cells, with the gating strategy depicted in Supplementary Fig. 1b and 2a). No difference was observed in the CD3⁺ population (Fig. 1a).

The fraction of CD4⁺ cells among CD3⁺ cell populations was significantly increased in the elderly, but the cell count was similar between young and elderly individuals (Fig. 1a; CD4⁺ T cells cluster, Supplementary Fig. 4a). For CD3⁺CD8⁺ T cells, the absolute counts and relative frequencies among the CD3⁺ cell populations was slightly decreased in the elderly, and this effect was insignificant (Fig. 1a; CD8⁺ T cells cluster, Supplementary Fig. 5a).

Naïve (CD45RA⁺CCR7⁺), central memory (CM; CD45RA⁺CCR7⁺), effector memory (EM; CD45RA⁺CCR7⁺), and terminally differentiated effector memory (TEMRA; CD45RA⁺CCR7⁺) cells were analyzed among the CD4⁺ and the CD8⁺ T cell subpopulations (Fig. 1a; clusters CD4⁺ T cells and CD8⁺ T cells). The gating strategy is depicted in Supplementary Fig. 1b. The relative fractions of naïve CD4⁺ and naïve CD8⁺ cells were decreased in the elderly, but only the absolute counts of naïve CD8⁺ were lower in the elderly, whereas the CD4⁺ cell counts remained similar (Supplementary Fig. 4c and Supplementary Fig. 5b). Similarly, the fraction and cell count of CD4⁺ recent thymic emigrants (RTE), based on the expression of CD31, was significantly lower in the elderly compared with the young, as expected (Hassan and Reen 2001; Kimmig et al. 2002).

The fraction, but not the corresponding cell counts, of the CM populations in CD4⁺ and CD8⁺ cells was increased in the elderly (Fig. 1a; the clusters CD4⁺ T cells and CD8⁺ T cells). The EM population remained similar between young and elderly individuals for both CD4⁺ and CD8⁺ with respect to both frequency and absolute counts (Fig. 1a, Supplementary Fig. 4e and Supplementary Fig. 5d). The fractions of TEMRA subpopulations in CD4⁺ and CD8⁺ cells were increased in the elderly (Fig. 1a, Supplementary Fig. 4f, and Supplementary Fig. 5e). This observation corresponded to an increase in the cell count of CD4⁺ TEMRA populations but not the CD8⁺ counterparts (Supplementary Fig. 4f and Supplementary Fig. 5e). Collectively these data demonstrate that the subpopulations of CD4⁺ and CD8⁺ T cells in the memory compartment are not affected with age in an equal manner.

Fig. 2 Responsiveness in CD4⁺ and CD8⁺ T cells. Cells were stimulated with indicated antigen for 6 h. Shown are individual measurements after subtraction of values of unstimulated cells and boxplots of indicated populations and total cell number/μl whole blood for **a** CD40L⁺ among CD4⁺ cells and **b** INFγ⁺ among CD8⁺ cells. **c** Distribution of number of co-expressed cytokines within the activated cells. **d** Distribution of individual cytokine combinations among the activated cells. The *numbers* above the plot-sections indicate the number of co-expressed cytokines. The box indicates the 25th, 50th, and 75th percentiles, the whiskers the minimum and maximum values excluding outliers. Gating was performed as described in Supplementary Fig. 1B. Wilcoxon rank-sum test was used to test for differences and *p* values < 0.05 are reported

Decreased numbers of adenovirus-specific and increased numbers of CMV-specific T cells in the elderly

The exhaustion of the T cell compartment in response to persistent infections might explain the immunosenescence observed in elderly individuals (Bulati et al. 2011; Haq and McElhaney 2014). To analyze potential age-dependent alterations in response to common pathogens, PBMCs were stimulated with peptides derived from Adenovirus, Aspergillus, CMV, and EBV (Fig. 2). The elderly group displayed a significantly lower fraction and count of Adenovirus-specific CD4⁺ and CD8⁺ T cells based on the expression of CD40L or INFγ (Fig. 2a, b). No difference in Aspergillus, CMV, or EBV-specific CD8⁺ T cells was observed (Fig. 2b). In contrast, a significantly higher fraction and cell number of CMV (pp65)-reactive CD4⁺ T cells was observed in the elderly (Fig. 2a).

Polyfunctional T cells, i.e., T cells that co-express two or more cytokines, are associated with protection against the progression of HIV-1 and mycobacterium tuberculosis (Betts et al. 2006; Beveridge et al. 2007). We therefore examined how the efficacy of the immune response, based on the polyfunctionality of antigen-specific T cells, is affected with age. To this end, we focused on the antigens showing age-dependent differences and the major T cell cytokines IL2, IL17, INFγ, and TNFα (Fig. 2c–e).

Among adenovirus-specific CD4⁺ T cells, an age-dependent decrease in cells producing two cytokines was observed (Fig. 2c), corresponding to a large increase in adenovirus-specific CD4⁺ cells producing none of the measured cytokines. Interestingly, for CMV-specific CD4⁺ T cells, the opposite results were observed. In the elderly, an increase of cells producing

two or three cytokines was observed compared with the young. This finding was concurrent with a decrease in cells producing none of the measured cytokines. The majority of adenovirus-specific CD8⁺ T cells in the young produced only a single cytokine. This fraction was increased in the elderly.

We further analyzed the composition of mono- and polyfunctional antigen-specific cells (Fig. 2d, e). The observed decrease in the proportion of two cytokine-producing, adenovirus-specific CD4⁺ T cells reflected a decrease in INF γ ⁺TNF α ⁺ cells (Fig. 2d). The increase in two and three cytokine-producing CMV-specific CD4⁺ cells was associated with a significant increase in IL2⁺INF γ ⁺TNF α ⁺ among the elderly and a strong, yet insignificant, increase in INF γ ⁺TNF α ⁺. The increase in adenovirus-specific, monofunctional CD8⁺ T cells reflected an insignificant increase in TNF α ⁺-producing cells (Fig. 2e). Taken together, these data demonstrate an age-dependent alteration in the polyfunctionality of antigen-specific T cells.

Fewer $\gamma\delta$ TCR⁺ T cells in the elderly

Significantly lower fractions of $\gamma\delta$ TCR⁺ T cells among CD3⁺ cells were observed in the elderly compared with the young. The cell count was significantly decreased (Fig. 1a; $\gamma\delta$ TCR⁺ T cells cluster, Supplementary Fig. 6a). To further explore the composition of the $\gamma\delta$ TCR⁺ T cells, the cells were stained for CD4 and CD8. The CD4⁺CD8⁺ population was the largest subpopulation of $\gamma\delta$ TCR⁺ T cells in both the young and the elderly (Supplementary Fig. 6b, c, and d). The fraction of CD4⁺CD8⁺ and of CD4⁺CD8⁺ among $\gamma\delta$ TCR⁺ T cells was higher in the elderly compared with the young (Fig. 1a; $\gamma\delta$ TCR⁺ T cells cluster, the gating strategy is depicted in Supplementary Fig. 2a). While the cell counts of CD4⁺CD8⁺ $\gamma\delta$ TCR⁺ T cells remained unperturbed with age, significantly lower counts of CD4⁺CD8⁺ and of CD4⁺CD8⁺ were observed in the elderly (Fig. 1a; Supplementary Fig. 6b, c, and d).

Ki67 was used to assess the basal proliferation rate of the $\gamma\delta$ TCR⁺ T cells and the three subpopulations CD4⁺CD8⁺, CD4⁺CD8⁺, and CD4⁺CD8⁺ (Supplementary Fig. 2a). The fraction of all Ki67⁺ cells among $\gamma\delta$ TCR⁺ T cells was increased in the elderly, while the corresponding cell count was decreased (Fig. 1a; Supplementary Fig. 6e). The increase of the fraction of proliferating cells among $\gamma\delta$ TCR⁺ T cells was not reflected in any of the measured subpopulations,

although tendencies for a higher fraction of Ki67⁺ cells were observed among CD4⁺CD8⁺ and CD4⁺CD8⁺ cells (Supplementary Fig. 6f, g, and h). The number of proliferating cells was decreased among $\gamma\delta$ TCR⁺CD4⁺CD8⁺ and in $\gamma\delta$ TCR⁺CD4⁺CD8⁺ cell populations (Supplementary Fig. 6f and h), while the count of $\gamma\delta$ TCR⁺CD4⁺CD8⁺Ki67⁺ cells was equal between the young and the elderly (Supplementary Fig. 6g). Taken together, these data show a general decrease in circulating $\gamma\delta$ TCR⁺ T cells in the elderly, while the proportion of proliferating cells was increased in this cell population. These differences are generally reflected in the CD4⁺CD8⁺ subpopulation.

CD4⁺Foxp3⁺Helios⁺ Tregs are affected by decreased recent thymic emigrants

Helios expression discriminates thymic-derived tTreg from peripherally derived pTreg, although recent evidence suggests that Helios is rather associated with induced tolerance (Thornton et al. 2010; Ross et al. 2014). Several reports describe an age-dependent increase in Tregs, however, little is known about the effect of aging on Helios expression in Foxp3⁺ Tregs. We therefore analyzed age-dependent alterations of this Zinc finger protein among CD4⁺Foxp3⁺ Tregs (Fig. 3; gating strategy in Supplementary Fig. 2b). The fraction and numbers of Foxp3⁺Helios⁺ among CD4⁺ cells did not differ between young and elderly individuals (Figs. 1a and 3a), and although Foxp3⁺Helios⁺ cells were increased in the elderly, in both fraction and cell number, this increase was not significant (Figs. 1a and 3b). Foxp3⁺Helios⁺CD31⁺ cells were strongly decreased in both frequency and number in the elderly (Fig. 3c), while no difference was observed for Foxp3⁺Helios⁺CD31⁺ cells (Fig. 3d).

Discussion

In the present study, we analyzed the effect of early aging (i.e., donors below 65 years) on the composition and responsiveness of the adaptive immune system. The innate immune system is addressed in part I of this two-part continuing PRIMAGE-study (Stervbo et al. 2015). Because CD4⁺ T cells have a high degree of functional plasticity (Coomes et al. 2013), we focused on larger cell populations, as alterations in these cells likely have physiological effects. As discussed elsewhere (Stervbo

Fig. 3 $CD4^+Foxp3^+$ Tregs in the elderly and in the young. Shown are individual measurements and boxplots of indicated populations and total cell number/ μ l whole blood for **a** $Foxp3^+Helios^+$ among $CD4^+$, **b** $Foxp3^+Helios^-$ among $CD4^+$, **c** $Foxp3^+Helios^+CD31^+$ among $CD4^+$, and **d** $Foxp3^+Helios^-CD31^+$ among $CD4^+$. The

box indicates the 25th, 50th, and 75th percentiles, the whiskers the minimum and maximum values excluding outliers. Gating was performed as described in Supplementary Fig. 2B. Wilcoxon rank-sum test was used to test for differences and p values <0.05 are reported

et al. 2015), the present study was focused on the alterations in the late middle-aged population and did not attempt to include gender as an additional component. Here, we show the first results from a comparison of the basal proliferation levels of $\gamma\delta TCR^+$ T cells in healthy young and elderly individuals and demonstrate a previously unappreciated alteration of $CD4^+CD8^-$ and $CD4^-CD8^-$ cells among $\gamma\delta TCR^+$ cell populations in the elderly.

We first analyzed the composition of the B cell compartment but did not identify any significant age-dependent differences with respect to the distribution of naïve and memory subsets. However, conflicting reports on age-dependent effects in the B cell compartment exist. Thus, for the overall pool of $CD19^+$ B cells, both unchanged and decreased cell numbers have been reported in the elderly (Chong et al. 2005; Caraux et al. 2010). An increase with age was also observed for $CD19^+IgD^+CD27^-$ naïve B cells (age 68–87 years), corresponding to a decrease in $CD27^+$ memory B cells (Chong et al. 2005). In contrast, increased values were also described for IgD^-CD27^+ memory B cells when comparing old (age 75–102 years) and young (20–55 years) individuals (Colonna-Romano et al. 2009), while the IgD^+CD27^+ pre-switched memory population

did not change, consistent with the observations in the present study (data not shown). $IgD^{Low/+}CD27^+$ post-switched memory B cells undergo expansion and contraction with age (Shi et al. 2003). Previous studies have shown that the B cell memory population increased until the age of 40 after which this cell population contracted. Collectively, these data indicate that age-dependent alterations in the composition of B cell subsets are effective at an age higher than that in the elderly group included in the present study.

As expected, we observed a decrease in the relative fractions of naïve $CD4^+$ and $CD8^+$ cells in the elderly, but only the absolute counts of naïve $CD8^+$ was lower in the elderly (Kang et al. 2004; Koch et al. 2008). The observed increase in the fraction of CM among $CD4^+$ cells is consistent with previous observations, while continuous stability has been reported for $CD8^+$ T cells (Kang et al. 2004; Koch et al. 2008). This discrepancy cannot be explained by differences in the definition of the aged group, as similar criteria have been applied (Koch et al. 2008). Consistent with Koch et al. (2008), we also detected comparable EM populations of $CD4^+$ and $CD8^+$ for young and elderly individuals. The observed increase in the fraction of TEMRA among $CD8^+$ T cells is consistent with previous findings, while the

increase in CD4⁺ T cells contrasts with the previously observed age-dependent stability of TEMRA cells (Koch et al. 2008). Altogether, these data are consistent with that of previous studies (Kang et al. 2004; Koch et al. 2008), showing that large heterogeneities exist in the age-dependent alterations within the T cell memory compartment.

Analysis of antigen-specific T cells revealed a significantly lower fraction and count of adenovirus-specific CD4⁺ and CD8⁺ T cells. This observation is consistent with previous observations showing decreased INF γ secretion in the PBMCs in elderly individuals (Olive et al. 2001; Sester et al. 2002). CMV-specific CD8⁺ T cells are increased in old individuals (65–101 years), but not in middle-aged/elderly (41–64 years) individuals, based on staining with HLA-A2 CMV pp65 pentamers (Pita-Lopez et al. 2009). Another report demonstrated similar levels of EBV- and CMV-specific CD8⁺ T cells between young (young 20–61) and old (75–82 years) individuals, identified through HLA-A2 tetramers to EBV BMLF1 and CMV pp65 epitopes, respectively (Colonna-Romano et al. 2007; Vescovini et al. 2014).

Here we observed no age dependence in CMV- and EBV-specific CD8⁺ T cells. In contrast, an increase in CMV-specific CD4⁺ T cells was observed. These data indicate an effective clearance of the Adenovirus infection in the elderly, while CMV infection persists with age, indicated as an expansion of CMV-specific clones (Stowe et al. 2007; Schwanninger et al. 2008). Indeed, memory T cells expand in the elderly, and the memory compartment is altered to contain larger pools of fewer clones (reviewed in Goronzy and Weyand 2013). The lower frequency of INF γ ⁺TNF α ⁺ double-producing cells in the elderly were observed after stimulation with Adenovirus, together with an increase of INF γ ⁺TNF α ⁺ double-positive cells after stimulation with pp65, indicating that the CD4⁺ T cells are functional in the elderly and not subjected to marked exhaustion. We previously identified CD8⁺ T cells with helper functions (Frentsch et al. 2013). However, no age-dependent differences in CD40L⁺ expression among INF γ -positive CD8⁺ T cells were observed in cells stimulated with a cocktail of the super antigens Staphylococcal Enterotoxin B and Staphylococcal Superantigen (data not shown).

The repertoire of $\gamma\delta$ TCR⁺ T cells contracts with age; thus, the polyclonal V δ population in children shifts to oligoclonality in the elderly and old individuals aged 57–88 (Giachino et al. 1994). This observation correlates with a decrease in the counts of $\gamma\delta$ TCR⁺ cells from

the age of 70 (Colonna-Romano et al. 2002; Argentati et al. 2002; Re et al. 2005; Andreu-Ballester et al. 2012). The results obtained in the present study are consistent with this notion and extend the onset of the effective contraction of the $\gamma\delta$ TCR⁺ population at the age of 53–67 years. The fraction of V δ 2⁺ among CD3⁺ cells gradually decreases from 30 years of age, and the alteration of $\gamma\delta$ TCR⁺ counts reflects an alteration of V δ 2⁺ among $\gamma\delta$ TCR⁺ cells (Caccamo et al. 2006; Wistuba-Hamprecht et al. 2013). In the present study, we observed that the fraction of $\gamma\delta$ TCR⁺ among CD3⁺ cells decreases among the elderly. This observation is in contrast to a previous report in which no changes in this fraction were observed from the age of 19 to 103 (Argentati et al. 2002).

To our knowledge, we have identified an unappreciated age-dependent increase of CD4⁺CD8[−] cells among $\gamma\delta$ TCR⁺ T cells in the elderly. $\gamma\delta$ TCR⁺ T cells provide B cell help (Wen et al. 1996), and particularly, CD4⁺CD8[−] cells are the IL-4-providing subset (Ferrick et al. 1995; Wen et al. 1998), indicating that B cell help might be stabilized through $\gamma\delta$ TCR⁺ T cells in the elderly.

In the present study, the fraction of Ki67-positive cells among $\gamma\delta$ TCR⁺ T cells was increased. This finding corresponded to a decrease in the count of proliferating $\gamma\delta$ TCR⁺ T cells, observed as a general decrease in the $\gamma\delta$ TCR⁺ T cell population. This finding could indicate that non-proliferating $\gamma\delta$ TCR⁺ T cells have a higher propensity for tissue residence than Ki67-positive cells. To the best of our knowledge, this is the first time age-dependent changes in basal proliferation levels of $\gamma\delta$ TCR⁺ T cells have been observed, although a similar decrease in proliferation has been observed for CD4⁺ and CD8⁺ T cells (Lee et al. 2012).

Several reports have demonstrated that the fraction and number of peripheral and tissue-located Tregs increase with age (Gregg et al. 2005; Vukmanovic-Stejic et al. 2006; Lages et al. 2008; Agius et al. 2009; Booth et al. 2010). Similar to the entire CD4⁺ T cell population, the fraction of regulatory recent thymic emigrants and Tregs with a naïve phenotype decreases with increasing Tregs with a memory phenotype (Gregg et al. 2005; Booth et al. 2010). We observed a strong decrease in Foxp3⁺Helios⁺CD31⁺ cells in the elderly, consistent with previous observations (Haas et al. 2007). The observed stability of Foxp3⁺Helios⁺ among CD4⁺ cells is also consistent with previous observations (Alexander et al. 2013).

Based on recent findings that show that the response to *Aspergillus* in healthy individuals is dominated with Tregs while severe allergic reactions are dominated with a memory T cell response (Bacher et al. 2013), it is tempting to speculate that the trend toward increased numbers of *Aspergillus*-specific CD4⁺ and CD8⁺ T cells reflects decreased Treg efficiency. However, this notion would disregard the prevailing observations that the suppressive function of Tregs remains unaffected with age (Gregg et al. 2005; Hwang et al. 2009).

Together with the data in the companion paper (Stervbo et al. 2015), it is clear that the dynamic changes of the adaptive immune system are heterogeneous and might counteract other detrimental changes. It will be interesting to identify the mechanisms that govern these heterogeneous changes and determine how these changes might affect immunosenescence.

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Author contributions CB, DR, SM, JM, MN, and AS performed the experiments. US, CB, DR, KJ, and UB performed the analysis. SO, NB, AG, AN, and AT conceptualized the overall strategy and developed clinical translation and implementation. All of the authors discussed the results and implications and commented on the manuscript at all stages. US drafted the manuscript.

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